Region directed mutagenesis of Varicella-Zoster virus thymidylate synthase

By

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Summary

Thymidylate synthase (TS) catalyses the reductive methylation of deoxyuridine monophosphate by 5,10-methylenetetrahydrofolate to produce thymidylate and dihydrofolate. In the reaction the enzyme is present initially as an open complex that closes down progressively as ligands bind. The binary complex which is formed as the substrate dUMP binds has an active site that is open and structurally very close to the unliganded state. It is upon binding of the folate cofactor that structural changes occur and the enzyme forms a closed ternary structure. In contrast to the movements of large domains seen in other proteins upon ligand binding, the changes in TS involves segmental accommodation in which segments of secondary structural elements all move in concert towards the active site. The two largest shifts involve the C-terminus and DRTG loop which moves 4Å to close over the active site.

Sequence data from 35 organisms reveal the protein to be one of the most highly conserved enzymes which makes it an ideal test bed for the design of species specific inhibitors. This thesis is concerned with the structure-function relationships in the TS of the human herpesvirus VZV. The highly conserved residues in the TS family were thought to be present to maintain enzyme function but mutagenesis studies of several family members have shown that almost all the conserved residues can be substituted without severe disruption of enzyme activity. It is thought that only four of these residues are critical to maintain catalytic activity. One possible explanation for the high conservation could be that TS interacts with other enzymes involved in DNA synthesis. Affinity chromatography was performed in this thesis to detect proteins interacting with TS but none were identified.

To identify residues which are critical for enzyme function a region-directed mutagenesis protocol was established using long oligonucleotides which were targeted to selected regions of the protein. Using this protocol a series of temperature sensitive mutants were produced in different regions of the enzyme. Temperature sensitive mutants are a means of identifying residues which are critical for protein stability and function. Initial studies were carried out on buried regions of the protein as it was predicted that residues involved in stability would be found there. The mutant enzymes were characterised by *in vivo* and *in vitro* assays and examined by molecular modelling to gain insights into the role of the mutated residue.

Following the initial experiments, this region-directed approach was used to target the highly mobile C-terminus and DRTG loop region of VZV TS. The DRTG loop is a highly conserved region in all TS species which moves up towards the C-terminus upon ligand binding resulting in Arg38 forming a hydrogen bond with the C-terminal carboxylate of the protein. Eight mutants were constructed in the DRTG loop region,

three of which contained base substitutions in the DRTG loop itself. The mutants all had reduced but significant TS activity.

Removal of the C-terminal residue from the prokaryotic enzymes results in complete inactivation which is believed to be due to the loss of a hydrogen bond network involving the C-terminal carboxylate group. By contrast removal of the C-terminal leucine from VZV TS results in an enzyme which retains ~3% of wild type activity. These findings have been investigated further by targeting the region directed approach to the C-terminus. Four temperature sensitive mutants were produced in this region, three of which had major reconstructions and one contained a point mutation. All mutants produced reduced but significant TS activity. Kinetic analysis of one of these mutants revealed that dUMP K_m was reduced three fold and folate K_m was greatly increased indicating that the mutant enzymes had difficulty forming the closed ternary complex. Studies with the inhibitor phenolphthalein were also consistent with this view. These results strengthen the previous findings that removal of the C-terminal residue did not result in the complete inactivation of VZV TS and confirm that there are significant differences between bacterial and VZV TS.

Site directed mutagenesis was used to produce an asparagine to aspartic acid mutation at Asn214 of VZV TS, a residue which is completely conserved in all TS sequences. This residue was initially thought to allow for the binding of substrate, but more recent studies have indicated that Asn214 is in fact conserved to discriminate between dUMP and dCMP binding to the enzyme. Experimental evidence has indicated that alteration of the corresponding residue, Asn229, of *L.casei* results in the enzyme converting from a dUMP methylase to a dCMP methylase. The N214D mutant from VZV was inhibited by the addition of increasing concentration of dCMP into the TS assay indicating that dCMP is competing with the normal dUMP substrate and confirming a role for Asn214 in dCMP/dUMP discrimination. This method of substrate differentiation could prove to be a general theme in protein-nucleic acid recognition.

Abbreviations

Amp	ampicillin
BrdUMP	5-bromo-2'-deoxyuridine 5'-monophosphate
BSA	bovine serum albumin
°C	degrees Celsius
CB3717	N^{10}-propargyl-5,8-dideazafolate
CD	circular dichromism
Ci	Curie
CIP	calf intestinal phosphatase
cm	centimetre
cpm	counts per minute
dATP	2'-deoxyadenosine 5'-phosphate
dCTP	2'-deoxycytidine 5'-phosphate
dGTP	2'-deoxyguanosine 5'-phosphate
dTTP	2'-deoxythymidine 5'-phosphate
DMSO	dimethyl sulphoxide
dNTP	2'-deoxynucleoside 5'-triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
dUMP	2'-deoxyuridine 5'-monophosphate
E	early
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EHV-1	Equine herpesvirus 1
EHV-2	Equine herpesvirus 2
EtBr	ethidium bromide
FdUMP	5-fluoro-2'-deoxyuridine 5'-monophosphate
FPGS	folylpolyglutamate synthase
FU	fluorouracil
hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid]
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
hr	hour

HSV-1	Herpes simples virus type 1
HSV-2	Herpes simplex virus type 2
HVS	Herpesvirus saimiri
IPTG	isopropyl-β-D-thiogalactosidase
IE	immediate early
kb(p)	kilobase pairs
K_{cat}	maximal catalytic rate
kDa	kilodaltons
K_m	Michaelis constant
L	late
L.casei	*Lactobacillus casei*
LB	Luria-Bertani broth
M	molar
MeWo	Melanoma Workshop
mg	milligrams
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
mRNA	messenger RNA
nm	nanometre(s)
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol
PMSF	phenyl methyl sulphonyl flouride
PRV	Pseudorabies virus
RFC	reduced folate carrier
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
ss	single stranded
SV40	Simian virus 40
TBS	tris buffered saline
TCA	trichloroacetic acid

TdR	thymidine
TEMED	N,N,N',N-tetramethylethylenediamine
TK	thymidine kinase
Tris	2-amino-2-(hydroxymethyl)1,3-propandiol
Triton X-100	octyl phenoxy polyethoxyethanol
tRNA	transfer RNA
TS	thymidylate synthase
TSB	transformation and storage buffer
UV	ultraviolet light
V	volt(s)
VZV	Varicella-zoster virus
wt	wild type
µCi	microCurie(s)
µg	microgram(s)
µl	microlitre(s)

One and three letter abbreviations for amino acids

Amino Acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1. Introduction

The biology and molecular biology of herpesviruses will be reviewed in the first section of this introduction, with special reference to varicella-zoster virus. The second section provides information on the structure and function of the enzyme thymidylate synthase.

1.1. Herpesviridae

1.1.1 Introduction

Herpesviruses are widely disseminated in nature, and most animal species have yielded at least one herpesvirus upon examination. Of nearly 100 herpesviruses that have been characterised, seven herpesviruses have been isolated from humans [herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and human herpesvirus 6 and 7 (HHV6 and HHV7)]. Recently Chang *et al.* (1994) identified an eighth herpesvirus which is associated with Kaposi's sarcoma, this has been classified as a gammaherpesvirus and is most closely related to herpesvirus saimiri (Moore *et al.*, 1996).

Membership of the family Herpesviridae is based on the architecture of the virion. A typical herpesvirion consists of a core containing a linear double-stranded DNA, which is packaged into an icosahedral capsid approximately 100-110nm in diameter. Initially the DNA was thought to be arranged as a toroid around a central protein "plug" (Furlong *et al.*, 1972), but cryoelectron microscopy studies provided no evidence for a toroidal arrangement of the DNA or for the existence of a central "plug". Recent studies have indicated that the capsids contain little or no protein and that the volume enclosed by the outer capsid shell is taken up entirely by DNA (Booy *et al.*, 1991). The capsid is surrounded by an amorphous sometimes asymmetrical material, which is in known as tegument, and this in turn is enclosed in an envelope containing glycoprotein spikes on its surface.

1.1.2. Molecular and biological properties of herpesviruses

Herpesviruses initiate infection by the attachment of the viral envelope to receptors on the cell surface. The virus envelope fuses with the plasma membrane, the nucleocapsid is released into the cytoplasm and migrates to the nucleus where the DNA enters via the nuclear pores. Viral DNA is replicated in the nucleus forming concatemers consisting of head to tail repeats of the genome. The DNA is then cleaved and packaged into preformed capsids which acquire tegument and envelope prior to leaving the infected cell.

In addition to the lytic cycle of infection, herpesviruses are able to establish a latent infection. In HSV the virus can persist in dorsal root ganglia in an apparently inactive state for varying durations, and then be reactivated by a variety of stimuli (reviewed by Roizman & Sears in Fields Virology 1996, Chapter 72, pp2231-2278). Thus after infection, viral DNA persists in the host for the entire lifetime of the individual.

1.1.3. Classification of Herpesviruses

The members of the family *Herpesviridae* have been classified into three subfamilies (i.e. the *Alpha,* the *Beta* and the *Gammaherpesvirinae*) on the basis of their biological properties such as host range in tissue culture, length of reproductive cycle, cytopathology and characteristics of latent infection (Roizman *et al.*, 1992).

The members of the subfamily *Alphaherpesvirinae* are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and the capacity to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily includes HSV-1 and 2, varicella-zoster virus (VZV), pseudorabies virus (PRV) and equine herpesvirus 1 (EHV-1).

A nonexclusive characteristic of the *Betaherpesvirinae* is their restricted host range. Their reproductive cycle is long and infection progresses slowly in culture. The infected cells frequently become enlarged and carrier cultures are readily established. The virus can be maintained in latent form in secretory glands, kidneys and other tissues. This subfamily includes human cytomegalovirus (HCMV) and murine cytomegalovirus.

The *Gammaherpesvirinae* only infect their natural host and cannot infect experimental animals outside the family or order. Viruses in this group are specific for either T- or B-lymphocytes, and infection is frequently arrested at a prelytic stage, with persistence and minimal expression of the viral genome (latent infection), or at a lytic stage, causing cell death without the production of infectious virus progeny. Members of the gammaherpesvirinae include Epstein-Barr virus (EBV), herpesvirus saimiri (HVS) and herpesvirus ateles (HVA).

1.1.4. Herpesvirus DNAs

Herpesviral genomes are highly diverse, they range in size from 125 to 230kbp with around 70 to 200 genes and they exhibit widely differing base compositions and patterns of repeated sequences. The variation in the size of the genome of any one herpesvirus appears to be minimal, but not insignificant. Thus, many viral DNAs contain terminal and internal reiterated sequences. Because of variablility in the number of these reiterations, the size of individual genomes may vary by more than 10kbp. Spontaneous deletions also occur and have been noted in both HSV, EBV and HCMV strains passaged

outside the human host (reviewed by Roizman in Fields Virology 1996 Chapter 71, p2221-22260).

The base composition of herpesvirus DNAs varies from 31 to 75% G+C. Furthermore, herpesvirus DNAs vary with respect to the extent of homogeneity of base sequence distribution across the length of the genome (Roizman *et al.*, 1992).

Probably the most interesting feature of herpesvirus DNAs is their sequence arrangement. There are six main types of genome structures, differing in the size and relative arrangement of repeated and unique sequences. According to this scheme the herpesviruses can be divided into six groups.

Complete sequencing of seven mammalian herpesviral genomes has shown that viruses from all three subfamilies contain a subset of 40 genes common to all the viruses, suggesting that these viruses share a common evolutionary origin. Detailed phylogenetic analysis has estimated that the three subfamilies arose approximately 180 to 220 million years ago (McGeoch *et al.*, 1995). Channel catfish virus, however, does not show such similarities to the mammalian and avian viruses (Davison, 1992), suggesting that fish herpesviruses form a distinct and diverse subfamily.

1.2. Varicella-Zoster Virus

1.2.1. Introduction

Varicella-zoster virus is the causative agent of two common well defined diseases: chickenpox (varicella) upon primary infection, and shingles (herpes zoster) after reactivation of the latent virus from dorsal root ganglia. Chickenpox is a highly contagious but commonly mild disease, usually of children, and shingles is a local vesicular condition caused by the reactivation of VZV within a dermatome. Normally, neither chickenpox nor shingles is life-threatening, although the latter is often extremely painful. Both diseases, however, may have serious consequences in immune-compromised patients (reviewed in Davison, 1991).

Primary VZV infection is presumed to be initiated by the inoculation of the respiratory mucosa with infectious virus transferred in respiratory droplets or by contact with infectious vesicular fluid from an infected individual (Nelson & St. Geme, 1966). During the incubation period, which lasts from 10-21 days, the virus probably spreads to regional lymph nodes and then causes primary viraemia with associated viral replication in the liver and spleen. A secondary cell-associated viraemic phase begins at 24 to 96 hours which results in the transport of the virus to skin and respiratory mucosal sites and it is the replication of the virus in the epidermal cells that causes the characteristic rash of varicella. The termination of viral replication requires the induction of VZV specific immunity. During this period of primary infection the virus gains access to the cells of the

trigeminal and dorsal root ganglia and establishes latency. The virus can remain in this latent state for up to several decades before being reactivated to produce shingles (reviewed by Arvin in Fields Virology 1996, Chapter 79 pp2547-2576).

1.2.2. Classification of VZV

Based on its morphology, VZV is classified as a member of the family Herpesviridae (Roizman *et al.*, 1992) and is further classified along with the herpes simplex viruses in the subfamily *Alphaherpesvirinae*, by virtue of its ability to replicate and spread quickly and efficiently. In common with other alphaherpesviruses, VZV establishes a life-long latent infection of sensory nerve ganglia, from which it can later reactivate.

The VZV particle, like that of all herpesviruses, is now appreciated as one comprising of four major elements; the core containing a linear double-stranded DNA, an icosahedral capsid, a tegument, and an envelope containing viral glycoprotein spikes on the surface. The enveloped particle is pleomorphic to spherical and 180 to 200nm in diameter.

1.2.3. Genome structure of VZV

The molecular biology of VZV has lagged behind that of the other human alphaherpesviruses, HSV-1 and HSV-2, due to the inability to obtain large quantities of cell-free infectious virus in tissue culture. VZV is extremely cell-associated and is usually passaged *in vitro* by seeding infected fibroblasts, rather than cell free virus, onto fresh cell monolayers. Therefore, most VZV research has followed routes that tend to avoid the need to work with cell-free virus and work has focused more on the genetic material of VZV.

The complete nucleotide sequence of the Dumas strain of the VZV genome was determined in 1986 by Davision and Scott. The genome is a linear double-stranded DNA molecule about 125,000bp in length and is thus one of the smallest herpesvirus genomes. It has a G+C content of 46%, rather lower than that of most herpesviruses (Ludwig *et al.*, 1972). The genome is usually present as a linear duplex in virions: however, Straus *et al.*, (1981) and Kinchington *et al.*, (1985) reported the presence of rare circular forms of DNA released from virions.

Restriction endonuclease mapping (Dumas *et al.*, 1981; Davison & Scott, 1986) revealed that the genome is composed of two unique regions, a unique long region (U_L, approx 100,000bp) and a unique short region [U_S, 5,232bp, Figure 1.1]. The unique long region is flanked by inverted repeat regions [terminal repeat long (TRL) and internal

repeat long (IRL), each 88.5bp] and the unique short region is flanked by inverted repeat regions [terminal repeat short (TRS) and internal repeat short (IRS), each 7,319.5bp].

The VZV genome exists predominantly in two isomeric forms (Dumas *et al.*, 1981; Ecker *et al.*, 1982) as the U_S region can be directed in either orientation in 50% of genomes. In contrast, the U_L region is directed in one orientation in 95% of genomes and in the opposite direction in 5% of genomes (Davison, 1984; Hayakawa & Hyman, 1987; Kinchington *et al.*, 1985).

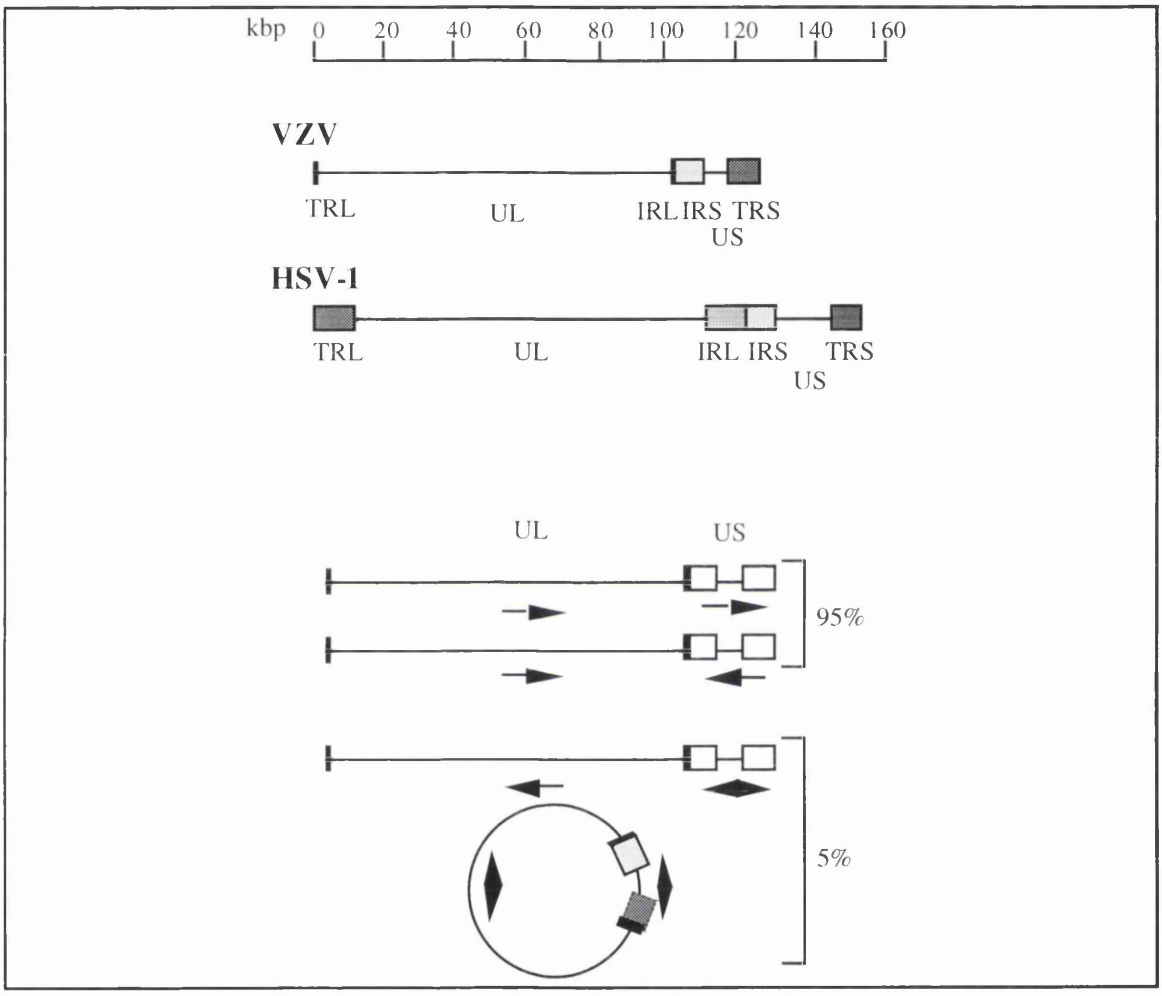


Figure 1.1. Comparison of VZV and HSV-1 genomes

The top panel shows schematics of the VZV (125kbp) and HSV-1 (152kbp) genomes and a scale in kbp is shown above. Inverted repeats (TRL/IRL and TRS/IRS) are shown as coloured boxes. The bottom panel shows the different isomers of the VZV genome; the two predominant forms contain the long segment in a single orientation, while the short segment is detected in an equimolar ratio in either of the two possible orientations, as indicated by the arrows. A small percentage of the VZV DNA molecules exist either in a circular form or with the long segment inverted (Davison, 1984; Hayakawa & Hyman, 1987; Kinchington *et al.*, 1985).

The DNA sequence of the Dumas strain of VZV was originally thought to contain at least 68 unique genes, three of which are present in two copies in the IRS and TRS regions and two (42 and 45) are probably expressed as spliced mRNA. Two additional genes have been proposed since the original sequence assignments were made (Davison & Scott, 1986; McGeoch *et al.*, 1993); ORF 9A (Barnett *et al.*, 1992) and ORF 33.5 which encodes an N-terminal truncated form of ORF 33 (Telford *et al.*, 1992). The arrangement of the open reading frames in the genome is shown in Figure 1.2. Although some of the genes have putative polyadenylation signals shortly after the ends of their ORF's, many of the genes are arranged in unidirectional families of up to four genes with a single putative polyadenylation signal at the 3' end of the family. This type of gene arrangement has been documented extensively for HSV (Wagner, 1985).

The DNA sequence of the Dumas strain of VZV contains five regions with repeat elements termed R1 to R5 (Davison & Scott, 1986). R1 to R4 are G-C rich like the G-C rich repeats present in HSV-1 (Watson *et al.*, 1981; Davison & Wilkie, 1981). In contrast, the reiterated element of R5 is longer and A+T rich. The R1, R2 and R3 regions are located in the U_L segment of the genome, and each is within the coding sequence of genes. In contrast R4 and R5 appear not to code for protein, the R4 region is located in both IRS and TRS regions between ORFs 62 and 63 and consists of multiple 27bp elements (Casey *et al.*, 1985) and the R5 region is located between ORFs 60 and 61 and consists of 88 and 24bp repeats (Hondo & Yogo, 1988). The Dumas strain only has a single copy of the R5 repeats, whereas other strains of VZV have multiple repeats. The variability in the number of repeats implies that the different strains of VZV have differing overall lengths of their genomes. The role of the reiterations in the growth of VZV is unknown but the relative lack of conservation of these features in position and sequence in different herpesvirus genomes suggests that some, perhaps all, may merely have accumulated in locations where they do not cause a selective disadvantage.

1.2.4. VZV and HSV-1 sequence homology

Hybridisation studies on VZV and HSV-1 DNA revealed that there was nucleotide homology between the two viruses. These studies indicate that large proportions of the VZV genome are collinear with the HSV-1 genome (Davison *et al.*, 1983). Subsequent comparison of sequences of HSV-1 and VZV allowed gene homologues to be identified. The functions of many VZV genes could therefore be predicted from studies on HSV homologues.

Within the U_L region of VZV, 56 of the 61 genes have homologues and 55 are located in similar regions of the genome. Five genes in the U_L region do not have HSV-1 counterparts. One gene in the U_L region, ORF 61, has its HSV-1 homologue, Vmw 110 located in the IRL/TRL region of HSV-1.

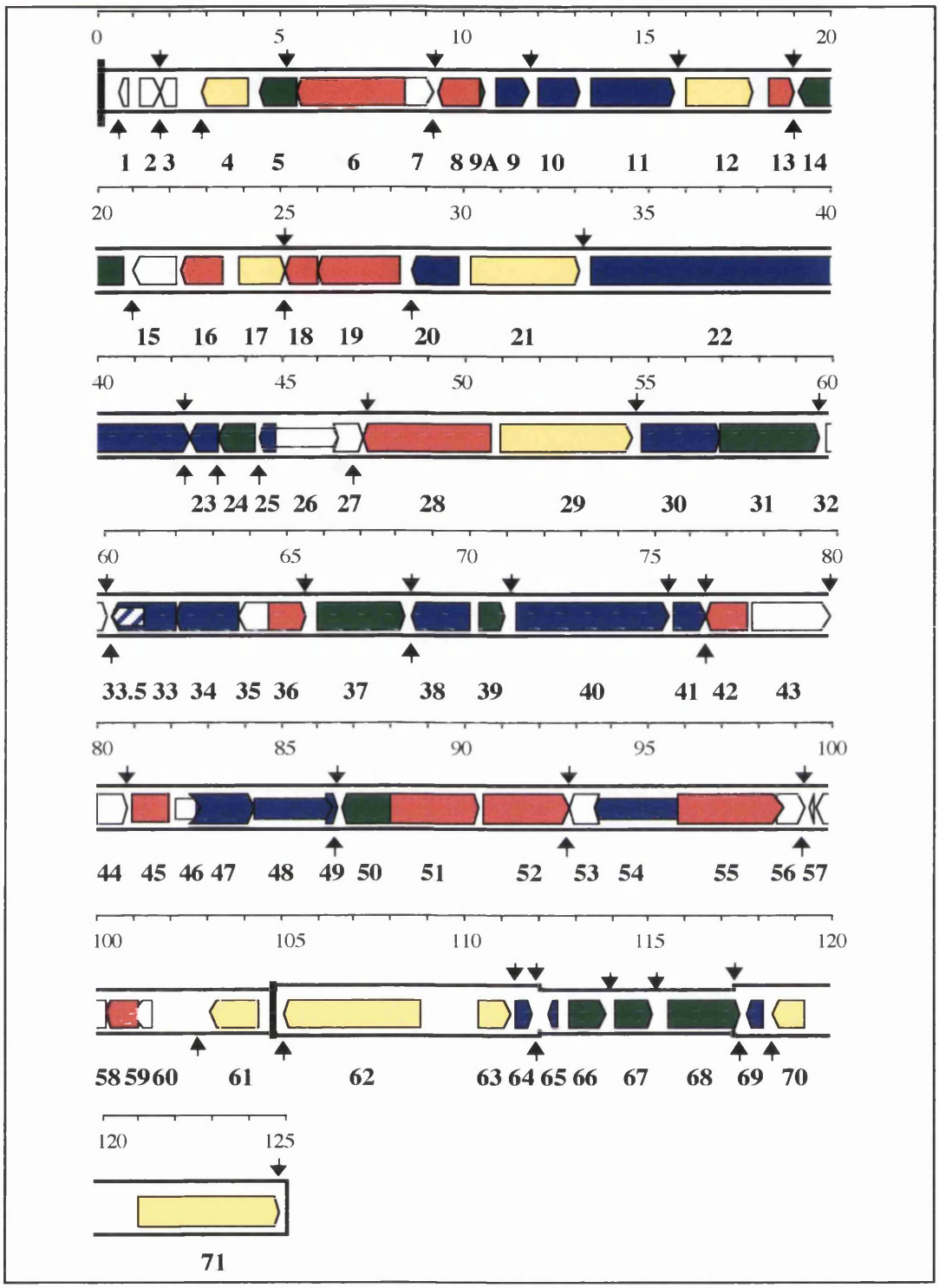


Figure 1.2. The arrangement of the open reading frames in the genome of VZV
Polyadenylation sites are indicated by the vertical arrows. The colour scheme is as follows: red indicates enzymes, blue proteins in the capsid, tegument or those involved in capsid maturation/DNA packaging, yellow shows proteins involved in transcription or translational control, green membrane proteins, and finally white indicates unknown function. (Data taken from Davison 1991, 1993: Ostrove 1990; McGeoch *et al.*, 1993).

In the U_S region of VZV, all four of the VZV genes have homologues within the U_S region of HSV-1, although the positions of the genes are rearranged relative to HSV-1 (Davison *et al.*, 1986). The three genes in the IRS/TRS regions of VZV (ORFs 62, 63 and 64) each have HSV-1 homologues; ORF 62 has its HSV homologue Vmw 175 located in the IRS/TRS repeat region of HSV-1, whereas ORFs 63 and 64 have their HSV-1 homologues located in the U_S region of HSV-1.

Five genes in VZV (ORFs 1, 2, 13, 32 and 57) do not have homologues in HSV-1. However, four of them (ORFs 1, 2, 32 and 57) have homologues in equine herpesvirus type 1, another member of the alphaherpesvirus family (Telford *et al.*, 1992). The fifth gene ORF 13, encodes the viral thymidylate synthase. Although no other alphaherpesvirus is known to encode this enzyme, it is encoded by four gammaherpesviruses [HHV8 (Moore *et al.*, 1996), EHV2 (Telford *et al.*, 1995), HVS (Horness *et al.*, 1986) and HVA (Richter *et al.*, 1988)].

1.2.5. Gene products of VZV involved in nucleotide metabolism

Comparison of the VZV ORFs with those of the more fully characterised HSV-1 proteins permitted many gene functions to be assigned. Additional information as to the likely function of the predicted VZV genes has also been gained from either sequence comparison with non-herpesvirus proteins or from experimental work (reviewed by Davison, 1991). The genetic relationship between VZV and HSV-1 forms the major basis for the properties or functions assigned to the VZV genes in Table 1.1.

VZV specifies a large number of enzymes involved in DNA synthesis. Pyrimidine deoxyribonucleoside kinase (dPyK), the product of ORF 36, was identified on the basis of its homology to HSV-1 thymidine kinase (Sawyer *et al.*, 1986). The VZV enzyme is a 70Kd dimer composed of two identical 35Kd subunits. The enzymological properties of VZV dPyK differ from those of HSV-1 TK (Ogino *et al.*, 1977; Cheng *et al.*, 1979; Sawyer *et al.*, 1986), particularly in that VZV phosphorylates deoxycytidine in preference to thymidine. The VZV enzyme, like TK, phosphorylates and therefore activates acyclovir. Acyclovir resistant strains of VZV have point mutations in the TK gene near the adenosine triphosphate (ATP) or thymidine binding site or mutations that result in a truncated protein (Sawyer *et al.*, 1988). In addition to specifying dPyK, which is involved in the salvage pathway for thymidine metabolism, VZV also encoded the enzyme thymidylate synthase which is involved in the pathway for the *de novo* synthesis of thymidylate. This enzyme bears strong homology to TS proteins from other organisms and several approaches have demonstrated that it is a functional enzyme (Thompson *et al.*, 1987).

VZV ORFs 18 and 19 are homologous to the small and large subunits of HSV-1 ribonucleotide reductase (RR) (Davison, 1986). This enzyme catalyses the conversion of

ribonucleoside diphosphates to their corresponding deoxyribonucleosides. Chemical inhibition of VZV RR by the inhibitor 2-acetylpyridine 5-[(dimethylamino) thio carbonyl] thiocarbonohydrazone (A1110U) potentiates the antiviral activity of acyclovir, presumably by decreasing intracellular pools of deoxyribonucleoside triphosphates and increasing the level of acyclovir triphosphate (Spector, 1989). This resultant 100-fold increase in the ratio of the concentrations of acyclovir triphosphate to dGTP facilitates the binding of the inhibitor to the target enzyme, DNA polymerase, resulting in increased inhibition of virus replication.

VZV also specifies deoxyuridine triphosphatase (dUTPase) an enzyme that catalyses the conversion of deoxyuridine triphosphate (dUTP) to its corresponding monophosphate (dUMP), providing both a mechanism to prevent incorporation of dUTP into DNA and a pool of dUMP for conversion to dTMP by thymidylate synthase. Uracil DNA glycosylase is encoded by ORF 59, this functions in DNA repair and acts to correct insertion of dUTP and excise deamination of cytosine residues in DNA (reviewed in Davison, 1991).

1.2.6. Regulation of VZV genes

Gene expression of many herpesviruses is co-ordinately regulated and sequentially ordered in a cascade fashion. The most extensive analysis has been done for HSV-1 genes which are classified as α, β and γ (Honess & Roizman, 1974, 1975) or immediate early (IE), early (E) and late (L) (Clements *et al.*,1977). The inability to obtain high-titre cell free VZV has made it difficult to achieve synchronous infection of cells in culture to determine the temporal classes of VZV genes. It is still unclear whether VZV follows a distinct cascade pattern during which the three classes of genes (immediate early, early and late) are sequentially expressed as found in other herpesviruses such as HSV-1 (Honess *et al.*, 1974).]

Gene regulation during HSV-1 infection in tissue culture has been extensively studied. The IE genes are transcribed by the unmodified host RNA polymerase II at the onset of infection. The promoter regions of each of the IE genes contain one or more copies of the conserved sequence TAATGARAT, which mediates stimulation of IE transcription by the virion tegument protein Vmw 65 (Ace *et al.*, 1989). Vmw 65 does not bind directly to DNA (Marsden *et al.*, 1987) but is incorporated into a multiprotein complex at TAATGARAT. In addition to Vmw 65 the complex contains the cellular factors Oct-1, which binds to TAATGARAT, and HCF, a large protein which is cleaved to a number of smaller products (Gerster & Roeder, 1988). Three of the immediate early proteins produced are important transactivators required for maximal expression of early and late genes (Everett, 1984a; O'Hare & Hayward, 1985; Sacks *et al.*, 1985). Two of these proteins Vmw 175 (IE 175 gene product) and Vmw 63 (UL54 gene product) are

Table 1. Functions assigned to VZV genes

(Taken from Davison 1991, 1993; Ostrove 1990; McGeoch *et al.*, 1993.)

VZV Gene	HSV-1 Counterpart	Gene function
2	—	Function Unknown
3	UL55	Function Unknown
4	UL54	IE protein; Post-translational regulator of gene expression
5	UL53	Possible membrane protein
6	UL52	Component of DNA-helicase-primase
7	UL51	Function Unknown
8	UL50	Deoxyuridine triphosphatase
9	UL49	Tegument protein
9A	UL49A	Possible membrane protein
10	UL48	Tegument protein; transactivates IE genes
11	UL47	Tegument protein
12	UL46	Modulates IE gene transactivaton by UL48 protein
13	—	Thymidylate Synthase
14	UL44	Membrane glycoprotein (gpV) -role in cell entry
15	UL43	Function Unknown, probable integral membrane protein
16	UL42	Subunit of DNA polymerase
17	UL41	Virion host shutoff protein
18	UL40	Ribonucleotide reductase small subunit (R2)
19	UL39	Ribonucleotide reductase large subunit (R1)
20	UL38	Capsid protein
21	UL37	Possible DNA binding function
22	UL36	Tegument protein
23	UL35	Capsid protein
24	UL34	Membrane-associated phosphoprotein
25	UL33	Role in capsid maturation/DNA packaging
26	UL32	Function Unknown
27	UL31	Function Unknown
28	UL30	Catalytic subunit of DNA polymerase
29	UL29	Single-stranded DNA binding protein
30	UL28	Role in capsid maturation/DNA packaging
33	UL26	Protease involved in virion maturation

VZV Gene	HSV-1 Counterpart	Gene function
33.5	UL26.5	Internal protein of immature capsids (processed by UL26
34	UL25	Virion protein
35	UL24	Function Unknown
36	UL23	Thymidine kinase
37	UL22	Membrane glycoprotein (gpIII) -role in cell entry
38	UL21	Role in virion morphogenesis
39	UL20	Integral membrane protein; role in virion egress
40	UL19	Major capsid protein
41	UL18	Capsid protein
42+45	UL15	Possible terminase
43	UL17	Function Unknown
44	UL16	Function Unknown
46	UL14	Function Unknown
47	UL13	Tegument protein; probable protein kinase
48	UL12	DNase-role in maturation and packaging of DNA
49	UL11	Myristylated tegument protein- role in envelopment and virion transport
50	UL10	Probable integral membrane protein
51	UL9	*Ori*-binding protein; DNA helicase
52	UL8	Component of DNA helicase-primase complex
53	UL7	Function Unknown
54	UL6	Role in virion morphogenesis
55	UL5	Component of DNA helicase-primase complex
56	UL4	Function Unknown
57	—	Function Unknown
58	UL3	Function Unknown
59	UL2	Uracil-DNA glycosylase
60	UL1	Function Unknown
61/71	RL2	IE protein; transcriptional regulator
62	RS1	IE protein; transcriptional regulator
63/70	US1	IE protein; transcriptional regulator
64/69	US2	Virion protein
65	US9	Tegument protein
66	US3	Protein kinase
67	US7	Membrane glycoprotein (gpIV)
68	US8	Membrane glycoprotein (gpI)

essential for normal viral growth. The third transcriptional activator, Vmw 110 is not essential for viral replication in tissue culture (Stow & Stow, 1986; Sacks & Schaffer, 1987). Genetic and biochemical evidence has shown that the HSV-1 IE polypeptide Vmw 175 is essential for the expression of early and late gene products and transactivates a number of early gene promoters in synergy with Vmw 110 protein but the role of the other IE proteins is still unknown (Courtney *et al.*, 1976; Marsden *et al.*, 1976; Watson & Clements, 1978, 1980; Preston, 1979a; Dixon & Schaffer, 1980; Preston, 1981; DeLuca *et al.*, 1985).

Four VZV IE proteins have been identified, three of which are phosphoproteins (reviewed by Cohen in Fields Virology 1996, Chapter 78 pp2525-2542.). The largest phosphoprotein has been assigned to ORF 62, whereas identification of the other proteins is uncertain at present. The product of VZV gene 62 is believed to be the VZV equivalent of HSV-1 Vmw 175 as the genes are located in equivalent positions and there is extensive predicted amino acid sequence conservation between them (McGeoch *et al.*, 1986). Also VZV ORF 62 has been shown to be a potent activator of gene expression in transient transfection assays (Everett & Dunlop, 1984; Everett, 1984; Inchauspe *et al.*, 1989) and the regions of the Vmw 175 polypeptide that have been shown to be the most important for its transactivation and repression phenotypes are the most highly conserved (Paterson & Everett, 1988a; DeLuca & Schaffer, 1988). The same regions are also highly conserved in the other alphaherpesvirus homologues of Vmw 175 (Grundy *et al.*, 1989; Cheung, 1989; Vleck *et al.*, 1989). The most compelling evidence that VZV IE 62 and HSV-1 Vmw 175 are functionally homologous is that HSV-1 mutants with lesions in Vmw 175 can be complemented by either transfected plasmid or transformed cell lines which express VZV IE 62 (Fesler *et al.*, 1988).

VZV ORF 10 is the homologue of HSV-1 Vmw 65. The two genes have similar locations within their respective viral genomes. The predicted amino acid sequences of VZV ORF 10 and Vmw 65 are highly homologous except that ORF 10 lacks the acidic carboxyl terminus of Vmw 65 which is required for transactivation (Davison & Scott, 1986; McGeoch *et al.*, 1988; Moriuchi *et al.*, 1993). VZV, like its HSV-1 homologue transactivates VZV ORF 62 and HSV-1 genes in transient expression assays (Moriuchi *et al.*, 1993), however, ORF 10 protein does not transactivate the putative VZV IE ORF 4 and ORF 61 genes (Kinchington *et al.*, 1994; Moriuchi *et al.*, 1994). Mutagenesis studies on the VZV ORF 62 promoter indicated that TAATGARAT-like elements contribute to transactivation of the VZV IE62 by the ORF 10 protein (Moriuchi *et al.*, 1995).

ORF 61 is an IE gene of VZV and is thought to be the HSV-1 Vmw 110 homologue, because both genes are located in similar parts of the genome, and the predicted gene products share a cysteine rich putative zinc-binding finger in the amino-terminal region and additional limited amino acid homology elsewhere (Perry *et al.*, 1986). However, the two proteins exhibit different properties in transient expression

assay systems *in vitro*, HSV-1 Vmw 110 transactivates HSV-1 genes, while VZV ORF 61 has been shown to be a transrepressor (Everett, 1984; O'Hare *et al.*, 1986). Experimental evidence has shown that cell lines expressing VZV ORF 61 complement the growth of an HSV-1 Vmw 110 deletion mutant and thereby verify that VZV ORF 61 is the functional homologue of HSV-1 Vmw 110 (Moriuchi *et al.*, 1992). The ability of VZV ORF 61 to complement a Vmw 110 deletion mutant of HSV-1 implies that despite marked differences in amino acid sequences and in activities in transient expression assays, the two proteins have similar functions during virus infection.

The VZV gene exhibiting the highest degree of similarity to the HSV-1 gene encoding Vmw 68 is VZV ORF 63 (Davison & Scott, 1986). Although the sizes of the two proteins are rather different, i.e. the VZV gene 63 product has a theoretical molecular mass of 30.5kDa, whereas its HSV-1 homologue has a molecular mass of 68kDa, and the total degree of amino acid conservation is low, they have one conserved domain with a degree of similarity ranging from 25-49% (Davison & McGeoch, 1986). The literature is unclear as to whether VZV ORF 63 by itself directly transregulates other VZV promoters since results from cotransfection experiments demonstrate that the VZV 63 protein strongly represses the expression of VZV gene 62. In addition, transient expression assays have shown that VZV gene 63 protein can activate the thymidine kinase gene which encodes a typical early protein, but it cannot affect the expression of late genes encoding glycoproteins I and II. The results from these transient expression experiments strongly suggest that the VZV gene 63 protein could play a pivotal role in the repression of IE gene expression as well as in the activation of early gene expression (Jackers *et al.*, 1992). Recent experimental evidence, however, has demonstrated that ORF 63 exerts only minimal effects on diverse VZV putative IE and E promoters and it was noted that it did not directly inhibit the ORF 62 promoter as had previously been reported (Kost *et al.*, 1995).

VZV ORF 4 protein shares considerable amino acid sequence homology with HSV-1 IE protein IE63, especially in the carboxy terminus. The carboxyl terminal region of IE63 is rich in cysteine and histidine residues and has been shown to bind zinc. While the carboxyl region of ORF 4 also contains cysteine and histidine residues, it is not known whether this region also binds zinc. The amino terminal regions of these two proteins have limited amino acid homology, however both are highly acidic. Despite structural similarities, VZV ORF 4 and HSV-1 IE63 behave differently in transient expression assays. ORF 4 protein transactivates a wide variety of target constructs. In contrast, IE63 acts as a transrepressor or a transactivator depending on the presence of different mRNA processing signals. Although IE63 is required for HSV-1 replication the role of ORF 4 in viral infection is not well understood. Cell lines expressing ORF 4 are unable to efficiently complement HSV-1 IE63 mutants (Moriuchi *et al.*, 1994).

1.2.7. VZV DNA replication

VZV DNA replication is thought to occur in four stages based on evidence from replication of the HSV-1 genome (Davison, 1984). Firstly linear DNA in the virion circularises. Since the VZV genome is not terminally redundant the simplest method for circularisation involves direct ligation of the termini to produce a novel L-S joint identical in sequence to the normal L-S joint. The circular molecule then begins to replicate, and isomerisation may occur by homologous recombination between the inverted repeats. More extensive replication of the circular molecule occurs to generate head-to-tail concatemers probably by a rolling circle mechanism (Ben-Porat & Rixon, 1979; Jacob *et al.*, 1979). Finally concatemers are cleaved to generate linear DNA for packaging into virions.

Origins of HSV-1 replication have been located in two distinct regions, namely close to the centre of U_L (ori_L) and within the inverted repeats IR_S and TR_S (ori_s). The HSV-1 origins lie betweeen the 5' ends of divergently transcribed genes. Two early genes which encode the viral DNA polymerase and major DNA binding protein flank ori_L (Quinn & McGeoch, 1985; Gibbs *et al.*, 1985), whereas the genes flanking ori_S belong to the IE class of genes. These specify the polypeptides Vmw 175 and either Vmw 68 or Vmw 12 (Wagner, 1985). Studies have revealed that VZV contains an origin of replication in IRS/TRS regions - termed ori_S located between ORFs 62 and 63 and ORFs 70 and 71 (Davison, 1986; Stow & Davison, 1986). In contrast to HSV-1, VZV does not appear to contain an origin of replication between its DNA polymerase and major DNA binding protein genes.

VZV ori_S contains a 45bp, almost perfect palindrome with the sequence $(TA)_{17}$ at its centre. Mutation of the palindrome results in the loss of replicative activity. HSV-1 has the sequence $(AT)_5$ and $(AT)_6$ in its ori_L and ori_S respectively. Adjacent to the VZV ori_S palindrome is the sequence CGTTCGCACTT, which is present in both the HSV-1 ori_S and ori_L (Stow & Davison, 1986). Further upstream from the ori_S palindrome is the sequence CATTCGCACTT. Transfection experiments using the VZV replication assay indicate that the 45bp palindrome and the sequence CGTTCGCACTT are essential for replication activity, whereas the sequence CATTCGCACT contributes additional activity but is not essential. A second sequence farther upstream from the other 2 sequence does not contribute to the replication activity (Stow *et al.*, 1990). Experiments have demonstrated that VZV is not able to support the replication of a plasmid containing HSV-1 ori_S; however HSV-1 is able to partially support the replication of a plasmid containing VZV ori_S (Stow & Davison, 1986).

Sequence analysis has revealed that the genome of VZV has homologues to all seven of the essential HSV DNA replication genes (McGeoch *et al.*, 1988; Wu *et al.*, 1988). VZV gene 51 is homologous to HSV UL9 which encodes a protein that binds to the viral DNA origins of replication and is thought to play an important role in the

initiation of viral DNA synthesis. The VZV origin binding protein, binds to the three sites adjacent to the palindrome at the sequences CGTTCGCACTT (located at two sites) and CATTCGCACTT (Chen & Olivo, 1994). The former sequence conforms to and the latter sequence contains a single base mismatch from the consensus sequence YGYTCGCACT used by the HSV-1 ori binding protein (Koff & Tegtmeyer, 1988).

1.2.8. Assembly and release of VZV from infected cells

Controversy exists as to how herpesviruses mature within and egress from cells. Added uncertainties exist for VZV replication, because the model needs to account for the inefficient formation and release of infectious progeny.

Two major and competing models for VZV egress have been proffered. Jones and Grose (1988) showed that naked particles traverse the nuclear membrane and become incorporated into and enveloped by large cytoplasmic vesicles. Virus-specific glycoproteins are released from the *trans*-Golgi network (TGN) and fuse with large cytoplasmic vesicles so their glycoprotein content is available to decorate the envelopes of newly arriving particles. The mature enveloped virions are then transported to the cellular membrane via the cytoplasmic vacuole. Exit of the virions at the cellular membrane takes place when cytoplasmic vacuoles harbouring VZV particles fuse and become incorporated into the cellular membrane. This model does not specify how nonenveloped nucleocapsids move from nucleoplasm to cytosol, how viral tegument gains access to the space between nucleocapsid and viral envelope or why enveloped viral particles are observed in perinuclear cisternae.

The competing model from Gershon and colleagues (Gershon *et al.*, 1973; Gershon *et al.*, 1994) proposed that nucleocapsids assemble in the nuclei of infected cells and bud through the nuclear membrane to reach the perinuclear cisterna-rough endoplasmic reticulum (RER). A temporary envelope is acquired from the inner nuclear membrane, this envelope fuses with the RER, delivering unenveloped nucleocapsids to the cytosol, where they travel until they reach the *trans*-golgi network. Glycoproteins of the viral envelope are synthesised in the RER independent of the nucleocapsids and tegument proteins are probably synthesised by free ribosomes. The tegument proteins probably bind to the tails of the glycoproteins and are transported through an intermediate compartment to reach the golgi stack, where high mannose proteins are modified to become complex oligosaccharides. The glycoproteins are finally transported to the *trans*-golgi network where they accumulate on the surface of a *trans*-golgi derived sac. The concave surface of the *trans*-golgi derived sac becomes the viral envelope, while the convex surface becomes a transport vesicle, which diverts virions from the secretory to the lysosomal pathway, delivering the virions to prelysosomes where they are subjected to degradative enzymes. It is proposed that VZV migrates to a lysosomal pathway because

its envelope glycoproteins are glycosylated with mannose residues which become phosphorylated at the six position (Gabel *et al.*, 1989). Mannose-6-phosphate chains render the evolving particles susceptible to binding and uptake by lysosomes bearing mannose-6-phosphate receptors. This model requires that an alternative pathway must be operative in some cell types *in vivo* to allow virus egress without lysosomal degradation. Therefore, this process explains the cell-associated nature of VZV *in vitro* but the mechanism by which the virus escapes diversion from the secretory to the lysosomal pathway *in vivo* remains to be determined.

1.2.9. VZV latency

A fundamental property of all herpesviruses is their ability to become latent, persist in an apparently inactive state for varying durations, and then be reactivated by certain stimuli. Thus, after infection, viral DNA persists in the host for the entire lifetime of the individual.

During HSV-1 infection, the virus accesses sensory neurons by direct spread at sites of mucosal or cutaneous inoculation. The viral nucleocapsids are then transported to the neuronal nucleus, where latency is established. During latency HSV expresses only a single family of latency-associated transcripts (LATs) (Croen *et al.*, 1987; Stevens *et al.*, 1987) that overlap extensively with, but are opposite in polarity to, the mRNA encoding the immediate early protein Vmw 110. Reactivation of the virus entails *de novo* replication of virus, centrifugal migration back down the axon, and spread to local mucocutaneous cells (Stevens & Cook, 1971). Reactivation is a common and often asymptomatic event with HSV-1 (Brock *et al.*, 1990), neither of which is true of VZV.

The nature of VZV latency has not been clarified as well as that of HSV-1. Whereas HSV reactivations recur numerous times, VZV infections rarely occur more than once. VZV latency is established during varicella, the primary viraemic infection. The virus may access neural tissues either haematogenously or by centripetal neural transport from mucocutaneous lesions. The VZV genome can be readily detected by the polymerase chain reaction in most human trigeminal ganglia and many dorsal root ganglia (Mahalingam *et al.*, 1992), it has also been found in human geniculate ganglia and olfactory bulbs (Furuta *et al.*, 1992; Liedtke *et al.*, 1993). The cellular localisation of the persistent virus is less well established. Initial *in situ* hybridisation studies have shown neuronal latency (Hyman *et al.*, 1983; Gilden *et al.*, 1987). With more refined probes VZV persistence was demonstrated in non-neuronal cells (Croen *et al.*, 1988; Meier *et al.*, 1993), this is in contrast to HSV which resides almost exclusively within neuronal cells during latency. The VZV genome possesses no obvious homologue of the HSV LAT-encoding sequences. Rather, *in situ* and Northern hybridisation studies argue for restricted transcription from multiple regions of the VZV genome during latency (Croen *et*

al., 1988). Therefore, VZV remains latent and can reactivate in the context of a nonneuronal cell that can support its replication and facilitate its spread to neuronal and nonneuronal cells. In this process, multiple sensory nerves become infected and convey virus to all parts of the cutaneous dermatome. HSV, however reactivates within a neuronal cell that is surrounded by satellite cells which are less capable of supporting its productive replication and so the virus spreads readily to the skin but it does not spread efficiently within the ganglia. Therefore, the relative infrequency of VZV reactivation may reflect its nonneuronal site of latency, rendering it less subject to the neural triggers that provoke HSV reactivation.

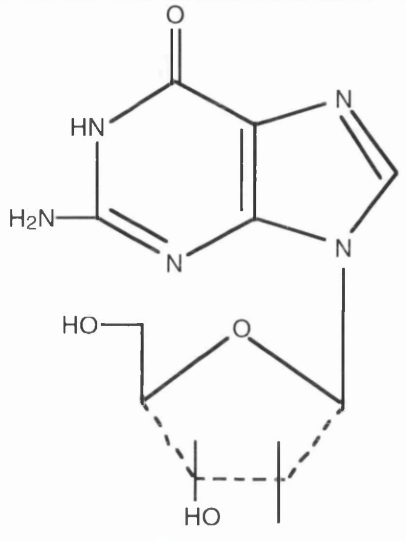
1.2.10. Antiviral therapy for VZV

Over the past thirty years considerable experimental work has been carried out to identify agents which are effective in the treatment of VZV. One reason that it has been so difficult to produce non-toxic antiviral agents is that viruses replicate inside cells. Therefore, anything which affects the DNA of the virus can possibly affect the DNA of the cell.

Initial studies demonstrated that human leukocyte interferon benefited immunocompromised patients with varicella or zoster with a clear reduction in disease severity (Arvin *et al.*, 1982). However, the precise mechanism by which the effects were mediated remains uncertain but it may involve non-specific augmentation of the deficient host responses to VZV or direct inhibition of virally driven transcription or translation. Experiments then moved onto nucleoside and pyrophosphate analogues which inhibit VZV replication. Adenosine arabinoside (vidarabine) was the first effective analogue, it proved to be a modestly potent but selective competitive inhibitor of VZV-encoded DNA polymerase (Whitley *et al.*, 1982).

Acyclovir, which is a nucleoside analogue, is now the drug of choice for VZV infections because it has higher specific activity against VZV replication *in vitro* and better clinical efficacy than vidarabine, it also elicits fewer adverse effects than the administration of vidarabine or interferon-alpha [reviewed by Elion, 1993 (Figure 1.3)]. The compound itself is closely related to the natural component of DNA, guanine deoxyribose, except that it lacks the 2'- and 3' carbons and the 3' hydroxyl of the deoxyribose ring. The compound blocks the replication of VZV by shutting down virus DNA synthesis through an interaction with the virus-coded enzyme DNA polymerase. Before blockade of DNA synthesis can occur, the compound has to be phosphorylated into its triphosphate form indicating that ACV is a pro-drug. The compound is selectively monophosphorylated by the VZV-encoded thymidine kinase (TK) and further modified by cellular enzymes (Fyfe *et al.*, 1978). It is clear that VZV-induced TK is very different from the cellular TK and this results in the high selectivity of acyclovir for the virus. The

triphosphate form of acyclovir, which is the active antiviral agent, behaves like a nucleoside triphosphate and competes with dGTP for the viral DNA polymerase. As the DNA chain begins to elongate, it eventually incorporates a molecule of acyclovir opposite cytosine on the template, this results in chain termination since acyclovir does not have 3'OH group to form a phosphodiester bond (Elion, 1983). This drug has potent activity against HSV, with reduced efficacy against VZV and relatively low activity against HCMV. The low activity against HCMV can be explained by the absence of a TK gene.



Acyclovir/Deoxyguanosine

Figure 1.3. Structure of acyclovir and deoxyguanosine
Structure of acyclovir is given in solid lines. Dashed lines represent the additional 2' and 3' carbons and 3'-hydroxyl group present in deoxyguanosine. (Taken from Elion, 1993).

There are three ways in which resistance to acyclovir can occur (reviewed in Elion, 1993). Most commonly a reduction in activity or deletion of the virus TK can occur, this is not a problem in immunocompetent patients but it does occur in highly immunocompromised patients after prolonged treatment. A herpesvirus TK with altered substrate specificity can also occur which would result in acyclovir becoming inefficiently phosphorylated. Finally the viral DNA polymerase can become mutated which results in the enzyme becoming insensitive to acyclovir-triphosphate.

Although in most respects acyclovir is an excellent drug, one drawback is its limited oral bioavailability. This restricts the levels of drugs that can be achieved in individuals following oral dosing and led to the idea that an oral prodrug might be developed to overcome this difficulty and lead to improved dosing convenience and potentially greater clinical effect. Research leading to new antiherpesvirus drugs has come

from three approaches. The first approach was directed towards improving the bioavailability of acyclovir by examining the potential of a variety of related prodrugs. Efforts were concentrated on examinination of simpler esters and 18 different amino acid esters were made. The L-valyl ester, L-valylacyclovir was the best prodrug [Beauchamp *et al.*, 1992, (Figure 1.4)]. It has the same broad spectrum of activity as the parent acyclovir but delivers higher levels of acyclovir and so increases the efficacy and dose convenience of the drug. The second approach was to examine a large number of 5-substituted pyrimidines for activity against those viruses which are not potently inhibited by acyclovir, i.e. HCMV. This research focused on the deoxypyrimidine nucleotides and led to the discovery of the potent, VZV specific compound 882C [Rahim *et al.*, 1992, (Figure 1.4)].

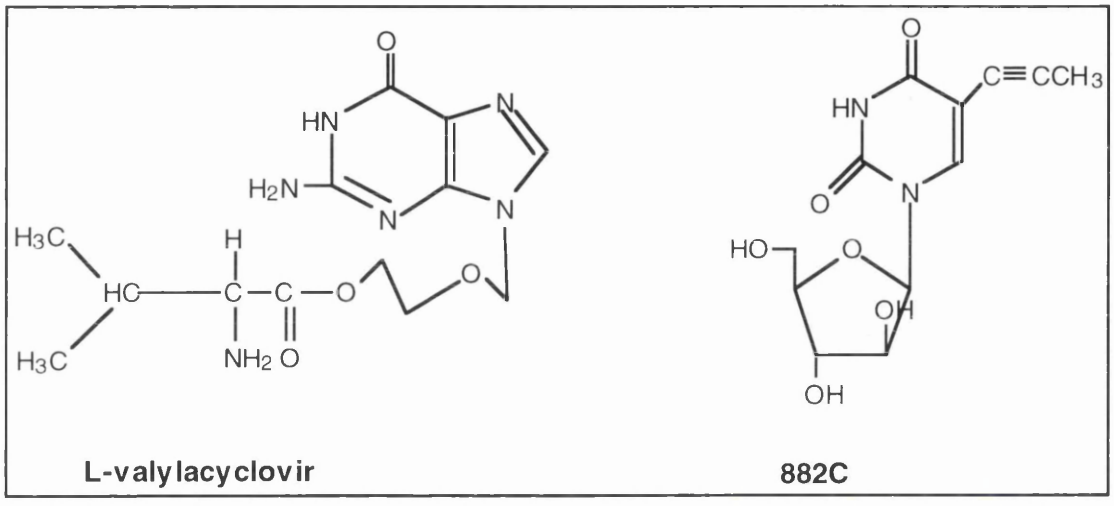


Figure 1.4. Structures of novel VZV inhibitors
The chemical structures are shown for the L-valyl ester prodrug, L-valylacyclovir and the 5-substituted pyrimidine 882C which is neither a guanine base or a chain terminator. (Taken from Darby, 1993, Purifoy *et al.*, 1993).

This drug demonstrates potent and highly specific VZV activity without significant activity against HSV or HCMV. In addition 882C is not toxic at the highest concentration tested. The specificity is achieved since 882C is converted to the 5'-monophosphate by VZV TK but not by cellular cytosolic TK. Furthermore, the monophosphate is specifically converted to the diphosphate by the activity of the VZV thymidylate kinase. The lack of activity of 882C for HSV correlates with the inability of the HSV TK to use 882C monophosphate as a substrate. It is the triphosphate of 882C that is the potent inhibitor of the VZV-specific DNA polymerase and this inhibition is probably the major mechanism of the antiviral activity. The potency of 882C has been found to be seven

times greater than that of acyclovir which indicates that the compound is extremely promising for the treatment of VZV infections. The third approach used in the search for new anti-herpesvirus compounds was to examine drug combinations with acyclovir (reviewed by Safrin *et al.*, 1993). This research led to the compound 348U, an inhibitor of herpes simplex virus ribonucleotide reductase (Spector *et al.*, 1992). This compound results in decreased pools of dGTP; in combination with acyclovir, this increases the competitive binding of acyclovir-triphosphate to the viral DNA polymerase.

1.3. Thymidylate synthase

1.3.1. Thymidylate synthase in DNA synthesis

The building blocks of DNA synthesis are the deoxyribonucleoside triphosphates; dATP, dGTP, dCTP and dTTP (Figure 1.5). The first three of these are synthesised by the direct reduction of their corresponding ribonucleotides in a reaction catalysed by ribonucleotide reductase. In eukaryotes and certain prokaryotes reduction occurs at the diphosphate level and is followed by phosphorylation of the dNDPs to dNTPs by the enzyme nucleoside diphosphate kinase. In other prokaryotes reduction of ribonucleotides occurs at the triphosphate level. Ribonucleotide reductase also mediates the synthesis of a fourth 2'-deoxyribonucleotide, namely dUDP.

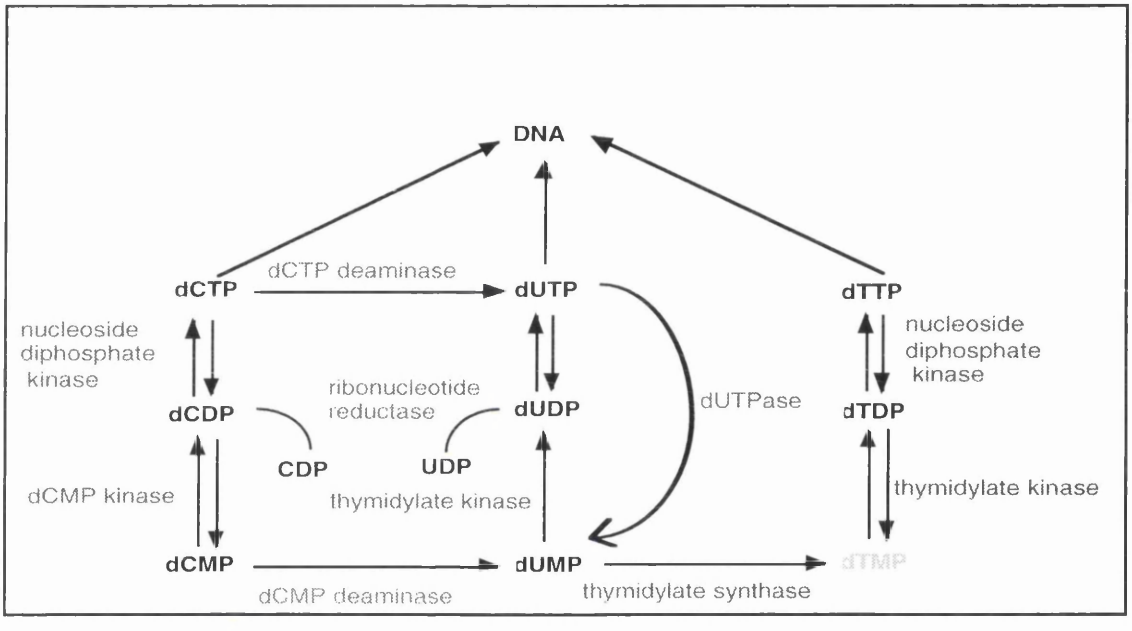


Figure 1.5. *De novo* pathway of pyrimidine deoxyribonucleotides synthesis. Enzymes are in red and thymidylate is given in blue. (Taken from Larsson, 1995).

As dTTP has no ribonucleotide counterpart, it cannot be synthesised in this way. Instead, it is synthesised *de novo* by the enzyme thymidylate synthase, which catalyses the formation of thymidylate via the reductive methylation of deoxyuridylate. dUMP is provided either by the hydrolysis of dUTP by the enzyme deoxyuridylate triphosphatase (dUTPase), or by the deamination of deoxycytidylate. A second means for the production of dTMP is via the salvage pathway where thymidine kinase catalyses the phosphorylation of thymidine. The phosporylation of dTMP is then catalysed by the enzyme thymidylate kinase and nucleoside diphosphate kinase catalyses the phosphorylation of dTDP to dTTP (Neuhard & Nygaard, 1987; O'Donovan & Neuhard, 1970).

1.3.2. The thymidylate cycle

TS is unique among those enzymes that use folate cofactors, in that for each molecule of dTMP formed one molecule of H_4folate is consumed (Figure 1.6). This reaction is the only one in Nature that results in the production of dihydrofolate which is an oxidised form of folate that cannot be used in any biological process. Thus to continue dTMP synthesis the tetrahydrofolate pools must be replenished. To regenerate the reduced folate pools, dihydrofolate reductase (DHFR) must rapidly reduce the dihydrofolate to a tetrahydrofolate form and the enzyme serine trans-hydroxymethylase completes the cycle by catalysing the formation of N^5, N^{10}-methylenetetrahydrofolate.

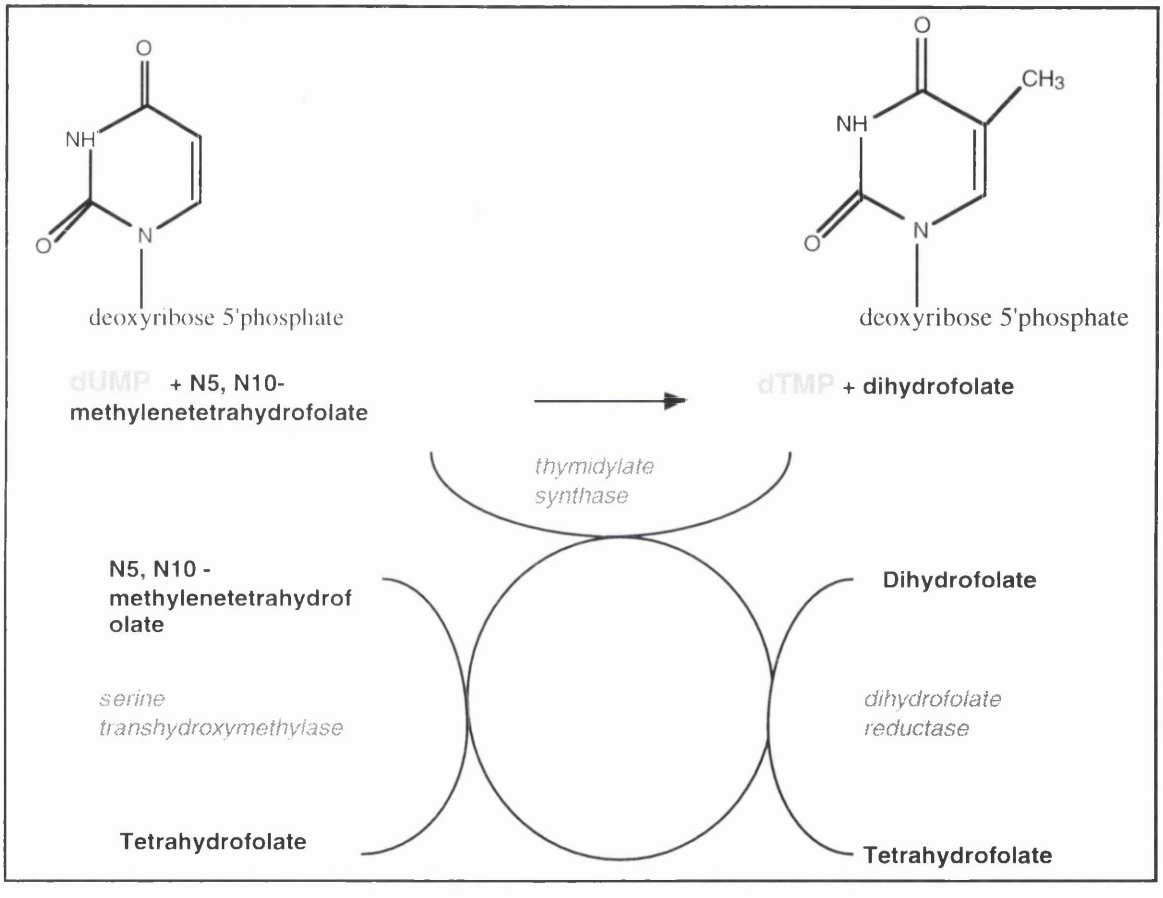


Figure 1.6. The thymidylate cycle

The reductive methylation of deoxyuridylate results in the production of thymidylate and dihydrofolate. This oxidised form of the cofactor cannot be used by the cell and is converted back to the reduced form via the enzymes dihydrofolate reductase(DHFR) and serine transhydroxymethlayse.

1.3.3. Structure of thymidylate synthase

The crystal structure of *L.casei* TS was solved by Hardy *et al.* in 1987. Subsequently, crystal structures of *E.coli* (Perry *et al.*, 1990; Matthews *et al.*, 1990), T4

phage (Finer-Moore et al., 1994), *Leishmania major* (Knighton *et al.*, 1994), human (Schiffer *et al.*, 1991) and *Pnuemocystis carinii* (Perry & Stroud, unpublished data) TSs were solved and confirmed the striking structural homology between these enzymes (Figure 1.7).

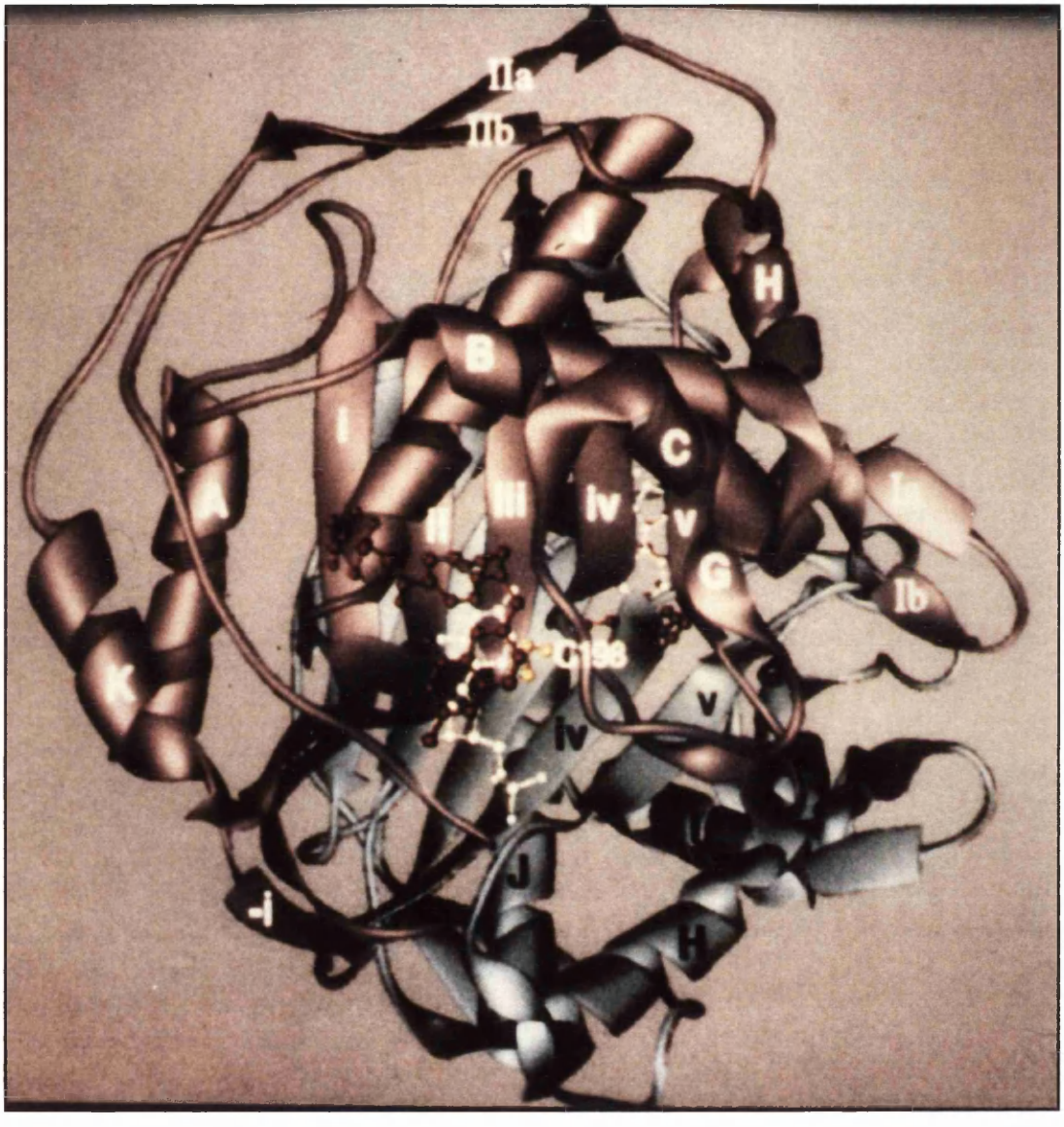


Figure 1.7. A dimer of *E.coli* thymidylate synthase
The monomers are shown in purple and blue. The β-strands and α-helices are labelled (refer to Figure 1.13). The active site cysteine is seen in yellow, dUMP is in white and folate is in red. (Taken from Montfort *et al.*, 1990).

Structures of binary complexes of TS with nucleotides, folate analogues, ternary complexes with nucleotides and folates, as well as complexes with novel inhibitors, have also been reported. The folate analogue CB3717 is used in many of the complexes studied

by crystallography as it prevents any reaction beyond that of enzyme with dUMP. Like CH_2H_4 folate it induces covalent bond formation between enzyme and dUMP however, CB3717 cannot form a covalent bond to dUMP analogous to the covalent bond between cofactor and dUMP in intermediate II of Figure 1.10.

The native enzyme is a symmetric, obligate dimer of chemically identical units of about 35kDa (see Figure 1.8).

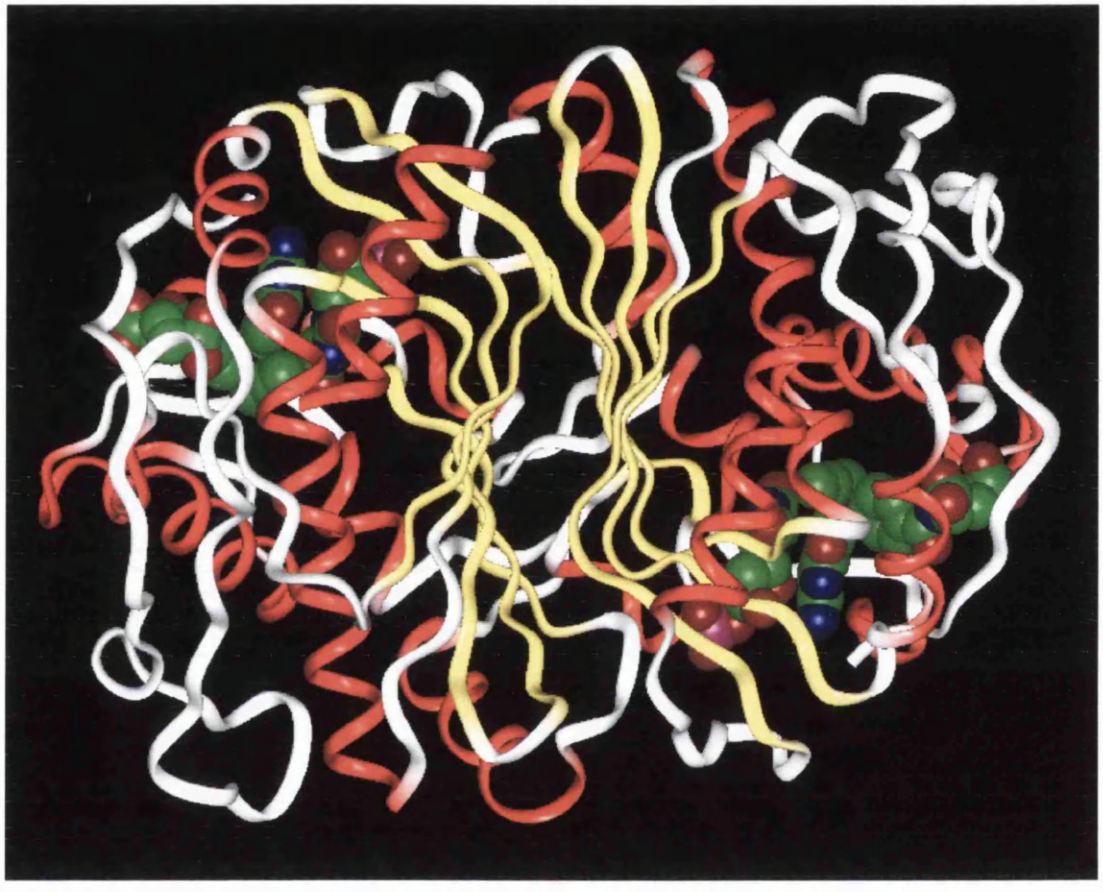


Figure 1.8. Proposed structure of thymidylate synthase dimer from varicella-zoster virus

The view is directly down the dimer interface between the two monomers. β-strands are coloured yellow, α-helices are red, connecting loops white and both substrate and cofactor are depicted as space filling models (Image generated by F.J. van Deursen).

The overall fold of the enzyme is common to all TSs, and is defined by a series 8 α-helices, 10 strands of β-sheet, and several segments of coil that connect the secondary structural elements. The subunit interface is formed by a 5-stranded twisted β-sheet composed of ~ 25 residues that packs against the same sheet formed by the other subunit of the dimer. Each of the two subunits folds into a three-layer domain. The first layer of

the domain is composed of the five β-strands that form the dimer interface. The central layer is made up exclusively of helices, a feature not normally seen in α/β domains (Richardson, 1981) and the third layer consists of several other helices and the carboxy-terminal region.

Several species of TS also contain inserts: there are six sites where these inserts are found around the TS core. They are all present in surface loops or in a smaller "variable" domain and probably do not influence the core structure. In particular, *L.casei* contains a 50 amino acid insertion (70 percent of the variable domain) which is arranged as three short α-helices in the variable domain (Hardy *et al.*, 1987). Since these insertions are not present in all species they cannot be relevant to fundamental aspects of structure or function, but may serve roles specific to the individual organism.

1.3.4. The active site and catalytic mechanism of TS

The substrate binding site of TS is a funnel-shaped cleft extending into the interior of each subunit (Figure 1.9). Components of each monomer contribute to each of the two active sites, which are separated widely on each side of the molecule. This accounts for the inability to dissociate the native enzyme into catalytically active monomers. Moreover it suggests a way for the active sites to communicate in the process of ligand binding. Each site contains a bound inorganic phosphate, this has been observed in all TS structures solved in the absence of nucleotides (Hardy *et al.*, 1987). The left wall of the cavity is formed by the sharply turning part of β-strands ii and iii and the loop between α-helix A and β-strand i. The ceiling is composed of residues form the exposed NH_2 terminus of helix J and the right wall by the end of β-strand iv. The back wall of the cavity is formed by four residues of the β-sheet of the other monomer (reviewed by Carreras & Santi, 1995, see Figure 1.7).

The catalytic cysteine residue (Cys198 in *L.casei*, Cys183 in VZV) lies in the NH_2 terminus of β-strand iv, close to the dimer interface, with thiol exposed on the right wall of the active site cavity. This thiol is the nucleophile that transiently adds to the 6-position of dUMP during catalysis and covalently binds to mechanism based inhibitors. In the *L.casei* structure three arginine residues [Arg178' (Arg 163 of VZV), Arg179' (Arg164 of VZV) and Arg218 (Arg203 of VZV), ' denotes residues from different subunit] and Ser219 (Ser204 of VZV) are at the base of the cavity co-ordinated to bound phosphate. Arg178' and Arg179' are not part of the same polypeptide chain as the other active site residues and are donated form the other subunit of the dimer for the purpose of ligating the bound phosphate (Hardy *et al.*, 1987).

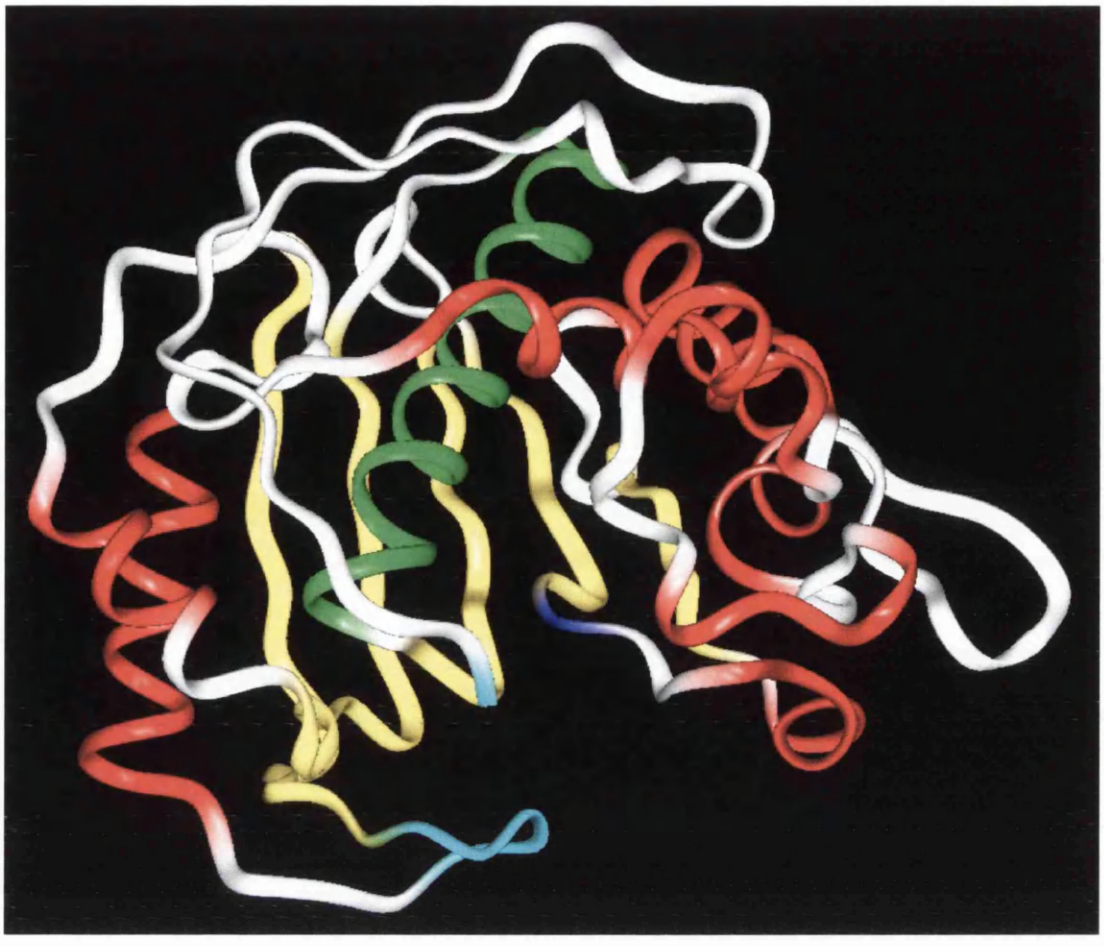


Figure 1.9. Proposed structure of a monomer of thymidylate synthase from varicella-zoster virus

β-strands are coloured yellow, α-helices are red, connecting loops white. The central J helix is green, and the active site cysteine is blue. The C-terminal residue and the DRTG loop are coloured cyan. Substrate and cofactor are not shown for clarity. The orientation is the same as in Figure 1.7 and at 90° to that in Figure 1.8. (Image generated by F.J. van Deursen).

The enzyme reaction occurring in the active site involves the initial formation of a covalent bond between the C-6 position of dUMP and the catalytic thiol (Cys198 of *L.casei*, Cys183 of VZV) of the enzyme to generate a nucleophilic centre at C-5, intermediate I (Figure 1.10). This is followed by covalent bond formation between C-5 of dUMP and the one carbon unit of CH_2H_4 folate. In general folate does not undergo direct reactions with nucleophiles but converts to a reactive species, which could be the 5- or 10-iminium ions formed by opening of the 5-membered imidazolidine ring of folate. In kinetic studies the 5-iminium ion was proposed because the N-5 is more basic than N-10, and N-10 is a better leaving group (Kallen & Jenecks, 1966). The joining of dUMP and

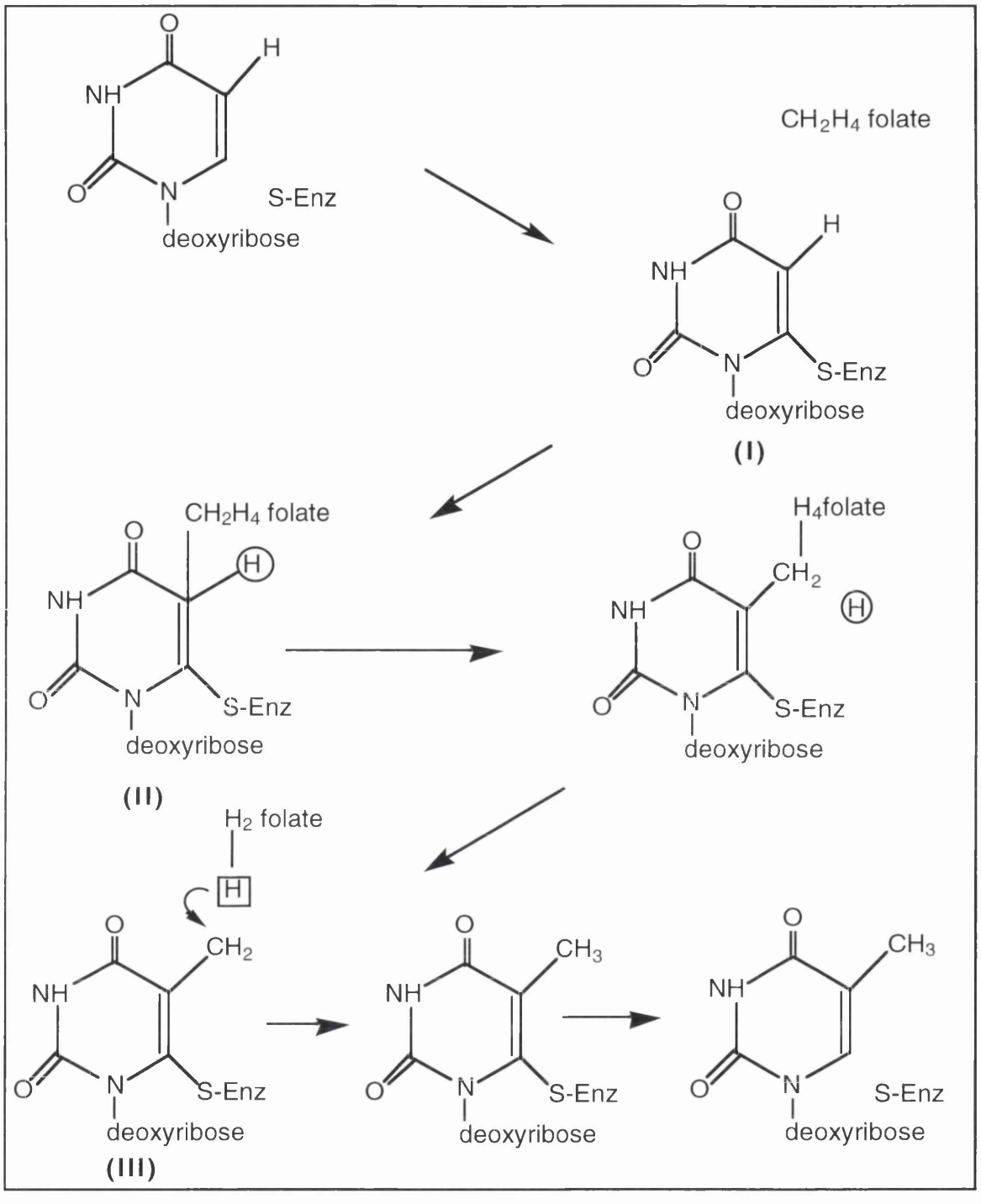


Figure 1.10. Chemical mechanism of TS

Formation of a covalent bond between the C-6 position of dUMP and the catalytic thiol of the enzyme activates C-5 of dUMP for condensation with the one carbon unit of the CH_2H_4 folate (I). A covalent bond is formed between the activated form of the cofactor and C-5 of dUMP (II). The C-5 proton is extracted, followed by β-elimination of folate to give intermediate III. Hydride transfer from non-covalent bound folate to exocyclic methylene and β-elimination of the enzyme provides the products, dihydrofolate, dTMP and active enzyme. (Adapted from Finer-Moore *et al.*, 1990).

folate is envisioned as a nucleophilic attack of C-5 of dUMP on the N-5 iminium ion of CH_2H_4 folate which results in the formation of intermediate II in which C-5 of dUMP is connected to N-5 of H_4folate by a methylene bridge (Figure 1.10). The C-5 proton of intermediate II is abstracted, followed by β-elimination of the folate to give the exocyclic intermediate III. Hydride transfer from non-covalently bound H_4folate to the exocyclic methylene intermediate III and β-elimination of the enzyme provide the products, H_2folate and dTMP, and the active enzyme (reviewed in Stroud & Finer-Moore, 1995).

1.3.5. Specific features of thymidylate synthase

The dimer interface

In addition to the active site of TS (described in section 1.3.3), a second distinctive feature of the enzyme is the subunit interface. This is formed by a five stranded twisted β-sheet composed of ~25 residues that packs against the same sheet formed by the other subunit of the dimer. In contrast to other proteins of known structure in which aligned β-sheets stack face to face with a left-handed rotation, sheets in the TS dimer are related by a right-handed twist (Hardy *et al.*, 1987; Matthews *et al.*, 1989). The common left-handed twist is thought to result from the fact that the natural twist between individual strands in a single sheet creates a deformed rectangular surface in which diagonally opposite corners point either both up or both down. When two such surfaces come together, the left-handed rotation of one relative to the other appears to be favoured by the fact that down-pointing corners of the top sheet can fold down over the edge of the lower sheet, and up-pointing portions of the lower sheets slip up over the edge of the top sheet (Chothia *et al.*, 1977; Cohen *et al.*, 1981). Therefore a rotation in the opposite direction would instead place down-pointing corners of the top sheet very close to up-pointing corners of the bottom sheet forcing interior portions of the sheet to separate creating an unfavourable cavity. However in TS it was noted that at just the point where a down corner of the top sheet would otherwise penetrate an up corner of the bottom sheet, the smaller of the two regular β-sheets on the bottom is arched backward by the series of stacked β-bulges in the lower sheet. Instead of impacting the top sheet, the bottom sheet slopes away in the same direction and with the same curvature as the top sheet, permitting a very close and favourable association between edges of the large top sheet and the bottom sheet while maintaining optimal side-chain packing throughout the central portion of the β-sandwich. These stacked β-bulges are locally stabilised by hydrogen bonding involving eight conserved residues. This structure anchors the phosphate of bound dUMP and controls the precise orientation of the catalytically essential active site cysteine.

The central J helix

Helix J is a six-turn, unusually hydrophobic element around which other secondary structures of the protein fold. It is even more hydrophobic than some membrane spanning helices. The helix packs diagonally across the β-sheet and is flanked by amphipathic helices A and H, which are on the edge of the middle layer, and helix B which packs on top of helix J on the opposite side from the β-sheet.

1.3.6. Ordered binding of substrate and cofactor

Intracellular pools of folate consists predominantly of polyglutamylated molecules with polyglutamyl groups of average length 4-7. Monoglutamated folates have relatively low affinity for TS while polyglutamates have much higher affinities, in some cases over 400 times greater (Lu *et al.*, 1984). Kinetic investigations by Darin and Aull (1978) suggested that when monoglutamated co-factor is used there is evidence that the ligands bind in an ordered fashion in which dUMP binds first followed by folate. The folate is proposed to bind in two steps to TS.dUMP complexes, first binding in a loose noncovalent adsorption complex, then forming a tight covalent complex (Santi *et al.*, 1987). Following catalysis, the oxidised folate is the first product to dissociate from the enzyme followed by the product dTMP. These results are explained by the vastly greater binding affinity of dUMP for TS compared to the affinity of the monoglutamated folate for TS.

However, the situation with respect to polyglutamated substrates is more complex. Galvin *et al.*, (1976) showed that whereas monoglutamated substrates bind to TS only in the presence of dUMP or a pyrimidine analogue, the pentaglutamate derivative forms binary complexes in the absence of pyrimidine. Furthermore Lu *et al.*, (1984) demonstrated that for tetraglutamate substrates the order of substrate binding and product release is reversed with binding of folate preceding that of dUMP and release of dTMP preceding that of dihydrofolate. Therefore, the polyglutamate group increases the affinity of tetraglutamate for TS so that the cofactor binds in the absence of dUMP.

1.3.7. Conformational changes associated with TS reaction

1.3.7.1. Binary complex formation

The active site of TS closes down progressively as ligands bind. In the binary complex, formed as the substrate dUMP binds, the active sites remain open and residues remain close to their positions in the unliganded state. Comparison of substrate bound and phosphate bound structures from *L.casei* TS have shown no significant conformational differences (Finer-Moore *et al.*, 1993). The phosphate of dUMP is co-ordinated by the

same highly conserved arginine residues and Ser219 (Ser204 of VZV) that ligate phosphate in the TS.Pi structure, as well as conserved Arg23 (Arg38 of VZV, refer to Figure 1.13). Each of these residues forms at least one hydrogen bond with the dUMP phosphate moiety, while Arg178' forms an additional bond with Tyr261 (Tyr246 of VZV), and Arg218 (Arg203 of VZV) forms additional H-bonds with the peptidyl carbonyl oxygens of both Arg178' (Arg163 of VZV) and Pro196 [Pro181 of VZV, (Fauman *et al.*, 1994)].

The only slight difference between the TS.Pi and TS.dUMP structures shows that Arg23 (Arg38 of VZV) has moved to interact with the phosphate moiety of dUMP and has become more ordered (Finer-Moore *et al.*, 1993). This movement may be in preparation for the conformational change where Arg23 forms a salt bridge with the C-terminal carboxylate following folate binding (see Figure 1.12).

The deoxyribose of dUMP interacts with Tyr261 (Tyr246 of VZV) and His259 (His244 of VZV), each of which forms hydrogen bonds with the 3'-hydroxyl of dUMP (Finer-Moore *et al.*, 1993, Montfort *et al.*, 1990). The C-2' carbon of dUMP lies in the back of the binding pocket close to the backbone amide of Asp221 (Asp206 of VZV) and the Ser219 (Ser 204 of VZV) side chain (refer to Figure 1.13)

The pyrimidine moiety of the substrate is involved in an extensive hydrogen bond network with Asp221 (Asp206 of VZV), Asn229 (Asn 214 of VZV), Glu60 (Glu75 of VZV), Trp82 (Trp97 of VZV), His199 (His184 of VZV) and several ordered water molecules. Asn229 forms the only direct contact between a TS side chain and the pyrimidine ring and assists in the discrimination between dUMP and dCMP [see Chapter 8, (Liu & Santi, 1992)].

The findings that dUMP binding has little effect on the conformation of TS is consistent with the observation that binding of dUMP has only a small effect on the UV light, circular dichroism and fluorescence spectra of TS (Donato *et al.*,1976; Leary *et al.*, 1975; Galivan *et al.*, 1975). It was suggested that because of the ordered mechanism of substrate binding, a portion of the conformational changes that occur when the binary complex is formed may be associated with the process of creating a binding site for folate.

1.3.7.2. Ternary complex formation

The spectral changes accompanying binary complex formation are small compared with those resulting from formation of the ternary complex as the UV and CD spectra alter considerably upon the addition of the N^5, N^{10}-CH_2FH_4 to the binary complex. Formation of the ternary complex results in the closing down of the enzyme and the production of a more compact structure of the protein, corresponding to an apparent decrease of 3.5% in the Stokes radius (Lockshin & Danenberg, 1980). This correlates well with the fluorescence quenching observed upon ternary complex formation (Bellisario *et al.*, 1976)

and thereby provides evidence that changes in the protein conformation, as opposed to changes in the environment of the ligand contribute to the majority of the observed variations in the fluorescence spectrum.

Studies on the crystal structure of TS bound in the ternary complex with both dUMP and folate show significant differences in the conformation of the protein compared to the unbound or dUMP bound structures (Montfort *et al.*, 1990; Matthews *et al.*, 1990; Kamb *et al.*, 1992). It is uncertain whether the cofactor and substrate together or the cofactor alone trigger the protein conformational change. Kamb *et al.*, (1992) solved the crystal structures of *E.coli* TS bound to either the cofactor analogue CB3717 or to a tighter binding polyglutamyl derivative of CB3717. These structures suggest that cofactor binding alone is sufficient to induce the conformational change in TS and dUMP binding is not required. The crystal structures further suggest that the dUMP binding site is accessible in the TS-cofactor binary complex. Conformational flexibility of the binary complex may permit dUMP to enter the active site of TS while the cofactor is bound.

Several structures of ternary complexes between *E.coli* TS, nucleotides, and folates or folate analogues have been reported (Montfort *et al.*, 1990; Matthews *et al.*, 1990; Matthews *et al.*, 1990; Fauman *et al.*, 1994). Interactions between TS and bound folates are similar in the various ternary complex structures. Initially the substrate binds at the bottom of the active site pocket covalently linked through C-6 to the active site Cys residue. The faces of the pyrimidine and ribose rings of the substrate are exposed to provide a complementary docking surface for the folate cofactor. The pterin ring of the folate binds to the surface of the exposed substrate and makes its largest contact with the bound pyrimidine of dUMP. The aromatic ring of the PABA moiety of the cofactor binds in a hydrophobic pocket of the enzyme (Montfort *et al.*, 1990; Matthews *et al.*, 1990b). The ternary complex structure of *E.coli* TS complexed with dUMP and tetraglutamated CB3717 shows the general position of the tetraglutamate group, which adopts an extended conformation to interact with a positively charged binding site on the protein surface (Kamb *et al.*, 1992). This same positively charged site may be responsible for reported interactions between TS and its mRNA [see section 1.3.9.7. (Chu *et al.*, 1991; Chu *et al.*, 1993)].

The ternary complexes formed upon ligand binding are more ordered and have undergone an extensive conformational change compared to the TS.dUMP structure. Sections of helices and sheets move as units, usually less than 1Å, in response to ligand binding in a process that has been termed segmental accommodation (Figure 1.11). Note that this mechanism avoids disruption of the favourable conformations and hydrogen-bond patterns found in these secondary structural elements.

Figure 1.11. Segmental accomodation by TS
A single monomer of *E.coli* TS is illustrated in the same orientation as in Figure 1.9. Residues shown in yellow deviate from the original position by less than 0.5Å upon ligand binding, whereas residues shown in blue undergo substantial movement. (Taken from Montfort *et al.*, 1990).

Two of the most pronounced structural differences noted in the ternary complexes of TS invlove changes in the surface loop (DRTG loop) containing Arg23 (*L.casei* numbering, Arg38 of VZV) and the C-terminal tetrapeptide. The C-terminal residue moves more than 4Å to interact with the side chain of conserved Thr24, thus forming a lid over the active site (Figure 1.12).

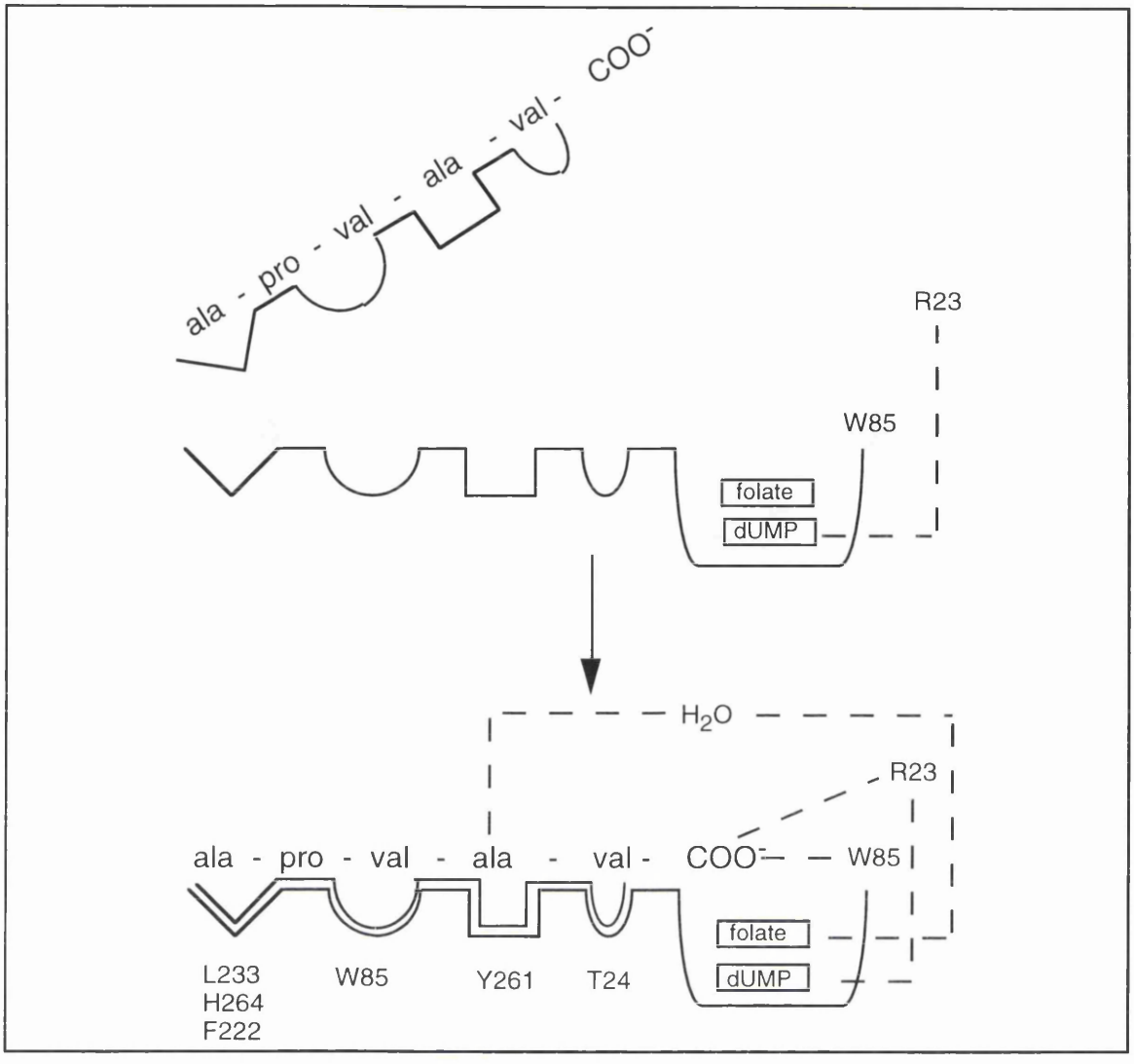


Figure 1.12. Interactions of the C-terminal residues from *L.casei* thymidylate synthase, before and after the ligand-induced conformational change. Dashed lines represent hydrogen bonds. Note the water mediated hydrogen bond contact between the folate and the main chain of Ala315 (Taken from Carreras & Santi, 1995).

The C-terminal carboxylate forms direct hydrogen bonds with Trp85 (Tyr100 is corresponding residue of VZV) and Arg23 (Arg38 of VZV), while the backbone carbonyl oxygen of Ala315 (Ala300 of VZV) forms direct and water mediated hydrogen bonds to the 2-NH_2 and 3-NH groups of the cofactor respectively. Not only does movement of the tetrapeptide over the active site further anchor the folate cofactor in its binding pocket and sequester it from solvent, but it also provides for the exact alignment of the substrate and cofactor through the intricate network of coupled hydrogen bonds (see Figure 1.12).

As in the TS.dUMP structure, nucleotides in the *L.casei* ternary complex structures show the phosphate group ligated by four arginine residues and Ser219 (Ser204 of VZV), and the 3'OH of the deoxyribose forms hydrogen bonds with Tyr261

(Tyr246 of VZV) and His259 (His244 of VZV). The positions of Asp221 (Asp206 of VZV), Asn229 (Asn241 of VZV), Glu60 (Glu75 of VZV), Trp82 (Trp97 of VZV), His199 (His184 of VZV), and the two ordered water molecules that comprise the hydrogen bond network co-ordinating the pyrimidine in binary complexes are preserved.

1.3.8. Stoichiometry of TS reaction

TS from all sources is an obligate homodimer with residues from both subunits forming two active sites (Hardy *et al.*, 1987). In the presence of cofactor, FdUMP binds to all TS, regardless of source, with a stoichiometry of approximately 2 mol/molecule of enzyme (Danenberg *et al.*, 1974; Danenberg & Danenberg, 1979; Plese & Dunlap, 1977; Santi & Danenberg, 1984). Evidence from *L.casei* TS indicates that the binding sites are not equivalent. The FdUMP.N^5,N^{10}-CH_2FH_4.enzyme complex with the two molecules of FdUMP per enzyme decayed within 24 hours to a complex with only one FdUMP molecule per enzyme, which was stable for a longer period of time (Aull *et al.*, 1974; Santi & Danenberg, 1984). Furthermore in the presence of triglutamated forms of the N^5, N^{10}-CH_2FH_4, the two binding sites show a twenty fold difference in affinity for FdUMP (Lockshin and Danenberg, 1979).

In the absence of cofactor, only a single molecule of dUMP or FdUMP is bound per mol of enzyme (Galivan *et al.*, 1976a; Beaudette *et al.*, 1980; Langenbach, 1976), although Plese and Dunlap (1978) reported 1.5 nucleotides were bound per enzyme molecule.

These observations have been proposed to reflect a sequential action of the catalytic sites, where the binding of one molecule of dUMP at either site results in minor conformational changes, and renders the second site inaccessible. This corresponds to the observed changes in the fluorescence and UV spectra (Donato *et al.*, 1976; Galivan *et al.*, 1975; Leary *et al.*, 1975). Binding of cofactor causes a much greater conformational change, thus providing access to the second site (Donato *et al.*, 1976; Danenberg *et al.*, 1974; Santi *et al.*, 1974; Manavlan *et al.*, 1986). A second molecule is then bound and the interaction of a second cofactor molecule completes the formation of the ternary complex (Santi & Danenberg, 1984).

1.3.9. Thymidylate Synthase from different sources: the TS family

To date sequences are known from more than 35 different species of TS (Figure 1.13 and 1.14). These include the human enzyme (which is a drug target for anti-cancer agents), thymidylate synthases from protozoa (which are generally bifunctional, with TS and DHFR present in a single protein), and TS from bacterial and viral species. It is thought that TS is encoded by many viruses to support the increased need for DNA

Figure 1.13. Alignment of 35 known TS sequences.

Numbering refers to VZV sequence. Residues shown in bold capitals are highly conserved in all 35 TS sequences. Residues shown in capitals and marked * are completely conserved. β-strands are indicated as ^^^^ and α-helices as ~~~~~~. The assignment of secondary structure is based on TS form *L.casei*. References for most of the TS sequences can be found in Carreras & Santi, 1995. Additional sequences have been isolated from GenBank ™/EMBL databank.

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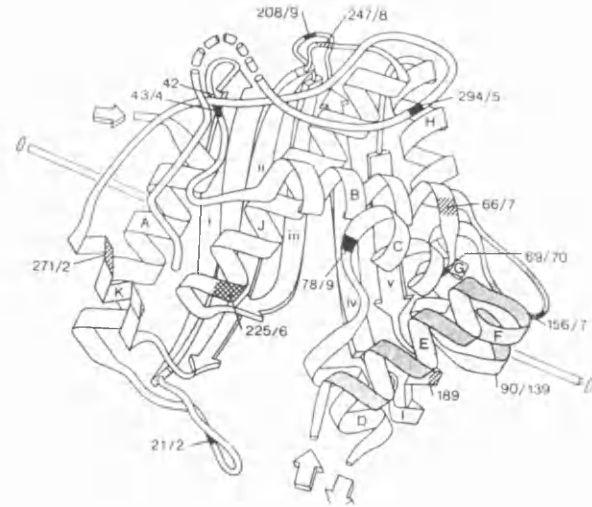
                          17      A      31  -i          37  42     i     54
                          |=============|  |^|           |   |^^^^^^^^^^^^|
VZV                   mgdlscwtkvpgftltgElQYLkqvddIlryGvrkr..........DRTGiGTlSlFG.mqarynLrne
HHV-8                        mvtgaetpheElQYlrqlreIlcrGsdrl..........DRTGiGTlSlFG.mqarysLrdh
EHV-2                             mvthcEhQYLntvreIlanGvrrg..........DRTGvGTlSvFG.dqakysLrgq
HVS                          msthteeqhgEhQYLsqvqhIlnyGsfkn..........DRTGtGTlSiFG.tqsrfsLene
HVA                              meelhaEhQYLsqvkhIlncGnfkh..........DRTGvGTlSvFG.mqsrysLekd
Human        mpvagselprrplppaaqerdaeprpphgElQYLgqiqhIlrcGvrkd..........DRTGtGTlSvFG.mqarysLrde
Mouse        mlvvgsel......qsdaqqlsaeaprhgElQYLrqvehIlrcGfkke..........DRTGtGTlSvFG.mqarysLrde
Rat          mlvegsel......qsgaqqprteapqhgElQYLrqvehImrcGfkke..........DRTGtGTlSvFG.mqarysLrde
S.cerevisiae                 mtmdgknkeEeQYLdlckrIideGefrp..........DRTGtGTlSlFappqlrfsLrdd
C.albicans                    mtvspntaEqaYLdlckrIideGehrp..........DRTGtGTkSlFappqlrfdLsnd
P.carinii                         mvnaeEqQYLnlvqyIinhGedrp..........DRTGtGTlSvFapsplkfsLrnk
C.neoformans        mtatiddqeknqrsnpcheEyQYLdlirrIinvGevrp..........DRTGtGTvalFappsfrfsLadn
A.thalianaX                 NH2-233-heEflYLnmvedIisnGnvkn..........DRTGtGTlSkFG.cqmkfnLrrs.
A.thalianaY                 NH2-232-heEylYLnlvkeIisnGnlkd..........DRTGtGTlSkFG.cqmkfnLrrn.
D.carota                    NH2-241-heEylYLglvenIisnGvtkn..........DRTrtGTvSiFG.cqmrfnLrks.
C.fasiculata                NH2-230-aeErQYLelidrImktGlvke..........DRTGvGTiSlFG.aqmfslrdnq
L.major                     NH2-231-heErQYLelidrImktGivke..........DRTGvGTiSlFG.aqmrfsLrdn
L.amazonensis               NH2-231-heErQYLelidrImktGiake..........DRTGvGTlSlFG.aqmrfsLrdn
L.tropica                   NH2-231-heErQYLelidrImktGivke..........DRTGvTTiSlFG.aqmrfsLrdn
P.chabaudi                  NH2-287-hpEyQYLniiydImhGnkqd..........DRTGvGvlSkFG.ymmkfnLsey
P.falciparum                NH2-322-hpEyQYLniiydImmnGnkqs..........DRTGvGvlSkFG.yimkfdLsqy
T.gondii                    NH2-321-heEfQYLdliadIinnGrtmd..........DRTGvGviSkFG.ctmrysLdqa
T.cruzi                     NH2-234-reEeQYLslvdrIireGnvkh..........DRTGvGTlSiFG.aqmrfsLrnn
E.coli                                mkQYLelmqkvldeGtqkn..........DRTGtGTlSiFG.hqmrfnLqdg
B.subtilisB                           mkQYkdfcrhvlehGekkg..........DRTGtGTiStFG.yqmrfnLreg
B.amyloliquefaciens   mifycvtllkdyfkrmkkmkQYkdlcrhvlenGekkg..........DRTGtGTiStFG.yqmrfnLqgg
S.flexneri                            mkQYLelmqkvldeGtqkn..........DRTGtGTlSiFG.hqmrfnLqdg
H.influenzae                          mkQYLelcrrIvseGewvan.........eRTGkhcltvinadleydvannq
M.genitalium                          mkQYLdlasyvlanGkkrk..........nRTdtdTlSvFG.yqmkfdLtns
L.lactis                      mtyadqvfkqniqnIldnGvfsenarpkyk...DgqmanskyvtG.sfvtydLqkg
L.casei                            mlEqpYLdlakkvldeGhfkp..........DRThtGTySiFG.hqmrfdLskg
S.aureus                      mynpfdeaYhglceeIleiGnrrd..........DRThtGTiSkFG.hqlrfdLtkg
B.subtilisA                   mtqfdkQYnsiikdIinnGisdeefdvrtkwdsDgTpahTlSvis.kqmrfdnse.
Phageφ3T                      mtqfdkQYnsiikdIinnGisdeefdvrtkwdsDgTpahTlSvms.kqmrfdnse.
Phage T4                              mkQYqdlikdIfenGyetd..........DRTGtGTialFG.sklrwdLtkg
  Consensus  ----------------------------E-QYL-----I---G-------------DRTG-GT-S-FG-------L---
                                               *
```



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                           68      B      82   86 C 93        99 D 104
                           |≈≈≈≈≈≈≈≈≈≈≈≈≈≈|    |≈≈≈≈≈≈|         |≈≈≈≈|
VZV                   FPLLTTKrVfwravveELLWFirGsTd..skeLaakd.....ihIWdiygsskflnrngfhkrht...............
HHV-8                 FPLLTTKrVfwrgvvqELLWFlkGsTd..sreLsrtg.....vkIWdkngsreflagrglahrre...............
EHV-2                 FPLLTTKrVfwrgvleELLWFirGsTd..sneLsarg.....vkIWdangsrdflaraglghrep...............
HVS                   FPLLTTKrVfwrgvveELLWFirGsTd..skeLsaag.....vhIWdangsrsfldklgfydrde...............
HVA                   FPLLTTKrVfwrgvveELLWFirGsTd..skeLaasg.....vhIWdangsrsyldklglfdree...............
Human                 FPLLTTKrVfwkgvleELLWFikGsTn..akeLsskg.....vkIWdangsrdfldslgfstree...............
Mouse                 FPLLTTKrVfwkgvleELLWFikGsTn..akeLsskg.....vrIWdangsrdfldslgfsarqe...............
Rat                   FPLLTTKrVfwkgvleELLWFikGsTn..akeLsskg.....vrIWdangsrdfldslgfsarqe...............
S.cerevisiae         tFPLLTTKkVftrgiilELLWFlaGdTd..anlLseqg.....vkIWdgngsreyldkmgfkdrkv...............
C.albicans           tFPLLTTKkVfskgiihELLWFvaGsTd..akiLsekg.....vkIWegngsrefldklglthrre...............
P.carinii            tFPLLTTKrVfirgvieELLWFirGeTd..slkLrekn.....ihIWdangsreyldsigltkrqe...............
C.neoformans         tlPLLTTKrVflrgviaELLWFvsGcTd..akmLssqg.....vgIWdgngskeflekvglghrre...............
A.thalianaX           FPLLTTKrVfwrgvveELLWFisGsTn..akvLqekg.....ihIWdgnasreyldgiglteree...............
A.thalianaY           FPLLTTKrVfwrgvveELLWFisGsTn..akvLqekg.....irIWdgnasrayldgiglteree...............
D.carota              FPLLTTKkVfwrgvveELLWFisGsTn..akiLkekg.....vnIWegngsreyldsigltdree...............
C.fasiculata         lPLLTTKrVfwrgvceELiWFlrGeTn..ahvLadkd.....ihIWdgngsrefldsrgltenke...............
L.major             rlPLLTTKrVfwrgvceELLWFlrGeTs..aqlLadkd.....ihIWdgngsrefldsrgltenke...............
L.amazonensis       rlPLLTTKrVfwrgvceELLWFlrGeTn..aqlLadkd.....ihIWdgngsrefldsrgltente...............
L.tropica           rlPLLTTKrVfwrgvceELLWFlrGeTs..aqlLtdkd.....ihIWdgngsrefldsrgltenke...............
P.chabaudi            FPLLTTKklfvrgiieELLWFirGeTn..gntLlekn.....vrIWeangtrefldnrklfhrev...............
P.falciparum          FPLLTTKklflrgiieELLWFirGeTn..gntLlnkn.....vrIWeangtrefldnrklfhrev...............
T.gondii              FPLLTTKrVfwkgvleELLWFirGdTn..anhLsekg.....vkIWdknvtrefldsrnlphrev...............
T.cruzi             rlPLLTTKrVfwrgvceELLWFlrGeTy..akkLsdkg.....vhIWddngsrrfldsrglteyee...............
E.coli                FPLvTTKrchlrsiihELLWFlqGdTn..iayLhenn.....vtIWdewaden.........................
B.subtilisB           FPmLTTKklhfksiahELLWFlkGdTn..vryLqeng.....vrIWnewaden.........................
B.amyloliquefaciens   FPmLTTKklhfksiahELLWFlkGdTn..vryLqeng.....vrIWnewaden.........................
S.flexneri            FPLvTTKrchlrsiihELLWFlqGdTn..igyLhenn.....vtIWddwaden.........................
H.infuenzae           FPLiTTrksywkaaiaEfLgyirGydn..aadfralg.....tktWdananenaawlanphrrgv...............
M.genitalium          FPLLTTKkVnwkaivhELLWFikGdTn..ikyLvdng.....vnIWnewpyenfkkspsfqnetlqefilkvktdnefak
L.lactis             eFPitTlrpipiksaikELmWiyqdqTs.elsvLeeky.....gvkyWgewgig...........................
L.casei               FPLLTTKkVpfgliksELLWFlhGdTn..irfLlqhr.....nhIWdewafekwvksdeyhgpdmtdfghrsqkdpefaa
S.aureus              FPLLTTKkVsfklvatELLWFikGdTn..iqyLlkyn.....nnIWnewafenyvqsddyhgpdmtdfghrsqqdpefne
B.subtilisA           vPLLTTKkVawktaikELLWiwqlksn..dvndLnmmg....v.IWdqwkqed...........................
PhageΦ3T              vPiLTTKkVawktaikELLWiwqlksn..dvteLnkmg....vhIWdqwkqed...........................
Phage T4              FPavTTKklawkaciaELiWFlsGsTnvndlrlLqhdsliqgktvWdenyenqakdlgyhs...................
    Consensus    -----FPLLTTK-V-------ELLWF--G-T------L----------IW-------------------------------
                      * *             *                          *
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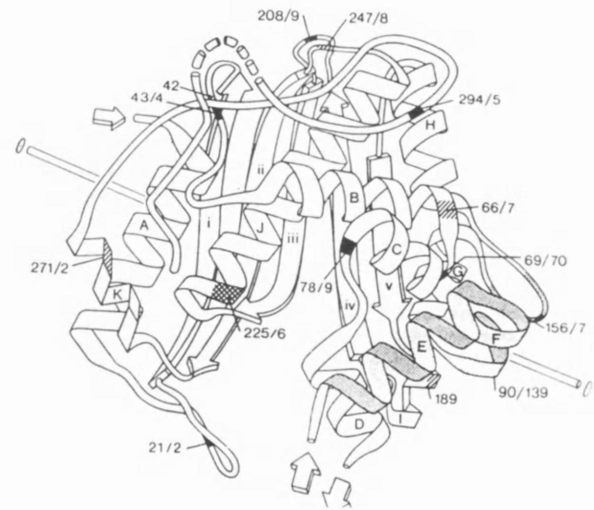
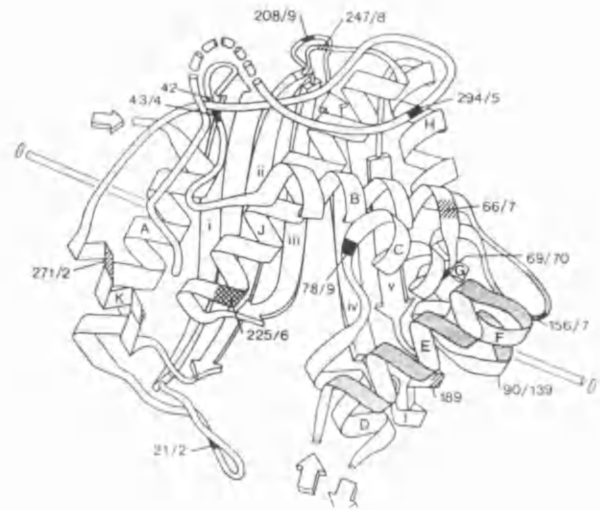


```
                             122 G 127                 148   H   159  165 v   173
                               |====|                    |=========|   |^^^^|  |==
VZV                          GDLGPiYGfQWRhfgaeykdcqsnylqqgiDQLqtvIdtiKtnPesRRmiissWNpkD
HHV-8                        GDLGPvYGfQWRhfgaayvdadadytgqgfDQLsyivdliKnnPhdRRiimcaWNpad
EHV-2                        GDLGPvYGfQWRhfgaayvdsktdyrgqgvDQLrdlIgeiKrnPesRRlvltaWNpaD
HVS                          GDLGPvYGfQWRhfgaeykgvgrdykgegvDQLkqlIdtiKtnPtdRRmlmcaWNvsD
HVA                          GDLGPvYGfQWRhfgaeyqglkhnyggegvDQLkqiIntihtnPtdRRmlmcaWNvlD
Human                        GDLGPvYGfQWRhfgaeyrdmesdysgqgvDQLqrvIdtiKtnPddRRiimcaWNprD
Mouse                        GDLGPvYGfQWRhfgaeykdmdsdysgqgvDQLqkvIdtiKtnPddRRiimcaWNpkD
Rat                          GDLGPvYGfQWRhfgadykdmdsdysgqgvDQLqkvIdtiKtnPddRRiimcaWNpkD
S.cerevisiae                 GDLGPvYGfQWRhfgakyktcdddytgqgiDQLkqvIhklKtnPydRRiimsaWNpaD
C.albicans                   GDLGPvYGfQWRhfgaeykdcdsdytgqgfDQLqdvIkklKtnPydRRiimsaWNppD
P.carinii                    GDLGPiYGfQWRhfgaeyidcktnyigqgvDQLaniIqkirtsPydRRlilsaWNpaD
C.neoformans                 GdLGPvYGfQWRhfgaeytdadgdykgkgvDQLqrvIdtiKnnPtdRRiilsaWNpkd
A.thalianaX                  GDLGPvYGfQWRhfgakytdmhadytgqgfDQLvdvIdkiKnnPddRRiimsaWNpsD
A.thalianaY                  GDLGPvYGfQWRhfgakytdmhadytgqgfDQLldvInkiKnnPddRRiimsaWNpsD
D.carota                     GDLGPiYGfQWRhfgarytdmhadysgqgfDQLldvIskiKnnPddRRiiqsaWNpsD
C.fasiculata                 mDLGPvYGfQWRhfgadykgfdany.degvDQiktivetlKtn..dRRllvtaWNpca
L.major                      mDLGPvYGfQWRhfgadykgfeanydgegvDQiklivetiKtnPndRRllvtaWNpca
L.amazonensis                mDLGPvYGfQWRhfgaeyrgleanydgegvDQikfivetiKanPndRRllftaWNpca
L.tropica                    mDLGPvYGfQWRhfgadykgfeanydgegvDQiklivetiKtnPndRRllvtaWNpca
P.chabaudi                   nDLGPiYGfQWRhfgaeytdmhadykdkgvDQLkniInliKndPtcRRiilcaWNvkD
P.falciparum                 nDLGPiYGfQWRhfgaeytnmydnyenkgvDQLkniInliKndPtsRRillcaWNvkD
T.gondii                     GDiGPgYGfQWRhfgaaykdmhtdytgqgvDQLknvIqmlrtnPtdRRmlmtaWNpaa
T.cruzi                      mDLGPvYGfQWRhfgaaythhdanydgqgvDQikaivetlKtnPddRRmlftaWNpsa
E.coli                       GDLGPvYGkQWRawp........tpdgrhiDQittvlnqlKndPdsRRiivsaWNvge
B.subtilisB                  GeLGPvYGsQWRswr........gadgetiDQisrlIediKtnPnsRRlivsaWNvge
B.amyloliquefaciens          GeLGPvYGsQWRswr........gadgetiDQisrlIhdiKtnPnsRRlivsaWNvge
S.flexneri                   GDLGPvfGkQWRawp........tpdgrhiDQittvlnqlKndPdsRRiivsaWNvge
H.influenzae                 dDmGrvYGvQgRawr........kpngetiDQLrkivnnltkgiddRgeiltffNpge
M.genitalium              qfaDLG.vYGkQWRnfn.............gvDQLkkvIqeiKenPnsRRlivssWNpse
L.lactis                     GtiGqrYGatvkky...............niigklleglaknPwnRRniinlWqyeD
L.casei  vyheemakfddrvlhddafaakyGDLGlvYGsQWRawh........tskgdtiDQLgdvIeqiKthPysRRlivsaWNpeD
S.aureus qykeemkkfkerilnddafakkyGnLGnvYGkQWRdwed........kngnhyDQLksvIqqiKtnPnsRRhivsaWNpte
B.subtilisA                  GtiGhaYGfQlgkkn.......rslngekvDQvdyllhqlKnnPssRRhitmlWNpde
Phageφ3t                     GtiGhaYGfQlgkkn.......rslngekvDQvdyllhqlKnnPssRRhitmlWNpdD
Phage T4                     GeLGPiYGkQWRdfg.............gvDQiievIdriKklPndRRqivsaWNpae
     Consensus    --------------------GDLGP-YG-QWR-----------------DQL---I---K--P--RR-----WN--D
                                        *   *                              *
```



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                               I   183  iv   192                   195  iii     207       J        229    235
                             ≈≈≈≈≈≈|    |^^^^^^^^^|                |^^^^^^^^^^| |≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈≈|    |^^^^
VZV                           ip.lMvLPPCHtlcQFYVang.................eLScqvYQRSgDMgLGVPFNIAgYaLLTyivAhvtglktGdli
HHV-8                         ls.lMALPPCHllcQFYVadg.................eLScqlYQRSgDMgLGVPFNIAsYsLLTymlAhvtglrpGeFi
EHV-2                         lp.aMALPPCHllcQFYVagg.................eLScqlYQRSgDMgLGVPFNIAsYsLLTymvAhltglepGdFi
HVS                           ip.kMvLPPCHvlsQFYVcdg.................kLScqlYQRSaDMgLGVPFNIAsYsLLTcmiAhvtnlvpGeFi
HVA                           vp.kMALPPCHvlsQFYVcdg.................kLScqlYQRSaDMgLGVPFNIAsYsLLTcmiAhvtdlvpGeFi
Human                         lp.lMALPPCHalcQFYVvns.................eLScqlYQRSgDMgLGVPFNIAsYaLLTymiAhitglkpGdFi
Mouse                         lp.lMALPPCHalcQFYVvng.................eLScqlYQRSgDMgLGVPFNIAsYaLLTymiAhitglqpGdFv
Rat                           lp.lMALPPCHalcQFYVvng.................eLScqlYQRSgDMgLGVPFNIAsYaLLTymiAhitglqpGdFv
S.cerevisiae                  fd.kMALPPCHifsQFYVsfpkeg........egsgkprLScllYQRScDMgLGVPFNIAsYaLLTrmiAkvvdmepGeFi
C.albicans                    fa.kMALPPCHvfcQFYVnfptsspdpnnpkqaktakpkLScllYQRScDMgLGVPFNIAsYaLLTkmiAhvvdmdcGeFi
P.carinii                     le.kMALPPCHmfcQFYVhips...........nnhrpeLScqlYQRScDMgLGVPFNIAsYaLLTcmiAhvcdldpGdFi
C.neoformans                  lp.lMALPPCHmfcQFfVslppadspgskp........kLSclmYQRScDlgLGVPFNiasYaLLThmiAlitdtepheFi
A.thalianaX                   lk.lMALPPCHmfaQFYVaeg.................eLScqmYQRSaDMgLGVPFNIAsYsLLTcmlAhvcdlvpGdFi
A.thalianaY                   lk.lMALPPCHmfaQFYVang.................eLScqmYQRSaDMgLGVPFNIAsYsLLTcilAhvcdlvpGdFi
D.carota                      lr.lMALPPCHmfaQFYVang.................eLScqmYQRSaDMgLGVPFNIAaYaLLTcmiAhvcdlvpGdFv
C.fasiculata                  lh.kMAvrPCHllgQFYVntqtk...............eLScmlYQRccDMgLGVPFNIAsYaLLTiliAkatglrpGelv
L.major                       lq.kMALPPCHllaQFYVntdts...............eLScmlYQRScDMgLGVPFNIAsYaLLTiliAkatglrpGelv
L.amazonensis                 lh.kMALPPCHllaQFYVnteks...............eLScmlYQRScDMgLGVPFNIAsYaLLTiliAkatglrpGelv
L.tropica                     lq.kMAvPPCHllaQFYVntdts...............eLScmlYQRScDMgLGVPFNIAsYaLLTiliAkatglrpGelv
P.chabaudi                    ld.gMALPPCHilcQFYVfdg.................kLScimYQRScDlgLGVPFNIAsYsifTymiAqvcnlqpaeFi
P.falciparum                     gMALPPCHilcQFYVfdg.................kLScimYQRScDlgLGVPFNIAsYsifThmiAqvcnlqpaqFi
T.gondii                      ld.eMALPPCHllcQFYVndqk................eLScimYQRScDvgLGVPFNIAsYsLLTlmvAhvcnlkpkeFi
E.coli                        ld.kMALaPCHaffQFYVadg.................kLScqlYQRScDvfLGlPFNIAsYaLLvhmmAqqcdlevGdFv
B.subtilisB                   id.kMALPPCHclfQFYVsdg.................kLScqlYQRSaDvfLGVPFNIAsYaLLTmiiAhvtglepGeFi
B.amyloliquefaciens           id.rMALPPCHclfQFYVadg.................kLScqlYQRSaDvfLGVPFNIAsYaLLTmmiAhvtglepGeFv
S.flexneri                    ld.kMALaPCHaffQFYVadg.................kLScqlYQRScDvfLGlPFNIAsYtLLvhmmAqqcdlevGdFv
H.influenzae                  fd.lgcLrPCmhthtFslgd..................tLhltsYQRScDvpLGlnFNqiqvftflalmAqitgkkaGkay
M.genitalium                  le.kMALaPCHslfQFYVeed.................kLSlqlYQRSgDifLGVPFNIAsYaLLvylvAhetklkpGyFi
L.lactis                      feetegLlPCafqtmfdvrrekdgqi............yLdatliQRSnDMlvahhiNamqYvaLqmmiAkhfswkvGkFf
L.casei                       vp.tMALPPCHtlyQFYVndg.................kLSlqlYQRSaDifLGVPFNIAsYaLLThlvAhecglevGeFi
S.aureus                      id.sMALPPCHtmfQFYVqeg.................kLncqlYQRSaDifLGVPFNIAsYaLLThlvAkecglevGeFi
B.subtilisA                   ld.aMALtPCvyetQwYVkhg.................kLhlevraRSnDMaLGnPFNvfqYnvLqrmiAqvtgyelGeyi
Phageϕ3t                      ld.aMALtPCvyetQwYVkqg.................kLhlevraRSnDMaLGnPFNvfqYnvLqrmiAqvtgyelGeyi
Phage T4                      lk.yMALPPCHmfyQFnVrng.................yLdlqwYQRSvDvfLGlPFNIAsYatLvhivAkmcnlipGdli
     Consensus              ------MALPPCH---QFYV--------------------LS---YQRS-DM-LGVPFNIA-Y-LLT---A-------G-F-
                                   **                                *        *  *        *            *
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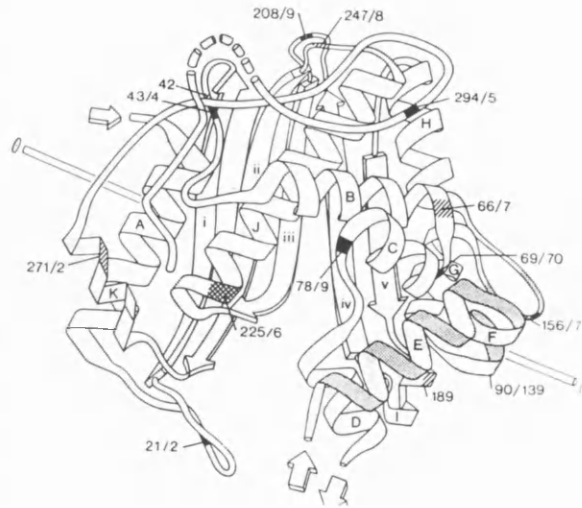

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              ii  248      K  258                                    283   289        301
          ^^^^^^^^^^^^^|   |=====|                                    |     |          |
VZV           HtmGDaHiYlnHidalkv.QLaRsPkpf.PcLkiirnvt...........dIndFkwdDfqldgYnP.hpplkmemal
HHV-8         HtlGDaHiYktHieplrl.QLtRtPrpf.PrLeilrsvs...........smeeFtpdDfrlvdYcp.hptIrmemav
EHV-2         HvlGDaHvYlnHveplkl.QLtRsPrpf.PrLrilrrve...........dIddFraeDfalegYhP.haaIpmemav
HVS           HtiGDaHiYvdHidalkm.QLtRtPrpf.PtLrfarnvs...........cIddFkadDiilenYnP.hpiIkmhmav
HVA           HtlGDaHvYvnHvdalte.QLtRtPrpf.PtLkfarkva...........sIddFkanDiilenYnP.ypsIkmpmav
Human         HtlGDaHiYlnHieplki.QLqRePrpf.PkLrilrkve...........kIddFkaeDfqiegYnP.hptIkmemav
Mouse         HtlGDaHiYlnHieplki.QLqRePrpf.PkLkilrkve...........tIddFkveDfqiegYnP.hptIkmemav
Rat           HtlGDaHiYlnHieplki.QLqRePrpf.PkLrilrkve...........tIddFkveDfqiegYnP.hptIkmemav
S.cerevisiae  HtlGDaHvYkdHidalke.QitRnPrpf.PkLkikrdvk...........dIddFkltDfeiedYnP.hprIqmkmsv
C.albicans    HtlGDaHvYldHidalke.QfeRiPkqf.PkLvikeerk.......neiksIddFkfeDfeivgYeP.yppIkmkmsv
P.carinii     HvmGDcHiYkdHiealqq.QLtRsPrpf.PtLslnrsit...........dIedFtldDfniqnYhP.yetIkmkmsi
C.neoformans  lqmGDaHvYrdHveplkt.QLeRePrdf.PkLkwarske........eigdIdgFkveDfvvegYkP.wgkIdmkmsa
A.thalianaX   HvlGDaHvYktHvrplqe.QLlnpPkpf.PvLkinpekk...........qIdsFvasDfdltgYdP.hkkIemkmav
A.thalianaY   HviGDaHvYknHvrplqe.QLenpPkpf.PvLkinpekk...........dIdsFvadDfeligYdP.hkkIdmkmav
D.carota      HsiGDaHvYsnHlsdlfetsfrmlPktf.PvLkinsgek...........dIdsFeaaDfkligYdP.hqkIemkmav
C.fasiculata  HtlGtaHvYsnHvealke.QLqRvPvaf.PvLvfkkere...........fledyestDmevvdYvP.yppIkmemav
L.major       HtlGDaHvYrnHvdalka.QLeRvPhaf.PtLifkkeerq...........yledyeltDmevidYvP.hpaIkmemav
L.amazonensis HtlGDaHvYrsHidalka.QLeRvPhaf.PtLvfkkeerq...........fledyelmDmevidYvP.hppIkmemav
L.tropica     HtlGDaHvYrnHvdalka.QLeRvPhaf.PtLifkkeerq...........yledyeltDmevidYvP.hpaIkmemav
P.chabaudi    HviGnaHvYnnHveslkv.QLnRtPypf.PtLklnpeik...........nIedFtisDftvqnYvh.hdkIsmdmaa
P.falciparum  HvlGnaHvYnnHidslki.QLnRiPypf.PtLklnpdik...........nIedFtisDftiqnYvh.hekIsmdmaa
T.gondii      HfmGntHvYtnHvealke.QLrRePrpf.Pivnilnker.........ikeIddFtaeDfevvgYvP.hgrIqmemav
T.cruzi       HtlGDaHvYsnHvepcne.QLkRvPraf.PyLvfrrere...........fledyeegDmevidYaP.yppIsmkmav
E.coli        wtgGDtHlYsnHmdqthl.QLsRePrpl.PkLiikrkpe...........sIfdyrfeDfeiegYdP.hpgIkapvai
B.subtilisB   HtfGDvHiYqnHieqvnl.QLeRdvrpl.PqLrfarkvd...........sIfnFafeDfiiedYdP.hphIkgavsv
B.amyloliquef HtfGDvHiYqnHveqvnl.QLtRdvrpl.PkLrfarnvd...........sIfdFafeDfiiedYdP.hphIkgavsv
S.flexneri    wtgGDtHlYtnHmdqthl.QLsRePrpl.PkLiikrkpe...........sIfdyrfeDfeiegYdP.hpgIkapvai
H.influenzae  HkivnaHiYedqlelmrdvQLkRePfpl.PkLeinpdik........tledletwvtmdDfkvvgYqs.hepIkypfsv
M.genitalium  HtlGDaHiYenHieqikl.QLtRttldp.Pqvvlksdks............IfaysfdDielvgYny.hpfIygrvav
L.lactis      yfvnnlHiYdnqfeqane.lmkRtasekePrLvlnvpdg..........tnffdikpeDfelvdYePvkpqlkfdlai
L.casei       HtfGDaHlYvnHldqike.QLsRtPrpa.PtLqlnpdkh...........dIfdFdmkDikllnYdP.ypaIkapvav
S.aureus      HtfGDaHiYsnHmdaiht.QLsRdsylp.PqLkintdks............IfdinyeDlelinYes.hpaIkapiav
B.subtilisA   fniGDcHvYtrHidnlki.QmeReqfea.PeLwinpevk...........dfydFtidDfklinYkh.gdkllfevav
Phageφ3t      fniGDcHvYtrHidnlki.QmeReqfea.PeLwinpevk...........dfynFtvdDfklinYkh.gdkllfevav
Phage T4      fsgGntHiYmnHveqcke.iLrRePkel.ceLvisglpykfrylstkeqlkyvlklrpkDfvlnnYvs.hppIkgkmav
    Consensus H--GD-H-Y--H-------QL-R-P----P-L-------------------I--F---D-----Y-P----I------
                  * *                                                 *     *
```
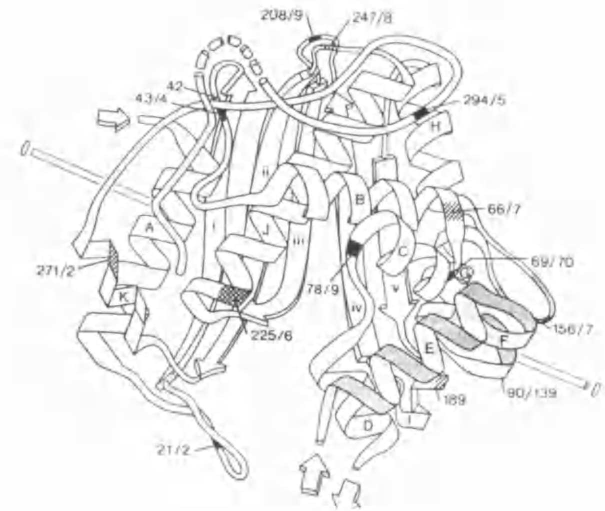



Herpesvirus
Varicella-zoster virus
Human herpesvirus 8
Herpesvirus saimiri
Herpesvirus ateles
Equine herpesvirus 2

Mammals
Human
Mouse
Rat

Yeast
Saccharomyces cerevisiae
Candida albicans

Fungi
Pneumocystis carinii
Cryptococcus neoformans

Plant
Arabidopsis thaliana X
Arabidopsis thaliana Y
Daucas carota

Protozoa
Leishmania major
Leishmania amazonesis
Leishmania tropica
Plasmodium chabaudi
Plasmodium falciparum
Toxoplasma gondi
Toxoplasma cruzi

Bacteria
Escherichia coli
Bacillus subtilis B
Bacillus amyloliquefaciens
Shigella flexneri
Haemophilus influenzae
Mycoplasma genitalium
Lactococcus lactis
Lactobacillus casei
Staphylococcus aureus
Bacillus subtilis A

Phage
Bacteriophage ϕ 3T
Bacteriophage T4

Figure 1.14. The TS family

synthesis during the viral life cycle although it is by no means widespread among large DNA viruses. There are only a few organisms that can adequately scavenge thymidylate from their environment and do not require TS, examples are the intracellular mycoplasmas.

A striking feature of this enzyme is the very high degree of sequence conservation between organisms. Approximately 10% (27 from a core of 275) of residues are completely conserved and a further 165 (60%) are greater than 80% conserved, thus placing TS amongst the most highly conserved enzymes (Perry *et al.*, 1990). This contrasts with dihydrofolate reductase, the enzyme which regenerates the folate cofactor oxidised by TS. Although amino acid sequences of DHFR have been reported from a smaller range of organisms, only 6% of residues (11 from a core of 170) are completely conserved with a further 41 residues (24%) being greater than 80% conserved (Lagosky *et al.*, 1987).

Examination of the 35 sequences shows a lack of alignment at the N-terminus and the bifunctional enzymes have an N-terminal DHFR domain. By contrast the C-terminal regions precisely align in all species, indicating the importance of this region. In all TS sequences there is a fully conserved proline and cytosine residue in the active site reflecting the requirement for orientation of the cysteine side-chain (Figure 1.13).

Generally the regions where there are extra amino acids fall outside the regions of secondary structure and do not upset α-helices or β-sheets. There are however three exceptions: a single amino acid insertion is present in β-strand i of the yeast enzymes, there is an insertion in helix I of *L.lactis* and in helix K of *H.influenzae*. In addition long insertions are present in *L.casei* and *S.aureus* as helices, termed helix E and helix F. Between helix G and helix H all eukaryotes have an insertion, this insertion is not present in the prokaryotic enzymes and may represent a eukaryotic-specific function. Inspection of Figure 1.13 reveals that the sequence of the insertion is relatively well conserved.

In the TS line-up *H.influenzae* and *M.genitalium* are often the sole divergent sequence at an otherwise highly or fully conserved position (an example is Asp of the DRTG loop). It is noteworthy that both sequences came from genomic sequencing projects using shot-gun sequence approaches rather than from sequencing of cloned and characterised TS genes. So it is possible that the error rate in these sequences is high.

The high degree of sequence conservation seen at the level of primary structure of TS is also evident at the tertiary structure level. A comparison of the 3-D structure of the native enzyme from *E.coli* and *L.casei* showed that they are 89% conserved in terms of corresponding atom types (3824 common atoms out of 4300; *E.coli* TS), despite sharing only 60% amino acid identity. While the high conservation of TS sequences is clearly related to the chemical reaction catalysed, it may also be linked to the interaction with other enzymes and to conserved conformational dynamics upon substrate binding.

1.3.9.1. Bacteria

The first studies of TS were performed on the enzyme isolated from *L.casei* (Friedkin *et al.*, 1962; Blakley., 1963). The isolation of a methotrexate resistant *L.casei* strain, that over-produced TS and DHFR meant that large amounts of highly purified enzyme were available for study (Crusberg *et al.*, 1970) and has resulted in the *L.casei* enzyme becoming the most intensively studied TS from all sources (Santi & Danenberg, 1984). In this organism the TS gene appears to be closely linked to DHFR on the chromosome with the TS sequence preceding that of DHFR.

In *B.subtilis* and *S.aureus* the genes encoding TS and DHFR are also closely linked. *B.subtilis* has two TS loci, *thyA* and *thyB* (Piggot & Hoch, 1985). *ThyB* gene encodes a heat-sensitive form of TS (Neuhard *et al.*, 1978) and can complement growth of both an *E.coli thyA* strain (Rubin *et al.*, 1978) and a *B.subtilis thyA thyB* strain (Iwakura *et al.*, 1988). The termination codon of *thyB* overlaps the initiation codon of *dfrA* (Iwakura *et al.*, 1988) by one nucleotide. Both genes are transcribed from the same promoter located upstream of *thyB* indicating that the genes belong to a single operon.

The nucleotide sequence of the gene for DHFR and the partial sequence of the gene for TS of *S.aureus* (Burdeska *et al.*, 1990) are identical to the complete nucleotide sequences of the genes for TS and DHFR in Tn4003, a transposon that confers resistance to trimethoprim (a DHFR inhibitor) in *S.aureus* (Rouch *et al.*, 1989). In both cases the genes are separated by only 44 nucleotides on the same strand suggesting they form part of the same operon.

By way of contrast the genes for TS (*thy A*) and DHFR (*frd*) are widely separated on the *E.coli* chromosome (Bachmann, 1983). However the two enzymes have been proposed to form part of a multienzyme complex that is responsible for channelling newly synthesised dNTPs into replicating DNA (Matthews *et al.*, 1978; 1988). Comparison of the *E.coli* and *L.casei* TS sequences reveals consistent homology over extensive regions. However a dramatic difference between the two sequences is reflected by the presence of a 51 amino acid stretch, present midway through the *L.casei* sequence which is completely absent from the *E.coli* enzyme (see Figure 1.13).

The gene coding for TS in *Lactococcus lactis* can complement the growth of an *E.coli thyA* strain, and its predicted amino acid sequence is the most widely diverged TS sequence known (Ross *et al.*, 1990a). This gene has been proposed as a safe selectable marker (an alternative to antibiotic resistance markers) for use in the generation of strains whose ultimate fate will be in food systems or for release into the environment (Ross *et al.*, 1990b).

1.3.9.2. Bacteriophage

Within the phage T4 genome the DHFR and TS coding sequences are adjacent and are arranged so that the termination codon of DHFR gene (located on the 5' side of the TS gene) overlaps the initiation codon of the TS gene. The two enzymes form part of a multi-enzyme aggregate, comprising both T4 and *E.coli* proteins and containing several more enzymes involved in dNTP synthesis which include dCMP hydroxymethylase, dCTPase-dUTPase and ribonucleotide reductase (Moen *et al.*, 1988). This complex is termed the dNTP synthetase complex and produces dNTPs in a manner tightly co-ordinated with DNA replication (Allen *et al.*, 1980; Chiu *et al.*, 1982; Matthews *et al.*, 1988; Moen *et al.*, 1988). Protein-protein interactions have been explored between two enzymes of the complex, deoxycytidylate deaminase and thymidylate synthase. These enzymes are functionally associated with one another since they catalyse sequential reactions. Experimental evidence has revealed that physical interactions occur between the enzymes in the presence or absence of other proteins and require one of two zinc binding sites which are present on the dCMP deaminase (McGaughey *et al.*, 1996).

In addition to their catalytic roles, TS and DHFR act as structural components of the T4 virion base plate (Capco *et al.*, 1973) an example of enzymes with a stable protein fold being recruited into a structural role. Analysis of mutants that lack both TS and DHFR has confirmed their role as components of the T4 virion, and also shows that these two genes are not essential for bacteriophage T4 viability (Wang & Matthews, 1989).

Phage Ø3T is a large temperate *Bacillus subtilis* phage which can complement Thy^- auxotrophs upon lysogeny since it encodes TS, the product of the *thy*P3 gene (Tucker, 1969). The *thy*P3 gene has been cloned, is functional in *E.coli* (Duncan *et al.*, 1977; Ehrlich *et al.*, 1976). The phage integrates into the *B.subtilis* chromosome by virtue of its homology to host *thyA* and flanking sequences to other regions of the *B.subtilis* genome.

1.3.9.3. Protozoa

TS and DHFR are distinct, monofunctional enzymes in most organisms. Generally TS is a dimer of identical subunits of about 35kDa each, and DHFR is a monomer of about 20kDa. In contrast, in protozoa TS and DHFR exist as a bifunctional protein (Bzik *et al.*, 1987; Ferone & Roland, 1980; Garrett *et el.*, 1984; Grumont *et al.*, 1986; Hughes *et al.*, 1989; Meek *et al.*, 1985). Both enzyme activities exist on a single polypeptide chain with the DHFR domain on the amino terminus and TS on the carboxy terminus. All bifunctional TSs have this arrangement suggesting the importance of a free C-terminus of TS for the enzyme reaction. The native protein is a dimer of two such subunits in the size range of 110-140kDa (Ivanetich & Santi, 1990).

A model has been constructed that describes the higher-order structure and function of the bifunctional protein (Grumont *et al.*, 1986). The dimeric structure of this enzyme probably involves important intermolecular contacts between the TS domains of each subunit; this is based on the high homology of the TS domain of TS-DHFR with other TSs, and the fact that all monofunctional TSs exist as stable dimers (Hardy *et al.*, 1987). Further, there is communication between the two TS domains, since a single proteolytic cleavage of one also destroys activity of the other without disruption of the subunit structure. The substrate dUMP is capable of forming a binary complex containing only one mole of dUMP per TS-DHFR dimer, indicating an interaction of the subunits (Garvey & Santi, 1987). In addition, the DHFR domains have been found to functionally communicate with each other as binding of methotrexate to one domain prevents binding to the other. Since elements of higher-order structure are conserved in the DHFR domain of TS-DHFR, it was suggested that the interactions between the DHFR domains contribute to dimer stability (Grumont *et al.*, 1986). Finally, a functional TS-DHFR interaction is implied by the substrate channelling of H_2folate from the active site of TS to that of DHFR as seen with *Leishmania tropica* (Meek *et al.*, 1985).

The TS-DHFR interaction provides the enzyme with a biological advantage as the H_2folate produced by the TS is channelled to DHFR faster than it is released into the medium (Meek *et al.*, 1985). Consequently the net rate of the sequential reactions is governed by the rate of TS, there is no accumulation of inhibitory H_2 folate and there is no depletion of H_4 folates, the bifunctional protein seems to be optimised for continued dTMP synthesis. Furthermore, the bifunctional DHFR-TS of Leishmania exhibits disparate binding of the usually stoichometric ligand methotrexate, with 0.5mol bound per mol of DHFR-TS subunit (Ferone & Roland, 1980; Meek *et al.*, 1985). It has also been shown that the DHFR and TS domains may be separately inhibited by agents selective for one activity or the other, suggesting that the two domains retain a level of independence (Ferone & Roland, 1980; Meek *et al.*, 1985).

1.3.9.4. Yeast

In the yeast *S.cerevisiae*, as well as in some other fungi, TS provides the only source of dTMP for replication. *S.cerevisiae* lacks the TK-mediated pathway (Grivell & Jackson, 1968) and the cell membrane is impermeable to phosphorylated thymine derivatives including dTMP. dTMP starvation in *S.cerevisiae* is lethal and induces DNA strand breakage, mitotic crossing-over, gene conversion and unequal sister chromatid exchange.

TS fluctuates periodically during the *S. cerevisiae* cell cycle (Storms *et al.*, 1984). The enzyme accumulates during S-period, peaks in mid to late S-phase and then drops suggesting that both periodic synthesis and instability of the enzyme contribute to the

activity profile. These observations imply that TS is precisely regulated and may represent one of the rare proteins in yeast whose expression is cell cycle dependent. A series of cell division cycle (cdc) mutants have ben produced which block *S.cerevisiae* at different stages of the cell cycle. cdc21 is the thymidylate synthase mutant (Storms *et al.*, 1984).

TS is thought to be part of a multienzyme complex in T4 phage (Chiu *et al.*, 1992), mammalian cells (Reddy & Pardee, 1980), and other cell systems. In yeast, a similar multiprotein complex has been described (Jazwinski & Edelman, 1984), however all components of this complex have not been identified. The introduction of a large insertion into the TS gene of *S.cerevisiae* has no effect on viability other than to create a thymidylate auxotroph phenotype which requires a second mutation to provide a dTMP uptake pathway, implying that if TS is part of the replication complex in yeast it is not a required structural component (Taylor *et al.*, 1987).

1.3.9.5. Herpesviruses

Varicella-zoster virus (Thompson *et al.*, 1987), herpesvirus saimiri (Honess *et al.*, 1986), herpesvirus ateles (Richter *et al.*, 1988), equine herpevirus 2 (Telford *et al.*, 1995) and HHV-8 are the only herpesviruses shown to date to have a TS gene (Moore *et al.*, 1996). The viruses belong to different subfamilies of the herpesviruses and this gene is apparently absent from other members of each subfamily.

A homology search of proteins predicted from completed DNA sequences of VZV revealed that the product of gene 13 was highly homologous to eukaryotic and prokaryotic TSs. Published sequences of EBV, a member of the gammaherpesvirinae, clearly contains no TS homologue (Baer *et al.*, 1984) and Honess *et al.*, (1986) were unable to detect novel TS activities in cells infected with HSV-1 or PRV, members of the alphaherpesvirinae. The origins of the VZV and HVS TS genes remain uncertain as there is no method to determine unambiguously whether the TS gene is an ancestral feature lost during divergence to herpesvirus subfamilies, or whether it has been acquired after divergence.

In addition to a TS, VZV and HVS specify a TK (Davison & Scott, 1984) which catalyses the phosphorylation of thymidine to thymidylate. Thus these viruses supplement cellular pathways for providing thymidylate *de novo* (using TS) and by thymidine salvage (TK). Little is known about the expression of virus TK and TS genes, but it seems plausible that the two enzymes are used at different stages of pathogenesis to provide thymidylate for DNA synthesis. The viability of TK-negative mutants of HSV-1 in tissue culture (Shiraki *et al.*, 1983; Dubbs & Kitt, 1974) indicates that a virus-coded function for the provision of thymidylate is not required, and therefore suggests that VZV TS may also be dispensable. The generation of VZV mutants from cosmid DNAs has shown that VZV

TS is not essential for replication *in vitro* (Cohen & Seidel, 1993) but it remains to be seen whether a similar situation occurs *in vivo.*

1.3.9.6. Plants

Dihydrofolate reductase has been purified and characterised from soybean seedlings (Reddy & Rao, 1976), domesticated carrot (Albani *et al.*, 1985) *Scenedesmus obliquus* (Backmann & Follmann., 1987) and wild carrot (Toth *et al*, 1987).

Analysis of TS from domesticated carrot *Daucas carota* revealed that TS copurified with DHFR and both activities migrated in non-denaturing PAGE as a band of 183kDa (Cella *et al.*, 1988). In mutant cell lines overproducing DHFR, TS activity paralleled DHFR increase. The ratio between TS and DHFR activities was constant during enzyme purification except for a sharp decrease of TS activity during the last step, possibly due to the extreme instability of TS (Cella *et al.*, 1988). Evidence of the bifunctionality of one of the enzymes has been strengthened by the sequencing of two oligopeptides, obtained by the tryptic digestion of the pure protein, which contain sequences similar to those found in the active sites of TS and DHFR (Cella *et al.*, 1991a). Therefore it was concluded that in domesticated carrot TS and DHFR exist as a bifunctional enzyme (Cell & Parisi 1993), similar to, albeit larger than those found in protozoa (Ivanetich & Santi, 1987).

A different situation with regard to DHFR and TS was found by Toth and colleagues (1987) in wild carrot suspension and cultured cells. In these cells TS and DHFR activities were localised on two different polypeptides which are part of an enzymatic complex formed by five different subunits. Further examination of wild carrot suspension by Balestrazzi and colleagues (1995) has shown that in addition to the monofunctional DHFR a bifunctional TS-DHFR is present. A DHFR-TS gene has recently been cloned from *Arabidopsis thaliana* (Lazar *et al.*, 1993) and immunochemical analysis has revealed the precence of a bifunctional DHFR-TS in *Nicotiana plumbaginifolia* and *Oryza sativa* (Balestrazzi *et al.*, 1995) indicating that a bifunctional protein may well be ubiquitous in plant cells.

In the case of the alga *Scenedesmus obliquus;* Bachmann and Folman (1987) copurified from a 30-fold overproducing strain DHFR and TS activities as a bifunctional protein. This bifunctional enzyme consists of two identical subunits of molecular mass of about 100kDa.

1.3.9.7. Mammals

Many studies have been carried out on the regulation of TS expression in mammalian cells. Initial studies focused on cell-cycle-directed events, these studies have shown that the maximal TS activity occurs during periods of active DNA synthesis

(Conrad, 1971; Navalgund *et al.*, 1980). In human, mouse and chinese hamster, TS enzyme and mRNA levels are low in quiescent cells but increase approximately 20-fold on stimulation to re-enter the cell cycle (Ayusawa *et al.*, 1986; Jenh *et al.*, 1985; Conrad & Ruddle, 1972). Moreover, the increase in TS enzyme levels that arises as cells enter S-phase appears to be regulated at both the transcriptional and post-transcriptional levels. Takeishi *et al.*, (1985) suggested the possibility of translational regulation of TS expression given the theoretical potential of three interconvertible secondary structures, each containing a stem loop structure in the 5' untranslated region (5' UTR) of the human TS mRNA.

More recently, there has been an increased interest in the translational regulatory mechanisms. A study was performed by Chu *et al.*, (1991) in which the addition of recombinant human TS protein to *in vitro* translation reactions inhibited translation of TS mRNA. This inhibition was specific in that recombinant TS protein had no effect on the *in vitro* translation of mRNA from other proteins. The inclusion of dUMP, F-dUMP, or CH_2H_4 folate to the *in vitro* translation reactions completely relieved the inhibition of TS mRNA translation by TS protein. These studies suggested that translation of TS mRNA is controlled by its own protein end product, in an autoregulatory manner.

Further studies on human TS, using an electrophoretic gel mobility shift assay, have indicated this translational autoregulatory process is mediated by an interaction between TS protein and two cis-acting elements on its corresponding TS mRNA (Chu *et al.*, 1993). The first site corresponds to a 30 nucleotide sequence that includes the translational start site contained within a putative stem loop structure, while the second site corresponds to a 100 nucleotide sequence located within the protein coding region. RNAs with either a deletion or mutation at the translational initiation region are unable to compete for TS protein binding. A similar study on *E.coli* TS mRNA confirmed a direct interaction between *E.coli* TS protein and *E.coli* TS mRNA (Voeller *et al.*, 1995). Two cis-acting sequences in the *E.coli* TS mRNA protein-coding region were identified, each of which binds TS with a relative affinity similar to that of full length *E.coli* TS mRNA. A third binding site was identified with an affinity for TS that was lower than that of the other two cis-acting elements. *E.coli* TS proteins with mutations in amino acids located within the nucleotide-binding region retained the ability to bind RNA while proteins with mutations at either the nucleotide active site cysteine or at amino acids located within the folate binding region were unable to bind TS mRNA (Voeller *et al.*, 1995). These results suggested that the folate binding site and/or critical cysteine groups my represent important RNA binding domains.

These findings relating to autoregulation of TS appear to have biological relevance in that they suggest a means whereby the level of this protein is finely controlled within a given cell and a means for malignant cells to protect themselves in response to a cytotoxic stress such as F-dUMP. F-dUMP may bind to TS and produce a conformational change

which inhibits its binding to TS mRNA. Translation of TS mRNA then proceeds uncontrolled, resulting in TS protein accumulation and drug resistance.

1.3.10. Crystal structure of human TS

The structure of unliganded human TS provides the first three-dimensional view of a mammalian TS (Schiffer *et al.*, 1995). The structure indicates a large degree of similarity in the overall fold to the bacterial enzymes, which is consistent with the high degree of sequence identity. The major exception is the active site loop. In *L.casei* TS structure, this region includes the end of β-strand v, a short α-helix, the I helix and the beginning of β-strand iv (refer to Figure 1.13). However, the entire active-site loop of the human TS structure is twisted by approximately 180° relative to the conformation in the *L.casei* enzyme structure. The structure of the human enzyme results in the active site cysteine residue being deeply buried in the β-sheet dimer interface which results in the cysteine residue undergoing a 180°C twist upon ligand binding. In all species of TS, ligand binding causes conformational changes which are implicated in correctly orienting substrate and cofactor, and in appropriately sequestering them from the external solvent environment. Therefore upon ligand binding the human enzyme must reorient the active site residues as well as undergoing the conformational changes which lead to the closure of the domains around the substrate and cofactor. A possible function for the orientation of the active site may be to protect the catalytic cysteine from being modified in the cell.

Human TS has a unique amino terminal sequence which is 27 residues longer than *L.casei* and contains eight proline residues and seven charged residues (Schiffer *et al.*, 1995). This amino terminal extension is longer than any other species of TS (refer to Figure 1.13). Secondary structure predictions have indicated that it is present as a randomly coiled structure. This extension, which is not present in VZV, may have a function unique to the human enzyme and it has been suggested that it may be present to enable TS to interact with other proteins in the cell, some of which are involved in DNA synthesis (see section 4.1).

Two other insertions are present in human TS, one of twelve residues and one of eight residues at positions 117-128 (human numbering) and 146-153 (refer to Figure 1.13). These sequences are highly conserved in all the currently sequenced eukaryotic TSs. As homologous insertions are not present in prokaryotes this suggests that they posses a eukaryote specific function (Hardy *et al.*, 1987; Perry *et al.*, 1990). Therefore the unique structures present in human TS can be used to assist in structure-based approaches to the development of species-specific inhibitors with antiproliferative activity against pathogenic TSs which do not inhibit the human enzyme.

1.4. Mutagenesis of thymidylate synthases

1.4.1. Structural plasticity of TS

TS is extremely permissive in the amino acid substitutions that it tolerates, including many for residues that are highly conserved. The accommodation of such substitutions may be due to the structural plasticity of the protein, i.e. the ability of the protein to adapt to changes in its primary structure, whilst maintaining its tertiary structure.

Perry *et al.*, (1990) investigated the structural plasticity of TS by comparing the *E.coli* and *L.casei* structures, in an attempt to generalise the effects of substitutions. A detailed study of the buried region of TS from both structures revealed three different mechanisms by which substitutions could be accommodated. The first is covariant accommodation, in which multiple sequence changes combine to minimise distortion of the main chain atoms and preserve the volume of the packed side chains at the same time. Covariant changes are seen at the centre of strand iii in the β-sheet, which is one of the most structurally conserved regions of the enzyme (refer to Figure 1.13). Phe255 in *L.casei* is replaced by Gly in *E.coli*. The surrounding space in *E.coli* is filled by side chains of Trp253 and Thr258, versus His253 and Ala258 in *L.casei* for a net change of only one atom in these three residues. A second covariant pair is Thr200, Leu201 of *L.casei* TS, replaced by Ala200, Phe 201 in *E.coli*, thus providing a similar side chain volume. In general, it has been found that when a substitution introduces three or more heavy atoms (carbon, oxygen, sulphur or nitrogen), this nearly always results in a second site or covariant change nearby (Perry *et al.*, 1990). Experimental data obtained from mutagenesis studies of the hydrophobic core of the bacteriophage lambda repressor protein, demonstrate that where a mutation increases the volume of the core in excess of a permitted level, second site mutations that can reduce the net volume, can restore activity (Lim & Sauer, 1991).

The second mechanism by which mutations can be accommodated is by the occurrence of local changes, whereby large effects observed close to the site of mutation dissipate as a function of distance. This is usually achieved by a mechanism that involves local rearrangements, often the repacking of adjacent residues, with only minimal effects on the overall architecture of the protein (Perry *et al.*, 1990).

A final mechanism by which plastic adaption can occur is by segmental accommodation, which is the concerted movement of individual elements of secondary structure. Concerted alterations are seen in the helices and external loops in TS which contain sites exhibiting the largest differences between *E.coli* and *L.casei* structures. The ensemble movement of helices B, C and G is the most prominent example of this process, where these structures have shifted together as a unit in *L.casei* TS relative to *E.coli*. This movement has occurred to accommodate the interactions between the 3-helix unit and the

helix D of *L.casei*, a helix that is absent from *E.coli* TS (Perry *et al.*, 1990). The maintenance of hydrophobic interactions in this helical region between the two TS proteins represents the preservation of packing constraints during evolution.

1.4.2. Construction of mutants

Numerous mutant thymidylate synthases have been reported. Some of the mutants were made by conventional site-directed mutagenesis techniques, where a single residue was targeted for a single substitution for a specific reason. Other experiments have used multiple substitutions to provide replacement sets of amino acids at a chosen position in the enzyme (Climie *et al.*, 1990; Michaels *et al.*, 1990; Kim *et al.*, 1992).

An *L.casei* TS gene was designed and chemically synthesised to contain 35 unique restriction sites and codons optimised for expression in *E.coli*. Replacement sets of mutant enzymes were created by the replacement of restriction fragments of the synthetic gene with fragments containing oligonucleotide mixtures coding for all 20 amino acids at targeted positions (Climie *et al.*, 1990). An important step in the procedure was the identification of catalytically active mutants by the genetic complementation of *E.coli* χ2913 which is deficient in TS. Individual mutant isolates were grown under nonselective conditions and then patched onto minimal agar lacking thymidine. Cell growth in the absence of exogenous thymidine identifies mutants that express catalytically active TS. Using this technique, Climie and co-workers identified 125 mutants at 12 different amino acid positions, at which residues had previously been predicted to play roles in the direct binding of, or lie in close proximity to dUMP.

Michaels and co-workers (1990) used a different approach to produce multiple mutants of *E.coli* TS. They used oligonucleotide site-directed mutagenesis to introduce amber nonsense codons at 20 sites in the *E.coli* TS gene and have made 12 or 13 amino acid substitutions at each site by transforming the mutants into suppressor strains. The suppressor strains have altered tRNAs that can insert an amino acid in response to an amber stop codon. Suppression of the amber mutation is commonly 25-50% efficient. In this way they have created over 245 variants of TS. The ability of different mutants to complement the growth of Thy$^-$ strains in the absence of thymine was used to determine their catalytic activity.

More recently Kim *et al.*, (1992) have carried out amino acid substitution analysis within the N-terminus of *E.coli* TS corresponding to residues 20-35 which contains a block of amino acids whose sequence have been well conserved among other known TS proteins. This procedure used suppression of amber mutations by tRNA suppressors and has yielded a bank of 124 new mutationally altered TS proteins.

With few exceptions the data obtained with the *E.coli* and *L.casei* enzymes by the two different approaches are in agreement; many of the key residues of TS can be substituted resulting in enzymes which still retain significant activity.

1.4.3. Thymidylate synthase assays

There are several assay systems available for the detection of TS. The *in vivo* complementation of *E.coli* with have a defective TS gene can be used as an initial screen. Individual mutant isolates are grown under nonselective conditions and then patched onto minimal agar lacking thymidine. Cell growth in the absence of exogenous thymidine identifies mutants that express catalytically active TS (Thompson *et al.*, 1987). This method provides a rapid, genetic method for the assessment of whether or not TS is active, but it is sensitive to the level and activity of the expressed enzyme.

There are several *in vitro* assay systems available for the detection of TS activity. The most commonly used is the spectrophotometric assay, that measures the increase in absorbance at 340nm concomitant with dihydrofolate production. It can be adapted to microplate formats for kinetic studies. This assay, however may be limited by interfering absorbance in crude extracts. The tritium release assay is a more sensitive isotopic method for detectintg TS activity *in vitro* (Roberts, 1966). It is based on the release of tritium, as tritiated water, from dUMP, specifically labelled in the 5'-position, when thymidylate is formed. Unreacted substrates are removed by absorption onto activated charcoal, and the radioactivity in water is measured. The amount of dTMP generated is directly equivalent to the amount of [^{3}H] released during the assay.

The cofactor independent dehalogenation of BrdUMP to form dUMP provides a spectrophotometric method for measuring the enzyme's ability to bind nucleotides and activate C-5 for substitution (Garret *et al.*, 1979) This reaction is slow compared to dTMP formation and suffers from low sensitivity.

1.4.4. Mutagenesis of residues interacting with substrates

1.4.4.1. Mutagenesis studies of residues interacting with phosphate of dUMP

Residues that interact with dUMP are highly conserved throughout the TS sequences. Nevertheless, a large variety of substitutions are permitted at such residues without a complete loss of enzyme activity.

X-ray structural studies on *L.casei* TS have shown that Arg23 (Arg38 of VZV), Arg178' (Arg163 of VZV), Arg179' (Arg164 of VZV), and Arg218 (Arg203 of VZV) form a positively charged binding site for the 5'-phosphate of dUMP (Hardy *et al.*, 1987). Arg 178' and Arg 179' are derived from a monomer different from the active site

they occupy and function in. Initial studies on T4 phage TS by Frasca *et al.*, (1988) reported that replacement of invariant Arg137 (corresponding to Arg179 of *L.casei*, Arg164 of VZV) by glycine resulted in an inactive enzyme and so concluded that this residue must be an essential residue in the binding of dUMP. In contrast, however, most replacements of Arg179' (Arg164 of VZV) from either *E.coli* or *L.casei* TS results in enzymes that complement Thy$^-$ *E.coli* (Michaels *et al.*, 1990; Climie *et al.*, 1990). Purified R179A, E, K and T mutants of *L.casei* showed relatively small differences in kinetic parameters compared to wild type TS. The most significant effect of all mutations is localised to a decrease in the net rate of association of TS with dUMP (Santi *et al.*, 1990), which decreases 10 to 20 fold relative to the wild type TS. The K_{cat} values were similar for mutants and only 4 fold lower than wild type, which indicated that once substrates are bound within the ternary complex the catalytic steps are only moderately affected by the nature of the residue at 179. This might be expected since Arg179 is positioned far from the reaction centre and there is no evidence that Arg179 plays a direct role in catalysis (Santi *et al.*, 1990). The high activity detected in the mutant enzymes is explained by "plasticity" of the enzyme and the compensatory actions of other arginine residues. In the crystal structure of the R179E mutant, the arginine residues Arg178' (Arg163 of VZV), and Arg218 (Arg203 of VZV) ligate the dUMP phosphate as in the wild type TS.dUMP complex, and Arg23 (Arg38 of VZV) has moved slightly to form a hydrogen bond with a phosphate oxygen different from that in the wild type TS.dUMP structure (Finer-Moore & Stroud, unpublished data cited in Carreras & Santi, 1995).

Substitutions for Arg178' (Arg163 of VZV) that allow complementation of Thy$^-$ *E.coli* include basic and other hydrophilic substitutions although proline is also accepted in *E.coli* TS (Climie *et al.*, 1990). The purified R178T mutant from *L.casei* is active by complementation and shows a 5-fold reduction in K_{cat}, a 1000 fold increase in dUMP K_m, and a 100 fold increase in folate K_m (Kawase & Santi, unpublished data cited in Carreras & Santi, 1995). The detrimental effect on the binding of dUMP can be explained by phosphate binding role of Arg178', but the effect on K_m of the cofactor has no known structural basis.

In addition to dUMP binding, Arg23 (Arg38 of VZV) forms a salt bridge with the C-terminal carboxylate following the structural change that occurs upon folate binding (Matthews *et al.*, 1990a; Montfort *et al.*, 1990; Matthews *et al.*, 1990b; Fauman *et al.*, 1994). Few members of the Arg23 replacement set complement Thy$^-$ *E.coli* (Climie *et al.*, 1990; Michaels *et al.*, 1990) and those members that do are structurally dissimilar. Studies with purified Arg23 mutants of *L.casei* TS show that all are weakly active *in vitro*, with 10 to 200 fold reductions in K_{cat}, up to 10 fold increase in dUMP K_m values and 4-30 fold increases in folate K_m values relative to those of wild type (Santi, unpublished data, cited in Carreras & Santi, 1995). The inability of the mutants to complement Thy$^-$ *E.coli* is best explained by their poor binding to substrates, which

would reduce K_{cat}/K_m below the essential level needed for cell growth. The effect of mutations on folate binding likely results from their inability to form the salt bridge with the C-terminal carboxylate (Matthews *et al.*, 1990a; Montfort *et al.*, 1990; Matthews *et al.*, 1990b; Fauman *et al.*, 1994).

The fourth arginine residue, Arg218 (Arg203 of VZV) cannot be mutated to any of the other amino acids without loss of function. This residue is thought to play a role in the stabilisation of the active site thiolate (Cys198 of *L.casei*, Cys183 of VZV) by ion pair formation.

Therefore, it is apparent that although Arg179 (Arg164 of VZV) is bound to the phosphate of dUMP, it is not essential for substrate binding or catalytic activity. It can be replaced by neutral (Ala, Thr), positive (Lys), or negative (Glu) amino acids without drastic changes in kinetic constants. The three other arginine residues that were proposed to interact with the phosphate group of the substrate would only accept limited substitutions. Among these arginine residues there appears to be a hierarchy of substitutability. Arg178' (Arg163 of VZV) can be replaced by any basic amino acids as well as tyrosine; there are few amino acids that can be substituted for Arg23 (Arg38 of VZV); and Arg218 (Arg203 of VZV) cannot be mutated to any other amino acid without loss of function (Michaels *et al.*, 1990).

1.4.4.2. Residues interacting with deoxyribose of dUMP

Residues interacting with the deoxyribose moiety of dUMP in *L.casei* TS include Tyr261 (Tyr246 of VZV) and His259 (His244 of VZV), which both form hydrogen bonds with the 3'-OH of dUMP. The Y261M mutant of *L.casei* was the only one of 14 mutants that complement Thy^- *E.coli* (Climie *et al.*, 1990)

The active site cysteine residue provides an essential nucleophilic catalyst for the TS reaction as it forms a covalent bond with C-6 of the deoxyribose group of dUMP. Mutagenesis of this completely conserved residue in *L.casei* (Cys198 of *L.casei*, Cys183 of VZV)) results in complete inactivation of the enzyme (Climie *et al.*, 1990). The corresponding active site nucleophile, Cys146 in *E.coli*, only accepts its original amino acid for full activity. However, partial activity was reported with the serine substitution (Michaels *et al.*, 1990). This correlates well with previous mutagenesis studies. Dev *et al.* (1988) reported that substitution of alanine, glycine or threonine at position 146 results in an inactive enzyme but a serine mutant retains a small amount of catalytic activity. Because alkoxides are capable nucleophilic catalysts in model reactions of TS, the weakly acidic serine might serve as a nucleophile in the TS reaction; however why this substitution is active in *E.coli* but not in *L.casei* is unknown.

1.4.4.3. Mutagenesis of residues interacting with pyrimidine of dUMP

His199 (His184 of VZV), Tyr146, Trp82 (Trp97 of VZV), Asn229 (Asn214 of VZV), Glu60 (Glu75 of VZV) and several highly ordered water molecules form a hydrogen bond network around the pyrimidine ring of dUMP (refer to Figure 1.13).

Tyr146 (Tyr123 of VZV) is a completely conserved residue and is located near His199 (His184 of VZV) and Asn229 (Asn214 of VZV) in *L.casei*. This residue forms a hydrogen bond with an ordered water molecule that is close to C-5 of dUMP in ternary complexes and may assist in removal of the C-5 proton (Matthews *et al.*, 1990; Fauman *et al.*, 1994). Three mutations have been produced in this residue and all were active by complementation, indicating that Tyr146 (Tyr123 of VZV) is not essential (Climie *et al.*, 1990).

His199 (His184 of VZV) is a highly conserved residue and lies in the active-site cavity. From crystal structures this was suggested as a candidate general base to assist the removal of the C-5 proton from dUMP. Of the His199 mutants isolated from *L.casei*, several retained catalytic activity indicating that this residue is not essential for catalysis. Similar findings were noted in *E.coli* (Dev *et al.*, 1989).

Asn229 (Asn214 of VZV) is completely conserved and lies in the hydrophobic J-helix which forms the core of TS. This is the only residue which makes direct contact between a protein side chain and the dUMP pyrimidine ring, forming a cyclic hydrogen bond network with 0-4 and NH-3 of dUMP. The highly conserved nature of this residue was thought initially to be to stabilise dUMP in the active site. A complete replacement set of mutants at position 229 of *L.casei* TS have been prepared, purified and characterised (Liu & Santi., 1993). Most of the Asn229 mutants of TS bind dUMP and catalyse dTMP formation as well as the wild type enzyme, thus Asn229 does not contribute to the binding of dUMP. Wild type TS binds to dUMP about 1000 fold tighter than dCMP. With the exception of N229Q, Asn229 mutants decrease the enzymes ability to discriminate between dUMP and dCMP [(Liu & Santi, 1992; Hardy & Nalivaika, 1992; Liu & Santi, 1993), refer to Chapter 8]. A similar situation was found in the corresponding residue (Asn177) from *E.coli*, the N177D mutant gave an initial velocity with dCMP which was 35 fold faster than that obtained with dUMP indicating that the mutant enzyme is more active on dCMP than dUMP (Hardy & Nalivaika, 1992).

Crystallographic studies have shown that the completely conserved Glu60 of *L.casei* TS (Glu75 of VZV) is involved in an extensive hydrogen bond network that includes several conserved side chains of the enzyme, ordered water molecules, and the pyrimidine ring of the substrate (Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990). Mutation of Glu60 leads to large losses in activity (Huang & Santi, 1994), purified *L.casei* E60L and E60A TS mutants catalysed tritium loss from [5-^{3}H] dUMP faster than dTMP formation, indicating formation of a steady-state intermediate with an acidic 5-hydrogen and a partitioning of the intermediate towards the reactants. A covalent complex

consisting of E60A or E60L TS, dUMP, and the cofactor CH_2H_4 folate was isolated by SDS PAGE and shown to be chemically and kinetically competent to form dTMP. These results provide proof of the formation of a covalent steady-state intermediate in the reaction pathway of TS and demonstrate that the rate determining step in the mutants occurs during conversion of the covalent intermediate to dTMP (Huang & Santi, 1994).

Finally, structure-function considerations have suggested that Glu60 serves a role in co-ordinating a hydrogen bond network that promotes proton transfer reactions at O-4 of dUMP and N-10 of the cofactor orienting the pyrimidine ring of substrate and the pterin ring of cofactor for hydride transfer (Huang & Santi, 1994).

1.4.4.5. Mutagenesis of residues interacting with CH_2H_4 folate

Although CH_2H_4folate is often described as a cofactor the fact that it is oxidised during the reaction means that it could be considered as a substrate. Folate binding to TS induces a conformational change in which the DRTG loop residues and the C-terminal residues move to cover the bound substrates. Residues involved in CH_2H_4folate binding that have been mutated include those of the C-terminus as well as Trp82 (Trp97 of VZV), Trp85 (Tyr100 is corresponding residue of VZV), Asp221 (Asp206 of VZV), Lys50 (Lys65 of VZV) and Lys51 (Arg66 is corresponding residue of VZV).

Trp82 (Trp97 of VZV) and Trp85 (Tyr100 of VZV) are conserved residues that pack against the bound folate analogue in ternary complexes. Trp82 has been replaced with several amino acids and some of the mutants, including W82F, were active (Kealey & Santi, unpublished data cited in Carreras & Santi, 1995). The corresponding residue, Trp80 in *E.coli* could not be restored to Thy^+ by any of the mutations made (Michaels *et al.*, 1990). Trp85 from *L.casei* has been replaced only with phenylalanine and this enzyme is active. However the W82F/W85F double mutant is inactive, suggesting that the presence of at least one of the tryptophans is essential.

The side chain of Asp221 (Asp206 of VZV) forms a hydrogen bond network with N-3 of the folate and may assist elimination of the cofactor from the TS.dUMP.CH_2H_4folate intermediate. Predictably substitutions at this position produce enzymes that do not complement Thy^- *E.coli,* though D221C is an exception. Purified D221A and D221N mutants are inactive but bind nucleotides with wild type affinity and dehalogenate BrdUMP more efficiently than wild type TS (D.Santi, unpublished data cited in Carreras & Santi, 1995). This is consistent with the role of Asp221 in the breakdown of TS.dUMP.CH_2H_4folate catalytic intermediate. Substitution of this residue in *E.coli* results in an enzyme that cannot complement Thy^- bacteria.

1.4.4.6. The C-terminal residue

Carboxypeptidase cleavage of the *L.casei* C-terminal valine from a single subunit of TS completely inactivated the enzyme and decreased the enzymes affinity for CH_2H_4folate (Aull *et al.*, 1974). Using mutagenesis Carreras *et al.* (1992) demonstrated that a single amino acid deletion (V316Am, where Am represents an amber stop codon) from both subunits at the C-terminus also abolishes product formation. In contrast to the bacterial enzymes it has been noted that removal of the C-terminal residue from VZV TS (L301Δ) results in an enzyme which retains a significant amount (3%) of wild type activity, in addition this mutant has increased debromination activity which reflects the lower K_m for dUMP (Harrison *et al.*, 1995).

Mutagenesis studies on the C-terminal residue of *L.casei* TS have shown that valine can be replaced with the 19 other amino acids: 14 of these mutants were active by genetic complementation and 18 were active by *in vitro* assay. In general the alterations at 316 had little effect on the K_m for dUMP, an increase in the K_m for folate cofactor, and a decrease in K_{cat}. These observations show that *L.casei* TS can tolerate the substitution of most amino acids for valine at the C-terminus without a complete loss of activity and that the position of the C-terminal carboxylate is important in both cofactor binding and catalysis. The apparently similar dUMP K_m values indicated that substrate binding is not significantly affected by mutations in the C-terminal residue. These results are consistent with crystallographic studies which show that free enzyme and the binary complex have similar structures and that the C-terminus is not directly involved in nucleotide binding in the binary complex (Hardy *et al.*, 1987; Perry *et al.*, 1990; Montfort *et al.*, 1990; Matthews *et al.*, 1990).

Although V316Am does not catalyse dTMP fomation it does catalyse the dehalogenation of BrdUMP, which is a partial reaction of TS which occurs in the absence of folate. The K_{cat} and K_m values for this reaction are not altered by mutations which affect folate binding and subsequent steps in the pathway (Climie *et al.*, 1992) suggesting that the conformational change of the C-terminus is not involved in the dehalogenation reaction. All that seems to be required of the enzyme is the ability to bind nucleotide and form a covalent adduct between the catalytic thiol of Cys198 and the nucleotide.

The V316Am mutant was also able to participate in the formation of the V316Am.dUMP.CH_2H_4 folate ternary complex. This complex was impaired in its ability to isomerise from the noncovalent to the covalent form, suggesting that the mutant is impaired in its ability to undergo the C-terminal conformational change. In support of this the crystal structure of the V316Am TS.FdUMP.CH_2H_4 folate complex showed that no covalent bonds were present between the enzyme and the ligands, and the enzyme had not undergone the folate-dependent conformational change (Santi, 1987; Climie *et al.*, 1992).

The loss of TS activity in the V316Am mutant can be rationalised in several ways. Firstly the position of the C-terminal carboxylate in the ternary complex of V316Am

would be moved by at least 3-4Å and disrupt the hydrogen bond network that facilitates CH_2H_4 folate binding. Secondly, loss of the residue at the C-terminal position would abolish side-chain mediated hydrophobic interactions that contribute to the conformational change of the enzyme. Finally truncation of TS would disrupt hydrogen bonding of the penultimate residue of TS to folate.

As with the V316Am TS, deletion mutants lacking 4, 8 and 10-terminal residues lack TS activity but dehalogenate BrdUMP with wild type efficiency (Carreras & Santi unpublished, cited in Carreras & Santi, 1995). Mutants lacking eight or ten C-terminal residues could not form covalent complexes with FdUMP and CH_2H_4 folate. The addition of an 8-residue synthetic peptide with the same sequence as the C-terminus did not revive activity of an A309Am (n-8) mutant. I will show in Chapter 7 that this is not the case for VZV TS.

14.4.7. Mutagenesis of the dimer interface of VZV TS

β-strand i forms part of the dimer interface and is the only region of secondary structure to have an insertion in more than one TS sequence (refer to Figure 1.13). From structural studies of TS, a highly conserved glycine residue (Gly48 in VZV TS) located in the β-kink region of β-strand i has been predicted to be essential. Harrison *et al.*, (1995) constructed a glycine to alanine mutation at position 48 in VZV TS. This mutant could complement TS deficient *E.coli* and demonstrated a two fold decrease in enzyme activity relative to the wild type enzyme. This highly conserved glycine residue does not appear to be essential for TS activity as yeast can tolerate alanine at this position and bacteriophage Ø3T can permit a serine, in addition Gly31 of *E.coli* TS can be replaced by an alanine or cysteine (Kim *et al.*, 1992).

TS from *Saccharomyces cerevisiae* has an insertion in β-strand i. A second mutant was constructed in VZV TS to determine whether the insertion seen in the yeast enzyme could be accommodated (Harrison *et al.*, 1995). The amino acid substitutions G48A and M49P which flank the extra amino acid in the *S. cerevisiae* TS sequence were used to create the mutant G48A/48P49/M49P which mimics the central portion of *S.cerevisiae* TS β-strand i. The sequence of β-strand i of this variant of VZV TS differs from that of *S. cerevisiae* by only three out of thirteen amino acids. The mutations were found to be accommodated in VZV TS as the variant could complement Thy^- *E.coli.*

TS from yeast, *Plasmodium* species and *Lactobacillus lactis* have an extra amino acid immediately adjacent to the carboxy-end of β-strand i. Experimental evidence has shown that VZV TS can accommodate an extra residue immediately adjacent to the carboxy-end of β-strand i , 58T59. It has been proposed that the insert in β-strand i of both yeast and the VZV TS mutant can be accommodated locally (within β-strand i) by the formation of a β-bulge. Richardson *et al.*, (1981) have shown that bulges form

irregularities, usually in antiparallel beta structures in which 2 consecutive hydrogen bonding residues on one strand are opposite a single residue on the other. The conformation of a β-bulge requires that a single residue on the opposite strand adopts a similar dihedral angle. The glycine which is situated at this position on the opposite strand in these species can readily adopt this conformation.

The mutant G48A/48P49/58T59 has also been studied and this produces more activity than the original G48A/48P49/M49P. This higher activity of the mutant suggests that the two changes together minimise the disruptive effects seen when the mutations are present singly (Harrison *et al.*, 1995). This is an example of covariant accommodation, where multiple sequence changes can combine to minimise distortion of the main chain atoms (Perry *et al.*, 1990).

1.4.5. Heterodimeric thymidylate synthase mutants

The active site of TS has residues derived from both subunits. Experimental studies have revealed that TS dimers can be reversibly folded and dissociated with only a small loss in activity, this has made it possible to produce heterodimeric enzymes with a single functional active site.

C198A and R178'F mutations are completely inactivate in *L.casei* TS. However, refolding C198A in the presence of R78'F resulted in the formation of heterodimers with one active subunit (R178' and C198), one inactive subunit (R178'F and C198A), half the activity of WT TS, and similar K_m values for substrate and cofactor (Pookanjanatavip *et al.*, 1992).

A similar method of heterodimer formation has been used to create active, asymmetric enzymes that have a C-terminal deletion on only one subunit of the dimer. It had previously been discovered that removal of the C-terminal amino acid from one subunit of TS results in complete inactivation of the enzyme (Aull *et al.*, 1974). Therefore Carreras *et al.*, (1994) attempted to form an active V316Am-TS heterodimer by refolding the WT subunit in the presence of increasing concentrations of inactive V316Am. The results revealed that the heterodimeric mutant was active and had kinetic parameters similar to the R178F'-C198A heterodimer. To further establish that the activity in the V316Am-TS was due to heterodimeric formation and not to WT homodimers forming, a further heterodimer was created from two completely inactive mutants; R178'F and V316Am. In the heterodimer, the R178'F mutation is part of the same active site as the V316Am mutation and prevents dUMP from binding to this active site. The second site of the heterodimer has no mutations and contains the wild type residues necessary for catalysis of dTMP formation. Refolding of the two inactive subunits produces an R178'F-V316Am heterodimer that catalyses dTMP formation at a rate that is 2-fold lower than the wild type enzyme. Since neither of the two mutants is active on its own, the

ability of inactive R178'F monomers to complement inactive V316Am monomers demonstrates that a TS heterodimer with a C-terminal deletion on one subunit can catalyse dTMP formation. This observation of active heterodimers with a C-terminal deletion of one subunit is not in accordance with previous studies of carboxypeptidase-inactivated TS (Aull *et al.*, 1974; Cisneros *et al.*, 1993).

1.5. Thymidylate synthase inhibitors

Thymidylate synthase is a target for drugs against proliferative diseases and cancer because it is required for the *de novo* synthesis of thymidylate and, hence, for DNA production. TS inhibition can be achieved with either fluoropyrimidines or other analogues, which are based on the structure of the substrate dUMP, or folate analogue compounds, which are based on the structure of the cofactor. Inhibition of TS by either antifolates or FdUMP leads to a depletion of dTTP and cessation of DNA synthesis. Ultimately DNA damage will occur by the misincorporation of dUTP and FdUTP. Increased excision of the fraudulent nucleotides might cause strand breaks.

1.5.1. Fluoropyrimidine inhibitors

The first compounds to have significant TS-inhibiting activity were the nucleoside analogues, predominantly 5-substituted-2'-deoxyuridine analogues. For these analogues to be active *in vivo*, they must first be enzymatically activated by being converted into the 5'-monophosphate derivative. The necessity for the phosphate group was shown by the fact that deoxyuridine is not a substrate for the purified enzyme (Reyes & Heidelberger, 1965) and exhibits a 500 fold lower binding activity than dUMP (Lockshin & Danenberg, 1981). In addition inorganic phosphate is a competitive inhibitor of nucleotide binding (Lockshin & Danenberg,1981). In *in vivo* studies, the necessity for the 5'-monophosphate group has been demonstrated by the fact that mutations in the activation step led to resistance.

5-monophosphate derivatives of 5-fluoro-2'-deoxyuridine, 5(triflouro-methyl)-2'-deoxyuridine (CF_3UdR), 5-nitro-2'-deoxyuridine (NO_2-UdR) and 5-ethynyl-2'-deoxyuridine (EYUdR) have emerged as the most potent inhibitors in both *in vitro* and *in vivo* experiments with nucleoside analogues (Figure 1.15).

FdUMP, the active form of FUdR, forms a covalent ternary complex with TS in the presence of the cofactor N^5,$N^{10}CH_2FH_4$, which is similar to the covalent catalytic intermediate. This complex is stable and terminates the reaction because the fluorine atom cannot be abstracted as occurs with C-5 hydrogen in the course of the normal TS reaction, presumably due to the strength of the C-5-F bond (Santi & Danenberg, 1984). The effects

of fluoropyrimidines on leukemic cells of mouse and humans have been examined in the presence folinic acid, a reduced folate (Keyomarsi & Moran, 1985). Growth rate experiments showed that cells exposed to moderate concentrations of FUrd were initially inhibited but recovered, whereas with cells exposed to both FUrd and folinic acid the initial growth inhibitory effects were sustained. Therefore folinic acid increased the intracellular levels of folate, resulting in the stabilization of the ternary complex and thus a higher degree and duration of TS inhibition.

In contrast to FdUMP, CF_3dUMP and NO_2dUMP can inhibit TS in the absence of N^5,N^{10},CH_2FH_4 but still interact with the active site cysteine residue forming a 5,6-dihydropyrimidine derivative. Their ability to interact with TS in the absence of cofactor is thought to be the basis of their tight binding to, and inhibition of the enzyme (Danenberg & Heidelberger, 1974; Wayata *et al.*, 1980).

EYUdR is a potent inhibitor of S-49 murine lymphoma cells and inhibits TS only in the presence of cofactor, by forming an inhibitory ternary complex (Barr *et a*l., 1981). This ternary complex was found to be much weaker than that formed by FdUMP (Danenberg *et al.*, 1981). Furthermore, the EYdUMP complex was disrupted upon denaturation of the enzyme in contrast to the FdUMP based complex which remains stable upon denaturation (Danenberg *et al.*, 1981).

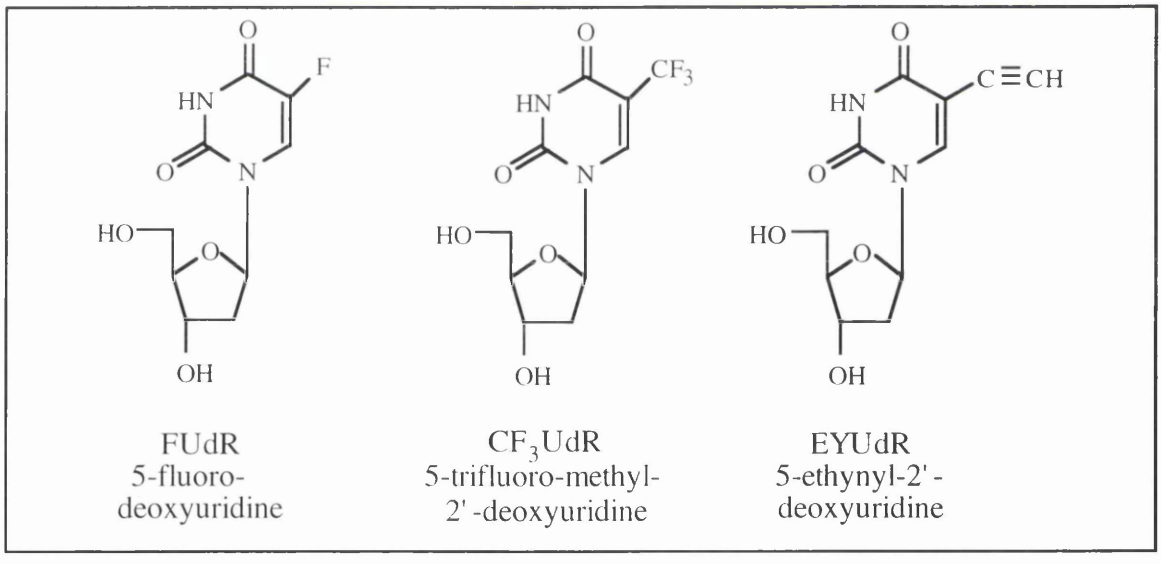


Figure 1.15. 5-substituted deoxyuridine analogue inhibitors of TS

These compounds has been used with limited success for the treatment of colorectal and other carcinomas. However, the fluoropyrimidines can also inhibit cell growth by incorporation into DNA and RNA.

1.5.2. Folate analogue inhibitors

Due to the limitations to the long term therapeutic uses of 5-FU, new TS drugs were designed which could elicit a thymineless condition, uncomplicated by other biochemical events, through direct inhibition of TS. The chemical approach exploited a quinazoline, rather than a pteridine nucleus, for the synthesis of a series of folate analogues. These compounds are less susceptible to metabolic degradation than fluoropyrimidines and are not incorporated into DNA or RNA (Harrap *et al.*, 1989). In addition tetrahydrofolate is a relatively large molecule providing a variety of sites amenable to manipulation in the design of specific inhibitors. To reach and target enzymes such as DHFR or TS, antifolates enter the cell via the reduced folate carrier (RFC) protein transport system on the cell membrane. Efficient or poor cellular antifolate uptake is determined by the molecular structure and polarity of the antifolate, the presence of reduced folates or antifolates, the intracellular folate depletion, and RFC deficiency (Jansen *et al.*, 1990). Once antifolates are inside the cell, glutamate residues are added by the enzyme folylpolyglutamate synthase (FPGS). These add more negative charges to the antifolate molecule and increase solubility, resulting in the agent's marked reduction in efflux from cells and its prolonged retention in tissues having high FPGS activity, such as the liver (Hoffbrand *et al.*, 1990). Moreover, polyglutamation increases the affinity of folate analogues for folate-dependent enzymes markedly and results in a potentiation of TS inhibition that may exceed by 100-fold that of the monoglutamated form (Calabresi & Chabner, 1990; Jackman *et al.*, 1990). Therefore, cellular uptake of antifolates via the RFC and the ability of antifolates to undergo polyglutamation have major roles in TS inhibition.

N^{10}-propargyl-5,8-dideazafolate (CB3717) was the first anti-folate compound to be developed. It is a 2-amino-4-hydroxy quinazoline carrying a propargyl on N^{10} that greatly increases the affinity of this compound for the active site of TS (Figure 1.16). This folate analogue was shown to be the tightest binding, monoglutamate folate-based TS inhibitor at the time of its discovery. With CB3717, the formation of a covalent bond between TS and dUMP is still induced. However, CB3717 cannot form a covalent bond with dUMP, as is the case with folate and dUMP, and methylene group transfer cannot occur. CB3717 does not use the RFC for cellular entry but undergoes extensive polyglutamation by FPGS. Maximal potentiation of TS inhibition was achieved by the triglutamate form of CB3717 (Calvert *et al.*, 1987). Phase I and II clinical studies demonstrated that CB3717 possesses clinical activity against ovarian, breast and liver cancers. However, severe renal toxic and profound malaise were noted even at lower doses and so further development of CB3717 was abandoned (Cantwell *et al.*, 1988; Sessa *et al.*, 1988; Vest *et al.*, 1988).

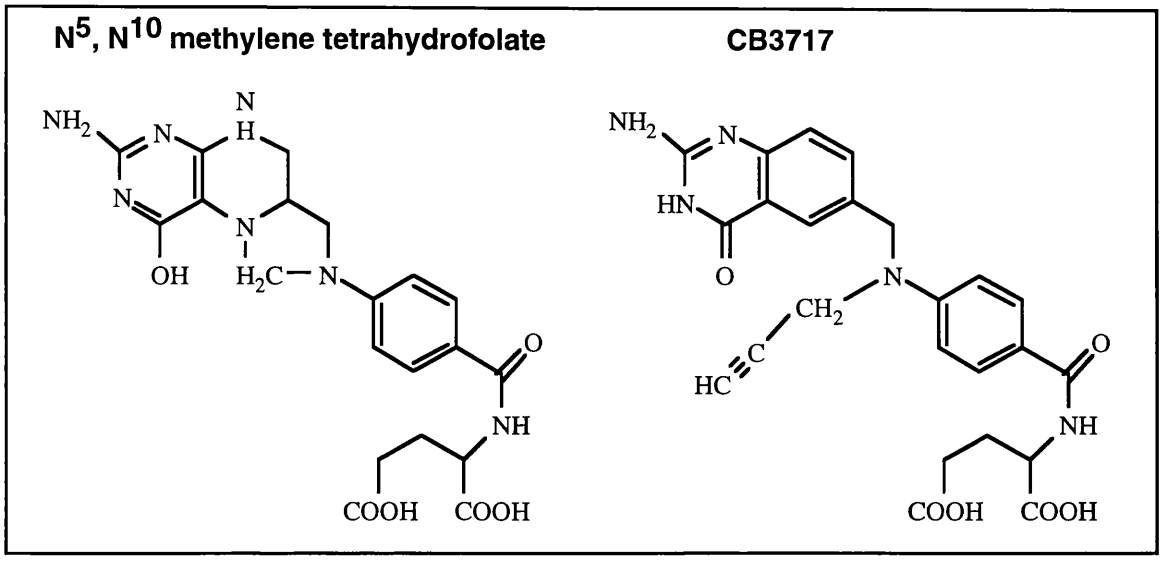
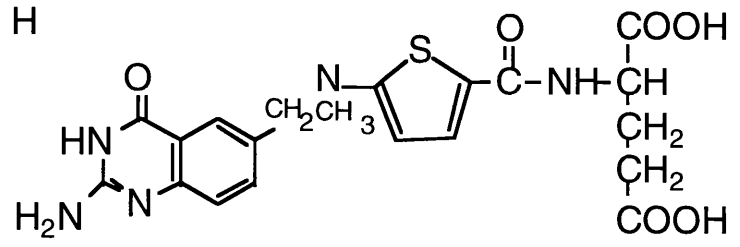


Figure 1.16. Chemical structure of N^5, N^{10} methylenetetrahydrofolate and 10-propargyl-5,8-dideazafolate (CB3717).

As the renal and hepatic toxic effects of CB3717 appeared to be due to its poor aqueous solubility (Newell *et al.*, 1982), further research focused on the synthesis of water soluble analogues. This led to the development of quinazoline compounds, in which the benzene ring of the amino benzoate residue has been replaced with various ring structures, including pyridine, thiophene, or thiazole (Jackman *et al.*, 1991; Marsham *et al.*, 1991). The N^{10}-methylthiophene analogue, ZD1694, was developed which selectively inhibits TS (Figure 1.17). Similar to CB3717, ZD1694 results in decreased TMP production, which leads to inhibition of DNA synthesis, resulting in cell death. In contrast to CB3717, ZD1694 uses the RFC for cellular entry, and thus higher intracellular levels are available for polyglutamation by FPGS. ZD1694 is an excellent substrate for FPGS, with an affinity 30 times higher than that of CB3717. Due to its effective use of the RFC and FPGS, this analogue is 500-fold more active in inhibiting cell growth than CB3717. This selective TS inhibitor has demonstrated antitumour effects in both preclinical and Phase II clinical trials and is undergoing Phase III evaluation. This drug, however has shown toxic effects to the bone marrow and small intestine.



ZD1694

Figure 1.17. Chemical structure of N^{10}-methylthiophene analogue, ZD1694

Antifolates that are potent TS inhibitors but do not require extensive polyglutamation to exert adequate cytotoxicity are presently under development; such agents can counteract reduced polyglutamation in tumours that express low or defective FPGS. Such an antifolate is ZD9331, a water-soluble quinazoline TS inhibitor that, like ZD1694, uses RFC for transport and enters cells readily but is not a substrate for FPGS as a result of a γ-tetrazole moiety that inhibits γ polyglutamation (Jackman *et al.*, 1994; Stephens *et al.*, 1994). Unlike antitumour doses of ZD1694 which produced toxic effects to the bone marrow and small intestine, antitumour doses of ZD9331 produced only haematological toxicity (Stephens *et al.*, 1994). This compound is in the preclinical stages of development.

1843U89 is a novel benzoquinazoline folate analogue in which the pteridine nucleus of the folate molecule is replaced by a lipophilic benzoquinazoline moiety. 1843U89 is an extremely potent, non-competitive TS inhibitor in enzyme assays, with kinetics suggesting binding to TS at a site independent of the substrate-binding site (Duch *et al.*, 1993). This analogue enters the cells via the RFC and, although an excellent substrate for FPGS, is metabolised only one step to the diglutamate form. In contrast to other antifolates, increasing the number of glutamate residues on 1843U89 enhances the TS binding and inhibitory actions of this compound only slightly. However, metabolism to the diglutamate form increases 1843U89 intracellular accumulation and retention markedly.

1.5.3. Bisubstrate analogues

Several bisubstrate analogues have been identified comprising a folate molecule covalently linked to a nucleotide analogue and designed to mimic the non-enzymic portion

of the catalytic intermediate of the TS reaction. These analogues have been shown to inhibit TS (Park *et al.*, 1979; Amarnath *et al.*, 1984).

Two such compounds, N^5-dUMP-N^{10}-propargyl-8-deazatetrahydrofolate and N^5-dUMP-8-deazatetrahydrofolate inhibit purified TS from murine leukemic cells in a competitive manner with respect to dUMP. Rather than entering into the active site of the enzyme to produce an inhibitory ternary complex, these compounds inhibit TS by blocking the nucleotide and folate binding sites.

1.5.4. *In vivo* regulators

Pyridoxal phosphate (PLP) reversibly inhibits TS from *L.casei.* The inhibition is competitive with respect to dUMP (Chen *et al.*, 1988, 1989). The observed changes in the absorption spectra of PLP resulting from the addition of TS (Chen *et al.*, 1989) were consistent with the formation of a thiohemiacetal by addition of the sulphydryl group to the carbonyl group of PLP. This suggests an interaction between the catalytic cysteine residue and the carbonyl group of PLP, similar to that formed between TS and dUMP during catalysis (Moore *et al.*, 1986; Chen *et al.*, 1989).

In addition, the common metabolite glyceraldehyde-3-phosphate was shown to act as a competitive inhibitor with respect to dUMP, with a K_i value of 12-13μm (Bures *et al.*, 1991). Although no evidence for the formation of a thiohemiacetal between the sulphydryl group of TS and the carbonyl group of G-3-P was given by Bures and co-workers (1991), such an intermediate is known to exist in the reaction catalysed by G-3-P dehydrogenase (Harris & Waters, 1976). They proposed that the mechanism of interaction with both enzymes is similar (Bures *et al.*, 1991).

Therefore PLP and G-3-P have a possible role in regulating DNA synthesis by modulating TS activity in both eukaryotic (Rode *et al.*, 1980; Chen *et al.*, 1989; Matherly *et al.*, 1989) and prokaryotic cells (Tolleson *et al.*, 1990). It has been observed that low levels of PLP are found in various rapidly proliferating cancer cells and the administration of pyridoxine or pyridoxal inhibits the proliferation of these cells both *in vitro* and *in vivo* (Thanassi *et al.*, 1985).

1.5.5. Anti-sense oligonucleotides

As the translation of human and *E.coli* TS mRNA is controlled by its own end product in a negative autoregulatory manner, an alternative approach to the inhibition of enzyme function other than the use of drugs is to specifically inhibit enzyme synthesis. The use of anti-sense oligonucleotides to arrest translation of specific mRNA molecules (Helene & Toulme, 1990) has been directed at the DHFR-TS mRNA of *Plasmodium falciparum.* A 49-mer targeted at the conserved region of TS gave 50% inhibition at 6μm

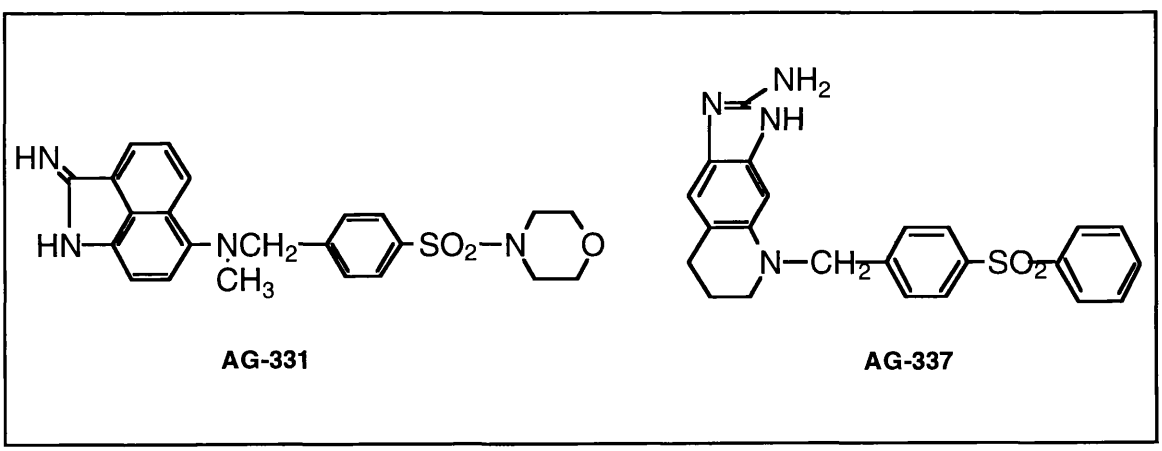
and 90% inhibition at 45μm (Sartorious & Franklin, 1991). Such an oligo is very specific to the DHFR-TS mRNA of *P.falciparum* (see section 1.3.9.3). There are delivery problems associated with the anti-sense oligonucleotides as they are charged and do not get into cells easily.

1.5.6. Oligopeptide inhibition of TS

Oligopeptides have been shown to inhibit VZV TS activity. A linear pentadecapeptide corresponding to the amino acid sequence of β-strand i (LPPCHTLCQFYVANG) inhibits TS *in vitro* (ICI_{50}=50μm). N- and C terminal deletions of this peptide have defined the active core as CHTLCQFY, which corresponds precisely to those amino acids at the interface of the two monomers. These oligos are thought to disrupt activity by preventing the correct formation of the dimer (H.Marsden & R.Thompson, personal communication).

1.5.7. Computer-assisted drug discovery

The X-ray structure of TS has been used in cycles of crystallographic analysis, synthesis, and inhibition assays to define high affinity heterocyclic inhibitors that resemble the cofactor. This led to the development of non-classical antifolates such as AG331 and AG337 which are lipophilic, lack a negatively charged glutamate side chain, and are extremely potent TS inhibitors (Figure 1.18).



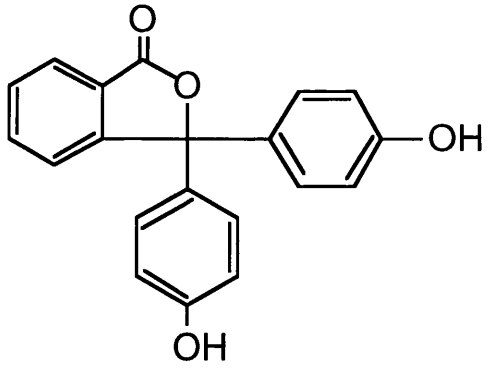
AG-331 **AG-337**

Figure 1.18. Chemical structures of AG331 and AG337

These drugs can enter cells readily without the RFC and thus have activity in tumour clones deficient in the carrier protein. Drug potency is not dependent on polyglutamation. These drugs can exert effective TS inhibition in FPGS-deficient cells,

however the prolonged intracellular retention of the new compounds conferred normally by polyglutamation is lost. Therefore, AG337 and AG331 require prolonged infusions to optimise antiproliferative effects.

In addition anti-TS drugs dissimilar to the substrate and cofactor remain attractive because they are less likely to have the side effects that are produced by the nucleotide and folate mimics. Shoichet *et al.,* (1993) sought novel inhibitors of TS with a computational screen targeted at the structure of *L.casei* TS determined at 2.3Å. A molecular docking programme (DOCK) was used to screen the Fine Chemical Directory (FCD) for molecules that are complementary to TS. Besides retrieving the substrate and several known inhibitors, DOCK proposed putative inhibitors previously unknown to bind to the enzyme. Three of these compounds inhibited TS at submillimolar concentrations. One of these inhibitors, sulisobenzone, crystallised with TS in two configurations that differ from DOCK favoured geometry. The structure of the complexes suggested another binding region in the active site that could be exploited. This region was probed with molecules sterically similar to sulisobenzone, which led to a family of phenolphthalein analogues that inhibit TS in the 1-30 micromolar range (Figure 1.19). These inhibitors do not resemble the substrates of the enzyme.



Phenolphthalein

Figure 1.19. Chemical structure of phenolphthalein

1.5.8. Resistance to TS inhibition

Three possible mechanisms of resistance to fluoropyrimidine inhibitors have been identified; prevention of activation, TS overproduction and structural alterations to TS. Characterisation of a variety of FU and FUdR resistant cell lines have shown that a significant decrease in any of a number of enzymes that prevent the formation of FdUMP or FUTP, prevent inhibition of TS.

Jasreboff and colleagues (1983) reported distinctly different properties of TS purified from an FdUrd resistant Ehrlich ascites carcinoma cell line when compared to the parent cell line. Both a higher K_i for FdUMP and a higher turnover number of the enzyme from the resistant line indicate a possible mechanism for resistance.

Another mechanism of resistance to FdUrd involving a change in the target enzyme was reported by Heidelberger and co-workers in 1960. In a cell free system it was shown that TS obtained from susceptible cells was inhibited by FdUMP, whereas the enzyme obtained from the resistant cells was not inhibited by FdUMP. The mechanism of resistance to fluoropyrimidines appears to involve the alteration of the active site in such a way that it can discriminate between dUMP and FdUMP. Considering the close proximity in size between the H atom and the F atom, it seems unlikely that the enzyme could distinguish between the two compounds on the substituted atom alone. However, substitution of fluorine in the 5-position of the pyrimidine increases considerably the acidity of the ring phenolic hydroxyl group, which might be sufficient for the enzyme to distinguish between dUMP and FdUMP (Heidelberger *et al.*, 1960).

The cause of resistance to FUdR in at least two murine cell lines has been shown to be due to 6- to 10-fold overproduction of TS (Baskin *et al.*, 1975). This form of resistance has also been observed for mouse FM3A mammary adenocarcinoma cells resistant to the folate analogue CB3717 (Danenberg & Danenberg, 1989). These elevated intracellular levels of TS "soak up" the inhibitor and provides enough extra enzyme activity to allow the cells to survive in normally lethal levels of inhibitor (Danenberg & Danenberg, 1989). Normally, the translation of human TS mRNA is controlled by its own end product in a negative autoregulatory manner (Chu *et al.*, 1993; Chu *et al.*, 1991). TS protein inhibits TS mRNA by binding of the protein to specific TS mRNA sequences critical for the initiation of translation. The intracellular level of TS protein is thus critical in the regulation of TS mRNA translation. It is possible that FdUMP, dUMP and selective TS inhibitors may bind to TS and produce a conformational change that inhibits binding to TS mRNA. Translation of TS mRNA would then proceed uncontrolled, resulting in TS accumulation and drug resistance (Chu *et al.*, 1991; Keyomarsi *et al.*, 1993). Observations have shown that following treatment of cells with the antifolate inhibitor ZD1694, TS protein levels accumulate by up to 40-fold, whereas TS mRNA levels remain unchanged. In addition coadministration of a translational but not a transcriptional inhibitor overcame the accumulation of TS protein. Therefore TS accumulation was potentiated by TS expression throughout the cell cycle after ZD1694 treatment. These effects were attributed to a loss of inhibition of TS mRNA translation, whereas gene transcription remained unchanged (Keyomarsi *et al.*, 1993).

High pre-treatment levels of TS have also been found to result in resistance to therapy. In 1995, Johnston *et al.* correlated TS protein levels and gene expression with the response of colorectal and gastric cancer to treatment with infusional 5-FU plus

leucovorin (a folate analogue). Both TS protein and TS gene expression were significant predictors of the response to 5-FU based therapy. Patients with responsive diseases had a low TS protein level whereas in patients whose tumours did not respond the protein levels were significantly higher. A similar pattern was noted for gene expression with higher TS mRNA levels found in non-responsive patients. The results suggest that the ability to predict the response and outcome based on TS expression in the primary tumour prior to or early in the course of treatment, may provide the opportunity to select patients who are most likely to benefit from 5-FU based therapy and the possibility to offer alternatives to those who will not benefit.

There are multiple mechanisms of resistance to the new selective TS inhibitors, with most of the studies being carried out on ZD1694. Potent inhibition of TS requires the active transport of the folate analogue via the RFC and subsequent polyglutamation by FPGS which results in increased affinity for TS and intracellular retention of the polyglutamate form. Therefore in drug-resistant cell lines there can be a decreased uptake of inhibitors via the RFC or failure to add polyglutamate (Freemantle *et al.*, 1993).

1.5.9. Improving drug efficacy

Several strategies have been undertaken in an attempt to improve the efficacy of TS inhibitors. To counteract TS induction, the development of an antisense RNA that targets the TS mRNA sequences responsible for TS translation has been suggested (Keyomarsi *et al.*, 1993). In another approach using gene therapy, cell lines resistant to TS inhibitors were transfected with a TS ribozyme using an EBV-based vector. This hammerhead ribozyme cleaves a sequence in the untranslated region of TS mRNA and the cells become sensitive to TS inhibition (Kobayashi *et al.*, 1995). IFN-γ has also been shown to enhance the sensitivity of human colon cancer cells to 5-FU (Chu *et al.*, 1990). Treatment using 5-FU alone resulted in the induction of TS and a decrease of sensitivity to 5-FU. However, the addition of IFN-γ to the treatment can reverse the development of resistance to 5-FU in the H630 cell line by inhibiting the overexpression of TS that results from 5-FU exposure. Other strategies have focused on potentiating TS inhibition or using agents synergistic with TS inhibitors. As folate analogues bind to TS at a site different from that of FdUMP, combining 5-FU with selective TS inhibitors could potentiate the actions of these agents. TS inhibition might be enhanced by using both a dUMP and a folate analogue to stabilise the ternary complex. The combination of AG331 or ZD1694 with 5-FU led to enhanced FdUMP binding to TS and increased the growth inhibitory effect of these compounds on colon cancer cell lines (Van der Wilt *et al.*, 1995).

Aims

There were three main aims to this thesis. TS has been found to be part of a multienzyme complex in T4 phage and bacteria. The first aim was to establish whether VZV TS was found to be associated with any other proteins involved in viral DNA synthesis in mammalian cells.

Previous mutagenesis studies on the highly conserved residues of TS had revealed that many of these residues were not essential for enzyme function. Therefore the second aim was to generate a series of random temperature sensitive mutants which may identify residues that are critical for enzyme function. Initially buried regions of the protein will be targeted as these are the regions in which the bulk of mutations leading to protein instability are expected to arise. Continuing on from the initial mutagenesis experiments the exposed C-terminus and DRTG loop region will be targeted as these are the regions which show most movement upon ligand binding. There appears to be a difference between the bacterial TSs and the VZV TS in their response to removal of the C-terminal residue. As described above the removal of the C-terminal residue from prokaryotic but not VZV TS led to complete inactivation of the enzyme. The identification of differences between the VZV and other enzymes could therefore assist the development of species specific inhibitors.

An asparagine to aspartic acid mutation in the completely conserved residue N229 of *L.casei* led to the enzyme converting from a dUMP hydroxymethylase to a dCMP hydroxymethylase, and similar results were observed in *E.coli*. Therefore, the final aim of my thesis was to produce the corresponding mutation in asparagine 214 of VZV in order to establish whether this enzyme behaved in a similar manner to the prokaryotic enzymes.

Chapter 2. Materials

2.1. Plasmids

Plasmid	Source
pBS$^-$	Stratagene
pET 23d	Novagen
pAD768	VZV TS-containing BamHI/HindIII cloned into pBS$^-$ (P.T. Harrison, PhD thesis, Glasgow University, 1992)

2.2. Bacteria and bacteriophage strains

Bacterial strains

Strain	Genotype
BL21(DE3)*	F$^-$, *ompT, hsdSB, gal, dcm* , (DE3)
BL21(DE3)pLysS	F$^-$, *ompT, hsdSB, gal, dcm,* (DE3), (CmR)
BW313	*relA1, spoT1, dut-1, ung-1, thi-1,* Hfr KL16 (PO45)
NM522*	*supE, thi*, D(lac-*pro*AB), *Dhsd*5(r-, m-). [F', *pro*AB,]
TG1*	*supE44, hsd5, thi1*, Δ(*Lac-pro*AB)/ F'[*tra*D36, *pro*AB$^+$, *lac*I^q, *lacZ* M15]
XL1*	*rec*A1, *end*A1, *gyr*A96, thi-1, *hsd*R17, *sup*E44, *rel*A1, lac[F'*pro* AB *lac*I^q, *lacZ* M15 Tn10 (Tet^r)]

Note: *thy A* derivatives were made of strains marked *

Bacteriophage

Bacteriophage M13 R408 and M13 K07 used in the production of single stranded DNA from phagemid vectors were laboratory stocks.

2.3. Bacterial growth media

Liquid media

Luria-Bertani Medium (LB)

Per litre:

NaCl	10g
Bactopeptone	10g
Yeast extract	5g

Adjusted to pH 7.0 prior to sterilisation

2xYT Medium (2YT)

Per litre:

Bactotryptone	16g
Yeast extract	10g
NaCl	5g

Adjusted to pH 7.0 prior to sterilisation

All LB media (both liquid and solid) was supplemented with 50μg/ml thymidine unless otherwise stated.

Minimal Medium (M9)	
Per 400 mls:	
dH_20	300ml
M9[x4] salts	100ml
15% casamino acids	20ml
20% glucose	4ml
1M $MgCl_2$	0.5ml
0.8mg/ml Vitamin B1	0.1ml

M9[x4] salts	
Per litre:	
$Na_2HPO_4.7H_20$	24g
KH_2PO_4	12g
NH_4Cl	4g
$NaCl_2$	2g

Solid media

LB

As above except supplemented with 15g Bacto-agar

M9

As above except 300ml dH_20 replaced by 300ml 2% Bacto-agar

M9T

As above except 300ml dH_20 replaced by 300ml 2% Bacto-agar with 20mls 1mg/ml thymine.

Top agar

0.6% bacto-agar in dH_20

2.4. Chemicals and reagents

All chemicals were purchased from BDH chemicals Ltd, Poole, Dorset, or from Sigma (London) Ltd unless given below.

Ammonium persulphate (APS)	Bio-Rad Laboratories
Ampicillin	Smithkline and Beecham
Bactopeptone	Difco
Casamino acids	Difco
Deoxynucleoside triphosphates	Pharmacia LKB
Ecoscint A	National Diagnostics
EN^{3}HANCE	Du Pont (U.K.) Ltd.
Hydroxyapatite solid	Bio-Rad Laboratories

IPTG	Calbiochem-Novabiochem Corporation
N,N,N',N-tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories
Organic solvents	May and Baker
Protein assay reagent	Bio-Rad Laboratories
Replicote™	BDH
S-Sepharose	Pharmacia LKB

2.5. Radiochemicals

All radiochemicals were purchased from Amersham International Ltd, Maidstone, UK.

Nucleotide	Activity	Concentration	Code
[α^{35}S]dATP	37Tbq/mmol	1mCi/mmol	(SJ1304)
5-[^{3}H]dUMP	622GBq/mmol	17.8Ci/mmol	(TRK287)

2.6. Enzymes and enzyme buffers

Calf intestinal phosphatase (CIP)	Life Technologies, Inchinnan Business Park, Scotland
DNase 1	Sigma
Klenow fragment of DNA polymerase	New England Biolabs, Bishops Stortford, UK.
Restriction enzymes	Boehringer Mannheim, Lewes, E. Sussex, UK. New England Biolabs, Bishops Stortford, UK.
RNase A	Sigma
Sequenase™	United States Biochemicals
T4 gene 32 protein	Pharmacia LKB, Uppsala, Sweden
T7 DNA polymerase	Pharmacia LKB, Uppsala, Sweden.
T4 Ligase	Life Technologies, Inchinnan Business Park, Scotland
T4 polynucleotide kinase	New England Biolabs, Bishops Stortford, UK
5xligation buffer	Life Technologies, Inchinnan Business Park, Scotland.

Buffer H	Boehringer Mannheim, Lewes, E. Sussex, UK

2.7. Buffers and solutions

Commonly used buffers and reagents

Enzyme dilution buffer	130mM Tris.HCl pH 7.5, 0.125mM DTT, 0.15% BSA
Ø/$CHCl_3$	Phenol/chloroform 1:1 ($^{v}/_{v}$)
TBE	90mM Tris.HCl, 90mM boric acid, 1mM EDTA
TE	10mM Tris.HCl pH 8.0, 1mM EDTA
TES buffer	50mM Tris.HCl pH 7.4, 5mM EDTA, 50mM $NaCl_2$
TM buffer	0.1M Tris.HCl pH 8.0, 0.1M $MgCl_2$

Reagents for DNA manipulations

Loading buffer	15% ($^{w}/_{v}$) ficoll, Bromophenol blue in 1xTBE
STET lysis buffer	8% ($^{w}/_{v}$) sucrose, 50mM Tris.HCl pH 8.0, 40mM EDTA, 1% ($^{v}/_{v}$)Triton X-100
Lysozyme solution	0.4% ($^{w}/_{v}$) egg white lysozyme, 50mM glucose, 25mM Tris.HCl pH 8.0, 10mM EDTA
TSB(transformation storage buffer)	7.4ml LB, 2ml 50% PEG 4000, 0.1ml 1M $MgSO_4$/1M $MgCl_2$, 0.5ml DMSO
NaOH/SDS solution	0.2M NaOH, 1% ($^{w}/_{v}$)SDS
Potassium acetate/acetic acid solution	60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5 ml dH_2O

Sequencing solutions

Labelling mix	1.5µM dGTP, 1.5µM dTTP, 1.5µM dCTP
Termination mixes (G, A, T, C):-	
G contained	8µM ddGTP, 80µM dGTP, 80µM dATP, 80µM dTTP, 80µM dCTP, 50mM NaCl
A contained	8µM ddATP, 80µM dGTP, 80µM dATP, 80µM dTTP, 80µM dCTP, 50mM NaCl

T contained	8μM ddTTP, 80μM dGTP, 80μM dATP, 80μM dTTP, 80μM dCTP, 50mM NaCl
C contained	8μM ddCTP, 80μM dGTP, 80μM dATP, 80μM dTTP, 80μM dCTP, 50mM NaCl
Annealing buffer (5xTM)	0.1M Tris.HCl pH 8.0, 0.1M $MgCl_2$
Stop solution	95% ($^v/_v$) deionised formamide, 20mM EDTA pH 7.5, 0.05% ($^w/_v$) Xylene cyanol FF, 0.05% ($^w/_v$) bromophenol blue

Reagents for oligonucleotide purification

Oligo elution buffer	500mM ammonium acetate, 10mM $MgCl_2$, 1mM EDTA, 0.1% ($^w/_v$) SDS
Oligo loading buffer	90% ($^v/_v$) deionised formamide in 1xTBE

Reagents for protein purification

Sonication buffer	50mM Tris.HCl pH7.4, 10mM DTT, 0.1% ($^v/_v$)Triton X-100
Sonication buffer B	20mM Hepes pH7.6, 25% ($^v/_v$) glycerol, 600mM NaCl, 1.5M $MgCl_2$, 0.2mM EDTA, 0.1% ($^v/_v$) NP40
1M potassium phosphate buffer	1M KH_2PO_4 mixed with 1M K_2HPO_4 to give pH 7
Potassium phosphate buffer	1M potassium phosphate pH 7, 1M DTT, 100% ($^v/_v$) glycerol

Reagents for protein gels

5x Electrode buffer	52mM Tris base, 53mM glycine, 0.1% SDS
Boiling mix	80mM Tris.HCl pH 6.8, 2% ($^w/_v$) SDS, 0.1M DTT 20% ($^v/_v$) glycerol, Bromophenol blue
Coomasie blue stain	1.25g Coomasie blue, 228ml methanol, 45ml acetic acid, made to 500ml with dH_20
Destain solution	50% ($^v/_v$) dH_20, 40% ($^v/_v$) methanol, 10% ($^v/_v$) acetic acid

Tritium release assay solutions

[2x] assay mix	128mM NaF, 260mM Tris.HCl pH 7.5, 64mM sucrose, 0.3% ($^{w}/_{v}$) BSA, 0.25mM DTT, 38mM HCHO, 1.4μM tetrahydrofolate
Stop solution (Tritium release)	3 volumes 33% ($^{v}/_{v}$) TCA, 1 volume 5mg/ml dUMP

Reagents for spectrophotometric assays

Spectro assay buffer	50mM TES pH7.4, 25mM $MgCl_2$, 6.5mM HCHO, 1mM EDTA, 75mM mercaptoethanol
Folate dilution buffer	30mM $NaHCO_3$, 15mM HCHO, 100mM mercaptoethanol

Reagents for protein binding assay

Buffer A	20mM Hepes.NaOH pH7.5, 10% ($^{v}/_{v}$) glycerol, 1mM DTT, 1mM EDTA, NaCl at varying concentrations
Coupling buffer	0.2M $NaHCHO_3$, 0.5M NaCl, pH 8.3
HiTrap Buffer A	0.5M ethanolamine, 0.5M NaCl, pH 8.3
HiTrap Buffer B	0.1M acetate, 0.5M NaCl pH7.4

Reagents for isolation and solubilization of inclusion bodies

Lysis buffer	50mM Tris.HCl pH 8.0, 25% ($^{w}/_{v}$) sucrose, 1mM EDTA
Detergent buffer	0.2M NaCl, 1% ($^{v}/_{v}$) deoxycholic acid, 1% ($^{v}/_{v}$) NP40
Triton X-100/EDTA solution	1% ($^{v}/_{v}$) Triton X-100, 1mM EDTA
Urea-containing buffer	50mM phosphate buffer with varying concentrations of urea

Chapter 3. Methods

3.1. Growth and maintenance of bacteria and bacteriophage

3.1.1. Overnight cultures

A well isolated single colony or 50µl of a glycerol stock was used to inoculate 5mls of L-broth in a 20ml universal, supplemented with the appropriate antibiotics and thymidine. The culture was incubated with vigorous shaking at 37°C overnight.

3.1.2. Bacterial glycerol stocks

A 10ml overnight culture of the appropriate clone was set up in a 20ml universal. Cells were harvested by centrifugation at 7000rpm for 5 minutes in a Beckman centrifuge. The cell pellet was resuspended in 5ml of 2% bactopeptone. 5mls 80% glycerol and 20µl 25mg/ml thymidine were added to this, mixed and stored at -70°C.

3.1.3. Antibiotics

Antibiotics were used at the following final concentrations, unless otherwise stated: Ampicillin (Amp) 100µg/ml, Kanamycin 75µg/ml, Chloramphenicol 25µg/ml.

3.1.4. Titration of helper phage

A 10ml overnight culture of the appropriate bacterial cells was set up. 3ml aliquots of molten top agar were equilibrated to 45°C in a waterbath. A series of 1 in 10 dilutions of the M13 R408 or M13 K07 helper phage were made in T2 buffer. 100µl cells and 100µl of the diluted helper phage (in the range 10^{-6} to 10^{-12}) were added to the equilibrated top agar, mixed together and immediately poured on to LB plates. Once the top agar layer had solidified the plates were incubated overnight at 37°C. Helper phage plaques were counted the next day and the titre calculated.

3.1.5. Growth of helper phage

An individual phage plaque was transferred into 0.5 ml LB. This was vortexed and incubated at 37°C to liberate phage. 0.25mls was used to infect 150mls of exponentially growing *E.coli* NM522 Thy^- cells which were incubated with vigorous shaking at 37°C overnight. The overnight culture was cleared of bacteria by centrifugation at 9K for 10 minutes at 4°C. The supernatant was decanted to a fresh

tube and recleared by a further centrifugation step prior to heat-inactivation at 65°C for 20 minutes. The phage stock was allowed to cool to room temperature and stored at 4°C. The titre of the helper phage was determined as described above.

3.2. Bacterial manipulation

3.2.1. DMSO-PEG 4000 transformation (Chung and Miller, 1988)

This method is the one routinely used for the transformation of bacterial cells. 20ml of LB was inoculated with 1ml of NM522 Thy^- bacterial cells and incubated at 37°C with vigorous shaking until A_{600} was ~ 0.3. Cells were pelleted by centrifugation at 9K for 5 minutes at 4°C and resuspended in 2ml transformation storage buffer (TSB). The resuspended cells were made competent by incubation on ice for 15 minutes.

10 to 100ng of DNA was added to 100µl of the competent cells and incubated on ice for 30 minutes. 900µl of LB (supplemented with thymidine) was added to the cells and incubated at 37°C for 1 to 1·5 hours. 100µl was spread on to LB plates supplemented with ampicillin and thymidine and incubated at 37°C overnight. A positive (~10ng plasmid DNA) and a negative (no DNA) control were also included in this experiment.

3.2.2. Calcium chloride transformation (Maniatis *et al.*, 1985)

In order to overcome some of the solubility problems that occurred during protein purification from BL21(DE3) cells, the vector containing the variant of interest was transformed into BL21 pLysS using the $CaCl_2$ technique. The strain transforms poorly using the DMSO PEG4000 technique. 10µl from an overnight culture of BL21 pLysS cells was inoculated into 10mls 2YT containing 25µg/ml chloramphenicol and grown for ~3 hours at 37°C. The cells were pelleted at 3K for 3 minutes in a Sorvall SS34 rotor (4°C) and the pellet was resuspended in 0.5ml ice-cold 0.1M $MgCl_2$. The cells were re-pelleted as before and resuspended in ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 minutes. 5µl of DNA was added to 100µl of the competent cells and incubated on ice for 20 minutes prior to heat shock at 42°C for 90 seconds. Cells were added to 0·5ml 2YT broth and incubated at 37°C, with vigorous shaking for 30 minutes. 100µl was spread onto LB plates containing ampicillin and chloramphenicol.

3.2.3. Electroporation

Bacterial electroporation was carried out as described in the Invitrogen Electroporator II manual, version 3.0.

Preparation of electrocompetent cells (Dower *et al.*, 1988)

500ml of LB was inoculated with a 10ml overnight culture of NM522 Thy^- and incubated at 37°C with vigorous shaking until A_{600} was 0.5-0.6. Cells were split into two centrifuge bottles and incubated on ice for 30 minutes and then pelleted by centrifugation at 3.5K for 15minutes in a Sorvall GS3 rotor. Supernatant was removed and the pellet was then thoroughly washed: the cells were resuspended (by shaking) in 50mls ice-cold water, 50ml volumes of water were continually added to the cells until a final volume of 500mls was reached. The suspended cells were centrifuged at 3.5K for 15 minutes and the pellets were resuspended in 10mls of ice-cooled 10% glycerol and combined. The cell suspension was then added to pre-cooled 30ml tubes and spun in a pre-cooled Sorvall SS34 rotor at 5800K for 15 minutes, the supernatant was decanted and pellet was resuspended in 1% glycerol by vortexing. The final cell suspension was aliquoted in 40µl amounts into microfuge tubes in a dry ice bath and stored at -70°C.

3.2.4. Generation of *thyA* strains of *E.coli* (Stacey & Simpson, 1965)

2mls minimal media supplemented with thymidine (TdR) and trimethoprim (20µg/ml) was inoculated with 40µl of an appropriate overnight culture and incubated, with vigorous shaking, at 37°C overnight. 40µl of this culture was used to inoculate 2mls of fresh media and incubated as before. A single loop of this culture was used to streak for single colonies on minimal media plates supplemented with TdR and incubated at 37°C overnight. Individual single colonies were patched onto minimal media plates and minimal media plates supplemented with TdR and incubated at 37°C overnight. Colonies growing only on minimal media plates supplemented with TdR were classified as Thy^- strains.

3.3. DNA isolation

3.3.1. STET lysis plasmid DNA preparation

1.5ml from a 5ml overnight culture was centrifuged for 40 seconds in the microfuge (12K) to pellet the cells. The cell pellet was resuspended in 200µl STET buffer and 20µl lysozyme (10mg/ml) was added. The solution was boiled for 40 seconds and centrifuged for 10 minutes at 12K. The debris and chromosomal DNA was present as a fluffy pellet and removed from the supernatant using a toothpick. 0·1 volumes 3M sodium acetate and 2 volumes isopropanol were added to the supernatant and placed in a dry ice/ethanol bath for 5 minutes. The DNA was precipitated by centrifugation at 12K for 10 minutes and the pellet was dried under vacuum. The lyophilised pellet was resuspended in 20µl dH_20.

3.3.2. Qiagen™ midi plasmid preparation

100mls of LB supplemented with ampicillin was inoculated with 10mls of an overnight culture of the appropriate plasmid containing *E.coli* strain and incubated in a 100ml conical flask overnight at 37°C with vigorous shaking.

The plasmid was isolated from the bacteria as described in the MIDI protocol of the Qiagen™ handbook.

3.3.3. DNA template preparation for double stranded sequencing (Maniatis *et al.*, 1985)

A single colony was inoculated into 5mls LB and grown at 37°C overnight. 1.5ml of the culture was spun down in microfuge for 1 minute and the cell pellet was resuspended in ice-cold 50mM glucose, 10mM EDTA and 20mM Tris.HCl pH 8.0. 50µl of lysozyme solution was added prior to incubation at room temperature for 5 minutes. 200µl of NaOH/SDS solution was added, the solution was mixed and incubated at room temperature for a further 5 minutes. 150µl of potassium acetate/acetic acid solution was added, the contents were mixed and incubated on ice for 15 minutes. The solution was centrifuged for 5 minutes in microfuge (12K) and the supernatant was removed to a fresh tube. 1ml of ethanol was added and the solution was incubated at room temperature for 1 minute. The DNA was pelleted by centrifugation at 12K for 5 minutes, the supernatant was discarded, 200µl ethanol was added and the solution incubated at room temperature for 1 minute. The DNA was repelleted by a further 5 minute centrifugation at 12K, the pellet was dried under vacuum and resuspended in 50µl water.

To denature the DNA, 4µl 2M NaOH and 4µl 2mM EDTA were added to the 50µl sample, mixed and incubated at 90°C for 5 minutes. 8µl 2M sodium acetate pH 5.0, 14µl of water and 160µl ethanol were then added prior to incubation at room temperature for 10 minutes. The denatured DNA was pelleted by centrifugation at 12K for 20 minutes and washed in 70% ethanol. The pellet was dried under vacuum and resuspended in 10µl dH_20.

3.3.4. ssDNA sequencing preparation (Katayama, 1990)

A single colony was inoculated into 5ml 2YT broth containing ampicillin (100µg/ml), thymidine (50µg/ml) and M13 K07 helper phage ($2x10^8$ pfu/ml) and incubated at 37°C with vigorous shaking for 2 hours. Kanamycin was added to a final concentration of 70µg/ml and the culture incubated at 37°C with vigorous shaking overnight.

3x 1.5ml samples from the 5ml overnight were centrifuged at 12K for 10 minutes in a microfuge to pellet the cells. 1ml of the supernatant was transferred to a fresh eppendorf tube and 300µl of a 2.5M NaCl/20% PEG 4000 solution was added, vortexed and incubated on ice for 20 minutes. The phage was pelleted by centrifugation in a microfuge at 12K for 5 minutes and the supernatant discarded. The phage pellets were resuspended and pooled together in 400µl 0.3M sodium acetate. An equal volume of Ø/$CHCl_3$ was added, the solution was vortexed vigorously and microfuged for 5 minutes. The top aqueous layer was transferred to a fresh eppendorf tube and 1ml ethanol added prior to incubation at room temperature for 30 minutes. The ssDNA was pelleted by centrifugation at 12K for 5 minutes and the supernatant carefully discarded. The ssDNA pellet was dried in the speedivac for 5 minutes, resuspended in 10µl TE buffer and stored at -20°C.

3.3.5. Uracil-ssDNA preparation (Künkel, 1985; Künkel *et al*, 1987)

Uracil-enriched ssDNA for use in mutagenesis experiments was prepared as follows. 10mls of 2YT (100µg/ml ampicillin and 100µg/ml uridine) was inoculated with 50µl glycerol stock of *E.coli* BW313 dut^- ung^- containing plasmid pAD768 and incubated at 37°C with vigorous shaking overnight. The 10ml culture was added to 200ml of 2YT supplemented with ampicillin and uridine (100µg/ml) in a 2 litre baffled flask and incubated at 37°C with vigorous shaking until A_{600} was 0.5. M13 R408 helper phage was added to a final pfu/ml of $5x10^9$ and the culture incubated at 37°C with vigorous shaking for a further 8 hours. The cells were pelleted by centrifugation at 7K for 20 minutes using a Sorvall GS3 rotor and the supernatant kept and spun again. To the final supernatant 0.25 volumes of 3.75M ammonium acetate/20% PEG 6000 solution was added, mixed, split in two and incubated on ice for 30 minutes. The phage precipitate was collected by centrifugation at 9K for 30 minutes using a Sorvall GS3 rotor. The phage pellet was allowed to air dry for 10 minutes and then resuspended in TE buffer (400µl per pellet). The protein was removed by chloroform extracting twice and Ø/$CHCl_3$ extracting 3 to 6 times or until the interface became clear. The aqueous phase was chloroform extracted one final time and removed to a fresh tube. 0.36 volumes 7.5M ammonium acetate and 2.5 volumes ethanol were added and the solution was incubated on ice for 20 minutes. The ssDNA was pelleted by centrifugation at 12K for 20 minutes, washed with 70% ethanol and dried under vacuum. The pellet was resuspended in 50µl dH_20. This ssDNA template was checked to ensure it was uracil enriched by transforming 1µl sample into a dut^+ ung^+ host (NM522) and a dut^- ung^- host (BW313). The concentration of ssDNA produced was estimated spectrophotometrically (1 A_{260} unit = 40µg/ml ssDNA).

3.4. DNA manipulations

3.4.1. Restriction enzyme digests

Restriction enzyme digests were usually carried out in 10 to 50µl reaction volumes. Restriction enzyme buffers are available commercially at 10x concentration and were diluted accordingly. Enzymes were added at 1 to 10 units (U) per µg DNA and incubations performed at 37°C for 3 hours or overnight.

When mini or STET prep DNA was digested, RNase was added to a final concentration of 20µg/ml for the last hour of the incubation. Digested DNA to be used in cloning experiments was always Ø/$CHCl_3$ extracted (to remove the restriction enzymes) and ethanol precipitated prior to use.

3.4.2. Removal of 5'-phosphate groups

1 unit of calf intestinal phosphatase (CIP) was added to the restriction digest reactions and incubated as normal. The DNA was extracted with Ø/$CHCl_3$ and ethanol precipitated prior to cloning.

3.4.3. DNA ligations

Ligations were usually performed in a total volume of 20µl. 100ng of vector and 17ng insert DNA were mixed with 4µl 5x ligation buffer, 1.5 units T4 DNA ligase and the volume made up to 20µl with dH_20. The ligation mix was incubated at 15°C overnight. 10µl of the ligated DNA was used for transformation of *E.coli.*

3.4.4. Isolation of DNA from agarose gels

DNA was isolated from agarose gels using a Pharmacia Sephaglass™ kit as per the manufacturer's instructions.

3.5. Oligonucleotides

3.5.1. Synthesis and purification

Oligonucleotides for sequencing primers and site-directed mutagenesis were synthesised on a Cruachem PS250 DNA synthesiser. Oligonucleotides were provided still attached to the column upon which they were synthesised. Removal of the oligonucleotide involved flushing the column with one column volume (~0.25ml) of concentrated ammonia solution, and incubating at room temperature for 20 minutes. This process was repeated until 1.5ml of concentrated ammonia had been flushed through the column. The oligonucleotides were incubated at 55°C for 5 hours to remove

the protecting group and subsequently dried overnight under vacuum. The pellet was resuspended in 50µl dH_20 and stored at -20°C until required.

15µl of the oligonucleotide was mixed with an 25µl of loading buffer (1xTBE, 90% deionised formamide) and boiled for 2 minutes. The sample was loaded on to a 15% polyacrylamide gel along with 5 to 10µl formamide dyes (in a separate well) as a marker track. Electrophoresis was performed at 400V in 1xTBE buffer. For oligonucleotides of length 15 to 30 bases the Bromophenol blue band was allowed to migrate approximately 7cm down the gel (~1 hour). For oligonucleotides 30 to 60 bases in length the Bromophenol blue band was allowed to migrate to approximately the end of the gel (~2-3 hours).

To isolate the oligonucleotide the gel was removed from the glass plates and laid on cling film. The DNA was visualised using the shadow casting technique, which involved illumination of the gel whilst on a thin layer chromatography (TLC) plate using a short-wave UV lamp. The DNA appears as a dark band against a uniform green fluorescent background. The slowest moving band towards the top of the gel was excised, cut into several smaller pieces and eluted in 0.5 ml oligonucleotide elution buffer overnight at 37°C. The eluted oligonucleotide was centrifuged at full speed in a microfuge for 5 minutes to remove any polyacrylamide. The supernatant was carefully removed to a fresh eppendorf tube and phenol/chloroform extracted once followed by a chloroform extraction. The DNA was precipitated with two volumes of ethanol, placed in a dry ice/ethanol bath for 10 minutes and centrifuged at full speed in the microfuge for 10 minutes. The DNA pellet was resuspended in 50µl of dH_20 and stored at -20°C prior to use. The concentration of DNA was determined spectrophotometrically (1 A_{260} unit = 33µg/ml oligonucleotide).

3.5.2. Spiked oligonucleotides

Spiked oligonucleotides for use in region directed mutagenesis were synthesised on the Biosearch 8600 DNA synthesiser using phosphoramidite mixes containing 96% of the normal nucleotide and 1.33% of each of the other three nucleotides (Ner *et al.*, 1988; McNeil & Smith, 1985).

3.5.3. Oligonucleotide sequences

A complete list and sequences of all the oligonucleotides used in this project are shown below.

Oligonucleotide for site-directed mutagenesis

Sequence	Mutation
TCC-AGC-AAT-GTC-GAA-CGG-TAC	N214D

Spiked oligonucleotides for region directed mutagenesis

Number	Sequence targeted (see Figure 6.1)	Size
RO1	CAA-ATT-GTA-TCG-AGC-TTG-CAT-TCC-AAA-TAA AGA-TAA-CGT-TCC-GAT-TCC-TGT-TCG-ATC	57mer
RO9	GGT-AAG-AAG-TGC-ATA-TCC-AGC-AAT-GTT-GAA CGG-TAC-CCC-AAG-GCC-CAT-ATC-CCC	54mer
RO11	ATC-TAT-ATG-ATT-CAA-GTA-AAT-ATG-TGC-ATC CCC-CAT-TGT-ATG-AAT-TAA-ATC-TCC	54mer
RO13	TCG-ATC-GCG-TTT-CCG-AAC-TCC-ATA-CCT-TAA AAT-ATC-ATC-CAC-TTG-TTT-TAA-GTA-CTG	57mer
RO15	CGC-TCC-AAA-ATG-TCT-CCA-CTG-GAA-GCC-GTA AAT-GGG-GCC-AAG-GTC-CCC-CGT-GTG-TCT	57mer
RO19	TTA-AAG-AGC-CAT-TTC-CAT-TTT-TAG-GGG-GGG GTG-TGG-ATT-ATA-TCC-ATC-AAG-CTG-AAA	57mer
RO20	AGC-CAT-TTC-CAT-TTT-TAG-GGG-GGG-GTG-TGG ATT ATA-TCC-ATC-AAG-CTG-AAA	51mer

Sequencing primer oligonucleotides

Number	Primer sequence (see Figure 6.1)
59	GTCTGTGTATATCAT
58	GTCCCATTTAAAGTC
60	CCATTGTATGAATTA
57	TGGAGGTAGTACCAT
61	TTTAATTGTATCTAT
62	CCACGACGGCCCTCC

3.6. Mutagenesis

3.6.1. Oligonucleotide site-directed mutagenesis

Oligonucleotide site directed mutagenesis was used to introduce a specific mutation at a defined site within the TS gene. The strand selection scheme of Künkel was employed using a uracil-enriched template strand which is degraded in a dut^+ ung^+ host strain (Künkel, 1985). 500pmole oligonucleotide was phosphorylated in 4µl 5x ligation buffer containing 10 units of T4 polynucleotide kinase at 37°C for 30 minutes.

The T4 kinase was inactivated by incubation at 65°C for 15 minutes. The phosphorylated oligonucleotide was diluted with dH_20 to a final concentration of 5pmole/μl. 1μl phosphorylated oligonucleotide was annealed to 0.1pmole U-ss DNA (estimated spectrophotometrically) in 10μl TM buffer at 37°C for 30 minutes. The annealed oligonucleotide was filled in by the addition of 1μl 5mM dNTPs (a 5mM solution of all four dNTPs), 5 units Klenow fragment of DNA polymerase I, 1 unit of T4 ligase and 2.0μg gene 32 protein in 20μl ligation buffer. After a 2 hour incubation a further 5U Klenow and 1U of ligase were added and incubated at room temperature for another 2 hours. The DNA was stored at -20°C.

3.6.2. Region directed mutagenesis

Spiked oligonucleotides were designed to have on average two base changes per oligonucleotide. They were used to introduce random mutations to a targeted region of the TS gene (see Figure 6.1). Mutagenesis was performed as outlined for site-directed mutagenesis (see section 3.6.1), again using the strand selection system of Künkel.

3.7. Mutant screening

3.7.1. Growth complementation assays

1 to 5μl mutagenised DNA from both the site-directed mutagenesis and region directed mutagenesis was used to transform NM522 Thy^- bacteria. Transformants were picked, using sterile toothpicks, patched onto M9 and M9T plates and incubated overnight in order to determine whether the colonies were Thy^+ or Thy^-.

3.7.2. Temperature sensitive clones

Clones obtained from region directed mutagenesis using spiked oligonucleotides were screened for temperature sensitivity. Amp^R colonies (grown at 37°C on LB agar plates) were picked, using the blunt end of a toothpick and patched onto three M9 and three M9T plates which had been divided into 50 patches by a template. The same toothpick was used to patch all six plates. The plates were incubated at three temperatures, 30°C, 37°C and 42°C for 24 hrs and the growth was scored. The plates were patched in the order 42°C, 37°C and 30°C to ensure that failure to grow at the high temperature was not due to an inadequate inoculum. Temperature sensitive mutants gave a Thy^+ phenotype at 30°C but a Thy^- phenotype at 37°C and 42°C.

3.7.3. Restriction digests

Candidate mutant clones were screened by restriction digest analysis of the plasmid DNA prior to sequencing to remove those clones that did not give the correct restriction pattern and contained "monster" plasmids arising from aberrant filling-in reactions. Restriction analysis was performed as described in section 3.4.1.

3.7.4. DNA sequencing

All DNA sequencing was performed in a 96 well micro-titre plate using a Sequenase™ kit. 3µl (~10-30ng) sequencing primer, was annealed to 0.1-1.0µg of DNA (ssDNA or dsDNA) in 10µl 1x reaction buffer by incubating at 37°C for 30 minutes in an eppendorf tube. The annealed reaction mix was transferred to a micro-titre plate and 1µl DTT, 2µl label mix (previously diluted 1 in 3), 1-2µl [α-^{35}S]dATP, and 2µl Sequenase enzyme (previously diluted 1 in 8) added. The plate was spun in a centrifuge at 1K for 30 seconds to mix the reagents and incubated at room temperature for 5 minutes. 3.5µl of this mix was transferred to a separate well containing 2.5µl of ddGTP. This was repeated for the other three dideoxy termination mixes (ddATP, ddTTP, ddCTP) and the plate sealed with a plate sealer (Flow Laboratories Ltd or Corning) and incubated at 37°C for 15 minutes. 4µl of stop solution was added to the sequencing reaction and the plate boiled for 2 minutes prior to loading.

Sequencing reactions were resolved on a 4% polyacrylamide (7.7M urea, 0.5x TBE gel). Prior to use both sequencing plates were coated with Repelcote™ and washed once with 100% ethanol before the gel was poured. Electrophoresis was performed at 60W in 0.5xTBE. For normal runs the bromophenol blue was used as a marker to terminate electrophoresis. For longer runs the gel was run for a further 1-2 hours. The sequencing gel was washed in 10% acetic acid for 30 minutes, transferred to 3MM paper and dried on a vacuum gel drier for 90 minutes. The gel was exposed to Kodak S 100 film overnight at room temperature.

3.8. Mutant characterisation

3.8.1. Crude TS extracts (Thompson *et al.*, 1987)

350ml LB supplemented with ampicillin (100µg/ml) and thymidine was inoculated with 10ml of an overnight culture of a suitable clone and incubated at 37°C (30°C for the temperature sensitive mutants) with vigorous shaking until A_{600} ~ 0·5. A small volume of culture was removed and kept on ice for a phenotype check (see later). The cells were pelleted by centrifugation at 7K for 10 minutes (Sorvall GS3 rotor) at 4°C. The supernatant was discarded and the pellet resuspended in 10ml TES buffer and

vortexed to disrupt any lumps. The suspension was transferred to a 15ml falcon tube and the cells were pelleted once more (MSE bench top centrifuge) by centrifugation at 3K for 10 minutes at 4°C. The supernatant was removed using a pipette and the pellet was frozen/thawed once, resuspended in 2 volumes of sonication buffer. Sonication was performed (using a Branson sonifier) with the microprobe on setting 5 and entailed 3x30 second bursts with 30 seconds cooling (on ice) between each burst. The crude sonicate was transferred to eppendorf tubes and spun at 12K for 10 minutes in a pre-cooled microfuge at 4°C. The supernatant was removed to fresh tubes and the protein concentration determined prior to being snap frozen in a dry ice/ethanol bath and stored at -70°C.

To check the phenotype of the extract a drained loop full of the culture was streaked to single colonies on a LB agar plate (supplemented with 50μg/ml thymidine only) and incubated at 30°C, 37°C or 42°C overnight. Single colonies were then patched on to M9, M9 plus thymidine and LB (supplemented with 100μg/ml ampicillin and 50μg/ml thymidine) agar plates and incubated at 30°C, 37°C or 42°C for temperature sensitive clones.

3.8.2. Protein concentration determination

A modified version of the Bradford assay was used to determine the protein concentration of crude and purified protein extracts. Assays were performed in micro-titre plates using an Anthos HTII plate reader at a wavelength of 620nm.

400μl of Bradford reagent was aliquoted into a series of wells and 10μl BSA protein standards were added with concentrations ranging from 0.2-1.6mg/ml. 1-10μl of the sample protein was added to a well and mixed prior to reading the plate. The plate reader plots a standard curve from which the amount of protein present in the sample can be calculated.

3.8.3. Tritium release experiments

3.8.3.1. Tritium release assay

This assay is based on the release of tritium as tritiated water from 5-[^{3}H] dUMP to the solvent (Roberts, 1966). 5μl of TS extract was mixed with 10μl [2x] assay mix, 0.4μCi 5-[^{3}H] dUMP and dH_20 to give a total volume of 40μl. The reaction mix was incubated at 30°C for 15 minutes and terminated by addition of stop solution and 940μl 2% charcoal suspension, which was used to absorb any unreacted [^{3}H] dUMP. The sample was vortexed and centrifuged for 5 minutes following which 500μl supernatant was removed into a scintillation vial. 5ml Ecoscint A was added prior to liquid scintillation counting in a Beckman LS5000CE scintillation counter. A total counts

control was included using 5µl dH_2O instead of extract and 940µl dH_20 instead of charcoal suspension. A background control was also included by replacing extract with 5µl dH_2O and adding 940µl charcoal, this value was subtracted from all experimental values. Assays were performed in duplicate on two or more independent extracts for each clone. The amount of extract used was adjusted so that less than 30-60% of the total counts are released. This ensures that assays are in the linear region of the reaction curve.

The amount of dTMP generated is directly equivalent to the amount of [^{3}H] released during the assay. The amount of [5-^{3}H] dUMP added to the reaction in pmol can be calculated from its specific activity. The total counts represents the equivalent amount of product (dTMP)

$$\text{pmol dTMP produced} = \frac{\text{sample CPM}}{\text{Total CPM}} \times \text{pmol 5 -[}^3\text{H] dUMP added}$$

From protein concentration, the specific activity of TS in the crude extracts can be expressed in terms of pmol dTMP produced per µg protein per minute.

3.8.3.2. Temperature profiles

The tritium release assay was also used to examine the temperature profiles of the temperature sensitive mutants. 70µl of the crude extracts were held at 42°C and at 5 minute intervals over a 30 minute period, 5µl samples were removed, added to the tritium release reaction mix at 30°C and assayed (section 3.8.3). A wild type sample was also assayed at the same time to give a control sample.

3.8.3.3. Phenolphthalein inhibition

Phenolphthalein was first identified as an inhibitor of TS using the DOCK computer programme (Shoichet *et al.*, 1993). This programme identifies molecules that will fit into the active site of enzymes and thus act as competitive inhibitors.

The tritium release assay was carried out as normal but with the addition of 50-500µM concentrations of phenolphthalein into the reaction mix. A stock solution of phenolphthalein was made at 1mM in 70% ethanol and a corresponding amount of ethanol was added to the control reactions.

3.8.3.4. Inhibition by folate analogues

Several inhibitors have been produced by ICI which resemble the structure of the folate cofactor, these folate analogues have all been derived from one parent compound. CB3717 (see Figure 1.16). These inhibitors were used at varying concentrations in the tritium release assay.

3.9. Construction of high level expression clones

To enable large quantities of protein to be purified from a bacterial culture system mutant TS genes were cloned into the vector pET23d under the control of the strong bacteriophage T7 promoter. This vector when transformed into the host *E.coli* strain BL21 (DE3) is inducible by IPTG allowing high levels of protein to be expressed.

An *NcoI-EcoRI* fragment from pAD768 which contained the TS coding region was ligated into an appropriately cleaved pET23d backbone. The ligation mix was transformed into NM522 Thy^- cells and the construct was validated by restriction digestion of mini-prep DNA. This step was required because of the poor transformation efficiency of BL21 (DE3) cells. DNA from the NM522 clones was used to transform the constructs into BL21 (DE3) or BL21 (DE3) pLysS cells.

3.10. Purification of mutant enzymes

3.10.1. Preparation and extraction

Both NM522 Thy^- bacteria and BL21 (DE3) Thy^- cells were used for preparation of extracts. A fresh single colony was picked into 20ml LB supplemented with 100μg/ml ampicillin and 50μg/ml thymidine and incubated at 37°C (or 30°C for temperature sensitive mutants) with vigorous shaking overnight. 2.5ml of this overnight was used to inoculate 750ml LB supplemented with 100μg/ml ampicillin and 50μg/ml thymidine in a 2 litre baffled flask. A total of 6 litres (8 flasks) was inoculated and incubated at 37°C or 30°C with vigorous shaking until the A_{600}~ 0.5. At this point 1ml was transferred to a universal and incubated at 37°C or 30°C as before. To the flasks IPTG was added to a final concentration of 0.2mM and incubation at 37°C or 30°C continued for a further 2-3 hours to allow induction of the protein to take place. 1ml of the induced culture was removed to a universal and along with the previous 1ml sample processed (as described in section 3.10.3) to check for protein induction.

The induced culture was centrifuged at 9K for 15 minutes (Sorvall GS3, 4°C) and the resulting pellets were resuspended in a total of 10ml TES buffer. A further 10ml of TES buffer was added and the suspension vortexed to disrupt any lumps. The cells were once again pelleted by centrifugation at 9K for 15 minutes (Sorvall GS3, 4°C) and the supernatant discarded. The bacterial pellet was resuspended in 15ml 50mM potassium

phosphate buffer pH 7.0 and vortexed vigorously prior to sonication. Sonication was performed (using a Branson sonifier) with the microprobe on setting 5 and entailed 3 x 30 second bursts with 30 seconds cooling (on ice) between each burst. The crude sonicate was centrifuged at 18K for 30 minutes (Sorvall SS34, 4°C) and the supernatant removed and kept at 4°C. The remaining cells were resuspended in a further 10ml 50mM potassium phosphate buffer pH 7.0, re-sonicated and spun as before. The two supernatants were combined and stored at -70°C prior to purification.

3.10.2. Preparation of extracts from BL21 pLys S cells

The cells were grown up and pelleted as in section 3.10.1. The combined pellet was frozen then thawed at room temperature and resuspended in 10ml sonication buffer B, 1mM DTT and 0.5mM PMSF were added to the buffer prior to sonication. The cell suspension was sonicated using the Branson sonifier on setting 5 for 10 seconds and the sonicate was transferred to a 5ml tube and pelleted at 35,000K for 20 minutes (ultracentrifuge, Sorvall T865 rotor). The supernatant was recovered and stored at -70°C.

3.10.3. Protein mini-gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed using a Bio-Rad MINI-PROTEAN™ II kit. Gels were cast according to the manufacturers instructions and consisted of a separating gel (12% polyacrylamide, 0.37M Tris.HCl pH 8.8) and a stacking gel (4% polyacrylamide, 0.125M Tris.HCl pH 6.8).

Bacterial cultures to be tested were initially pelleted by centrifugation of 1ml of the appropriate culture in a microfuge (12K) for 1 minute. The supernatant was removed and the pellet resuspended in 50µl dH_2O and then 50µl of boiling mix added. The sample was boiled for 2 minutes prior to loading on the gel. Electrophoresis was performed at 220V in 1x electrode buffer for 45 minutes or until the Bromophenol blue dye front had reached the bottom of the gel. The gel was removed from the casting plates and the top stacking layer carefully discarded. The gel was stained using a Coomassie blue stain for 20 minutes and then destained for 20 minutes. The destain was discarded and the process repeated. Destained gels were air dried overnight between a sheet of porous cellophane (Bio-Rad).

3.10.4. Hydroxylapatite chromatography

Protein to be purified was initially loaded onto a hydroxylapatite (HTP) column and then an S-Sepharose column. All chromatographic procedures were performed at

4°C. A 30ml column was poured by resuspending ~12g hydroxylapatite in 3 volumes of 50mM potassium phosphate buffer pH 7.0 and mixing by gentle swirling. The hydroxylapatite was allowed to settle and the buffer decanted, fresh buffer was added and the process repeated. The washed hydroxylapatite was finally resuspended in an equal volume of 50mM potassium phosphate buffer pH 7.0 and poured in to the column and allowed to settle. To wash and equilibrate the column, two column volumes of 50mM potassium phosphate buffer pH 7.0 were pumped through the column at 1ml/minute.

Samples were kept from all stages of the chromatography (loading, washing and eluting) for analysis on a protein mini-gel (see section 3.10.3). The supernatant usually appeared viscous after sonication and was thus diluted to a final volume of 50ml with 50mM potassium phosphate buffer pH 7.0. The sample was added onto the column at 0·5ml/minute, and the flow through collected. The unbound protein was removed by washing (1ml/minute) the column with 60mls (2 volumes) of 50mM followed by 100mM potassium phosphate buffer pH 7.0. The TS protein was eluted from the hydroxylapatite by washing the column in 2 volumes 300mM potassium phosphate buffer pH 7.0 at 1ml/minute.

Samples were collected from every stage of the HTP column and 10µl aliquots were mixed with 5µl boiling mix and run on a protein gel as before (section 3.10.3) in order to determine whether TS was eluted in the 300mM wash.

3.10.5. Ammonium sulphate precipitation and dialysis

The partially purified TS protein from the hydroxylapatite column was precipitated with 70% ($^{w}/_{v}$) ammonium sulphate. Solid ammonium sulphate was added slowly to the protein solution while stirring on ice and the solution was left stirring for a further 60 minutes. The precipitate was pelleted by centrifugation (Sorvall SS34, 4°C) at 10K for 40 minutes and the pellets were resuspended carefully without frothing in 4ml of 50mM potassium phosphate buffer pH 7.0. The protein solution was dialysed overnight against 1 litre of 50mM potassium phosphate buffer pH 7.0 using Spectra/Por (Mol Wt. cut off 10,000) dialysis tubing. The dialysed protein solution was centrifuged (12K, microfuge) to remove any sediment and the supernatant stored at 4°C prior to loading on to an S-sepharose column.

3.10.6. S-sepharose chromatography

All chromatographic procedures were performed at 4°C. A 10ml S-sepharose column was poured using fast flow S-sepharose suspension (Pharmacia). As before samples were kept from all stages of the chromatography (loading, washing and

eluting) for analysis on a protein mini-gel. The column was washed and equilibrated with 4 column volumes of 50mM potassium phosphate buffer pH 7.0 at 1ml/minute. The dialysed protein sample was loaded directly on to the top of the column and pumped on at 0.5ml/minute. The unbound protein was washed from the column with 2 volumes 50mM potassium phosphate buffer pH 7.0 at 1ml/min. The TS protein was eluted from the column using a salt gradient. The gradient was achieved using mixing chambers which contained 20ml 50mM potassium phosphate buffer pH 7.0 in one chamber and 50mM potassium phosphate buffer pH 7.0 plus 400mM KCl in the other. The gradient was applied to the column at 0·5ml per minute, and fractions collected every 2 minutes.

The Bradford assay was used to visualise which fractions contained the TS protein. These fractions were tested for activity (tritium release assay) and stored at -70°C until required.

3.11. Purification of protein from inclusion bodies

3.11.1. Isolation of inclusion bodies from total cell extracts

Bacterial cells expressing the protein were harvested by centrifugation at 7K for 10 minutes (Sorvall GS3) and resuspended in 10mls lysis buffer. Lysozyme was dissolved in a small volume of lysis buffer and added to a final concentration of 0·1mg/ml. The cell suspension was incubated on ice for 30 minutes and then freeze/ thawed, as the solution was thawed it became viscous. $MgCl_2$, $MnCl_2$ and DNase I were added to the suspension to final concentrations of 10mM, 1mM and 10µg/ml respectively and the viscosity of the solution decreased as the DNA was digested by DNase I. An equal volume of detergent buffer was added to the lysate and centrifuged at 5,900K for 10 minutes (Sorvall SS34). The supernatant was transferred to a fresh tube without disturbing the white tight pellet (inclusion bodies) and upper "jelly-like layer" (membrane proteins). The pellet was resuspended in Triton X-100 solution, to remove most of the membrane proteins, and the inclusion bodies were spun down. This procedure was repeated until the jelly-like was no longer seen.

3.11.2. Solubilisation of inclusion bodies

The inclusion bodies were solubilised in 10 volumes of 8M urea buffer using a pipette and the sample was centrifuged at 20K for 1 hour (Sorvall SS34). A large jelly-like pellet was obtained and the supernatant was collected. The pellet was resuspended in urea-containing buffer, centrifuged again at 20K and the supernatant collected. The supernatants were analysed by SDS-PAGE.

3.11.3. Re-folding of proteins

To remove the urea and allow the protein to refold, the supernatant containing solubilised inclusion bodies was dialysed against decreasing concentrations of urea in 50mM phosphate buffer. The supernatant was dialysed against 50mM phosphate buffer containing 8M urea, 4M urea and 2M urea for 1 hour in each case. Finally the supernatant was dialysed overnight against 50mM phosphate buffer containing no urea. Insoluble material was removed by centrifugation and a sample of the supernatant was collected in each case and run on a mini protein gel (section 3.10.3).

3.12. Kinetic characterisation of mutants

Steady-state kinetic constants were determined using the spectrophotometric assay as described (Pogolotti *et al.*, 1986). Reactions were set up in microplate wells and the change in absorption at 340nm was monitored on an Anthos Labtec HTII microplate reader. 100µl of spectro assay buffer was added to the plate, the concentrations of dUMP and folate were varied and dH_2O was added to give a final volume of 200µl. When dUMP concentration was varied the folate was constant at 880µM; when folate was varied, the dUMP concentration was constant at 1.25 mM.

3.13. Protein binding assays

3.13.1. ^{35}S-Methionine labelled cells

A small flask of MeWo cells were grown in Dulbecco's medium at 37°C until sub-confluent and 1ml of stock VZV was added. 3-4 days post infection the infected cells were transferred to a sub-confluent medium flask and incubated at 37°C until plaques had developed. The cells were then transferred to a large flask. Having allowed plaques to form in the large flask, the medium was decanted and Eagles methionine free medium was added and the cells were incubated at 37°C for 1.5 hours. Following incubation the medium was decanted, fresh medium containing 50µCi/ml ^{35}S-methionine was added and the cells were incubated overnight at 37°C to enable labelling to take place. Cells were removed from the incubator after ~16 hours, the medium was decanted and the cells were washed twice with phosphate buffer saline (PBS). 3mls of Buffer A was added to the flask and the cells were scraped off and pipetted into a 15ml falcon tube, this was repeated using 2ml of buffer. The cells were incubated at 4°C for 1 hour and centrifuged at 3K for 20 minutes (Sorvall SS34, 4°C). The supernatant was removed and the pellet stored at -20°C.

3.13.2. Preparation of labelled cell extracts (Boehmer and Lehman, 1993)

The labelled cells were resuspended in Buffer A (1.2M NaCl), transferred to a Dounce homogeniser and lysed with 10 strokes of the tight fitting pestle. The lysed cells were cleared by centrifugation at 55,000rpm for 60 minutes (ultracentrifuge, Sorvall T865 rotor). Nucleic acid was precipitated from supernatant by the drop-wise addition of 0.05vol of 0.3M spermidineHCl/1MNaCl/0.31 vol of 5% streptomycin sulphate both in buffer A. After a 30 minute incubation on ice the nucleic acid precipitate was removed by centrifugation at 25,000rpm for 30 minutes (ultracentrifuge, Sorvall T865 rotor). The supernatant was dialysed against 500ml buffer A (0.1M NaCl) overnight. After dialysis, the precipitate was removed by centrifugation at 55,000rpm for 30 minutes (ultracentrifuge, Sorvall T865 rotor), and the supernatant was stored at -70°C (~500µl). A sample was removed from the supernatant and retained for gel analysis.

3.13.3. SDS-PAGE analysis of radiolabelled samples

A 50ml SDS-PAGE gel was cast consisting of a separating gel (12% polyacrylamide, 0.37M Tris.HCl pH 8.8) and a stacking gel (4% polyacrylamide, 0.125M Tris.HCl pH 6.8). 10µl of the labelled extracts were added to boiling mix and water to give a final volume of 50µl, and boiled for 2 minutes prior to loading. Electrophoresis was performed at 150V in 1 x electrode buffer until the bromophenol blue dye front had reached the bottom of the gel (4-5hours). The gel was removed from the casting plates and the top stacking layer carefully discarded. The gel was stained using a Coomassie blue stain for 20 minutes. The gel was then destained for 20 minutes, the destain discarded and the process repeated. Following destaining the gel was rinsed in dH_2O and placed in EN^{3}HANCE to improve the detection of the radiolabelled extracts. After 30 minutes the gel was removed and washed for 20 minutes in dH_2O, this process was repeated three times. The gel was dried under vacuum for 1 hour and placed against X-ray film in a cassette case at -70°C, initially overnight and then longer if required.

3.13.4. HiTrap affinity column

Purified protein was bound to a 1ml NHS activated HiTrap column as in the instruction booklet for Pharmacia HiTrap™ affinity columns. The viral infected or mock infected cells were applied and the column was incubated at 37°C for 30 minutes. The column was washed with 2 volumes of buffer F (0.1M NaCl) and developed with 10ml linear gradients of NaCl (0.1-1M) followed by elution with 1ml at 1.0M and 4mls

at 0.1M NaCl. Samples were removed at each stage of the process and retained for gel analysis (section 3.10.3).

3.13.5. Precipitation of samples from HiTrap column.

10μg/ml of BSA and 1 volume of 40% TCA were added to the 2ml samples collected from the column. The fractions were precipitated on ice, centrifuged at 12K for 15 minutes in a microfuge and the supernatant was removed. The remaining pellet was rinsed in ethanol followed by ethanol/ether and dried under vacuum for 5 minutes. The pellets were resuspended in boiling mix, boiled for 2 minutes and analysed by SDS-PAGE (section 3.10.3).

3.14. Molecular modelling

Two models of VZV thymidylate synthase, one of the ternary complex and the other of the native enzyme with bound phosphate, have been constructed by Celia Schiffer and Robert Stroud (personal communication). The models are based upon the co-ordinates of the ternary complexes of *E.coli* TS with CB3717 and dUMP (Montfort *et al.*, 1990) and the co-ordinates of the *E.coli* TS structure with bound phosphate respectively (Perry *et al.*, 1990). All residues from the *E.coli* sequence that differed from the equivalent residue of the VZV sequence was substituted by the latter using the graphics programme INSIGHT (Dayringer *et al.*, 1986). The substituted residues were positioned in a conformation which overlapped as much as possible with that of the residue existing in the *E.coli* structure. Where a VZV residue was larger than one it replaced, it was placed in a conformation which corresponds to the most frequent found for that amino acid in protein structures. The model lacks the first 19 amino acids from the VZV sequence, and the two short insertion sequences common to all eukaryotic thymidylate synthases (residues 105-116 and 134-141; VZV numbering), that are not found in the *E.coli* enzyme. At the time the model was generated, these regions of the human TS being studied by this group (Schiffer *et al.*, 1991) were unresolved. The model of the VZV native TS was refined by 200 cycles of conjugate gradient minimisation with the refinement package of XPLOR. Due to difficulties in the initial stage of the energy minimisation, the α-carbon atoms were constrained to stay fixed, thus allowing only movement of side chains to remove any energetically unfavourable contacts.

Computer analysis of DNA and protein sequences were performed on the Micro Vax computer using the software package from the University of Wisconsin Genetics Computer Group (GCG Inc., Wis.). The models of the VZV TS native enzyme and the ternary complex (generated as above) were visualised and manipulated using INSIGHT

II (Biosym technologies) run on a Silicon Graphics and Indigo workstation and an Iris Workstation.

In collaboration with Adrian Lapthorne (Chemistry), Setor was used to introduce and evaluate point mutations in the VZV ternary model (Evans, 1993). In the absence of the crystal structure detailed modelling of the mutations was deemed inappropriate.

Chapter 4. Protein-protein interactions of VZV TS

The results and discussion are divided into five chapters. The first chapter deals with the examination of protein-protein interactions of TS by affinity chromatography. The second chapter then carries on to develop an improved protocol for obtaining purified TS in a soluble form. Chapters 6 and 7 investigate the effect of mutations, initially on buried regions of the TS protein and then on the more solvent exposed C-terminus and DRTG loop region. The final chapter examines an arginine residue which has been found to discriminate between dCMP and dUMP binding in bacterial TS and other enzymes.

Protein-Protein interactions of VZV TS

TS is one of the most highly conserved enzymes known and its high degree of sequence conservation was initially thought to be due to constraints on enzyme structure and function. The enzyme, however, is extremely permissive in the amino acid substitutions that it tolerates, and many residues that are highly conserved and/or important for structure or function can be mutated without loss of enzyme activity. The observation that so many residues of the protein are not essential for enzyme function raises the question of why these residues are conserved. There are several possible explanations for this conservation paradox. Because no mutant has been reported that catalyses dTMP formation more efficiently than the wild type TS, conservation may simply result from optimisation of TS activity (Carreras & Santi, 1995). Alternatively, some residues may be conserved for reasons other than catalysis, such as mRNA binding (Chu *et al.*, 1991,1993). The most likely explanation, however, for the high conservation is that TS interacts with other enzymes involved in DNA precursor biosynthesis and forms part of a multienzyme complex (Reddy *et al.*, 1980). A similar situation exists for the histone proteins as they also show high sequence conservation. These proteins are assembled into an octamer around which DNA is wrapped to form a nucleosome core. It is thought that the high conservation of these proteins is to allow the proteins to interact with the DNA in the nucleosome and with each other (Luger *et al.*, 1997).

Using protein affinity chromatography, Wheeler and colleagues (1992) have identified a multienzyme complex in bacteriophage T4. Purified deoxycytidylate hydroxymethylase from T4 was immobilised onto an affinity column and whole cell extracts were applied to the column. The column retained several proteins that were identified by SDS-PAGE as the products of specific T4 genes (Wheeler *et al.*, 1992). These include several enzymes involved in DNA precursor biosynthesis, thymidylate synthase, dCMP deaminase, dihydrofolate reductase, dCTPase-dUTPase and ribonucleotide reductase. Two of the enzymes in the complex are functionally associated

with one another as they catalyse sequential reactions; the dUMP that is produced by the enzymatic deamination of dCMP serves as the substrate for thymine nucleotide synthesis via the dTMP synthase reaction. McGaughey *et al.* (1996) have explored protein-protein interactions of the phage-coded forms of these two enzymes. An extract of radiolabelled T4 proteins was passed through a column of immobilised enzyme (either dTMP synthase or dCMP deaminase) and the specifically bound proteins were identified. In addition to binding strongly to each other several other proteins were identified which include dihydrofolate reductase and the single-stranded DNA-binding protein (McGaughey *et al.*, 1986). As protein affinity chromatography experiments on T4 phage TS have identified protein-protein interactions, a similar study was performed on immobilised VZV TS to identify interacting proteins. It was expected that several enzymes involved in dNTP biosynthesis might be identified.

4.1. Detection of proteins interacting with TS

Protein affinity chromatography was used to look for any specific interactions between TS and other proteins involved in dNTP synthesis. VZV TS was purified (refer to Chapter 3, 3.10) and covalently attached to an NHS-activated Sepharose Hi-Trap affinity column. Confluent ^{35}S-methionine labelled MeWo cells were infected with VZV-infected cells and incubated for 2-3 days, cell extracts were then prepared. As a comparison ^{35}S-methionine labelled mock infected cell extracts were also prepared. The extracts were then applied separately to the column and bound proteins were eluted using a linear salt gradient. Fractions obtained from the column were examined by scintillation counting and revealed that most of the radiolabelled proteins had come through the column in the buffer wash and only a small amount of radioactivity was present in the 0.1-0.4M NaCl washes (Figure 4.1). The samples containing radioactivity were precipitated with BSA and examined by SDS-PAGE (refer to Chapter 3, 3.13). The autoradiographs in Figures 4.2 and 4.3 reveal that for VZV and mock infected cells no proteins appeared to bind to TS on the column and all of the proteins came through in the buffer wash. Autoradiographs were exposed for periods of up to three weeks but there were still no bands.

4.2. Coupling and activity of bound TS

There are several possible explanations for the absence of any detectable protein-protein interactions. Firstly it could be that TS had not coupled to the column efficiently. To test the efficiency of the coupling procedure, the concentration of TS protein in the load on and flow through were examined by the Bradford assay. TS protein loaded onto

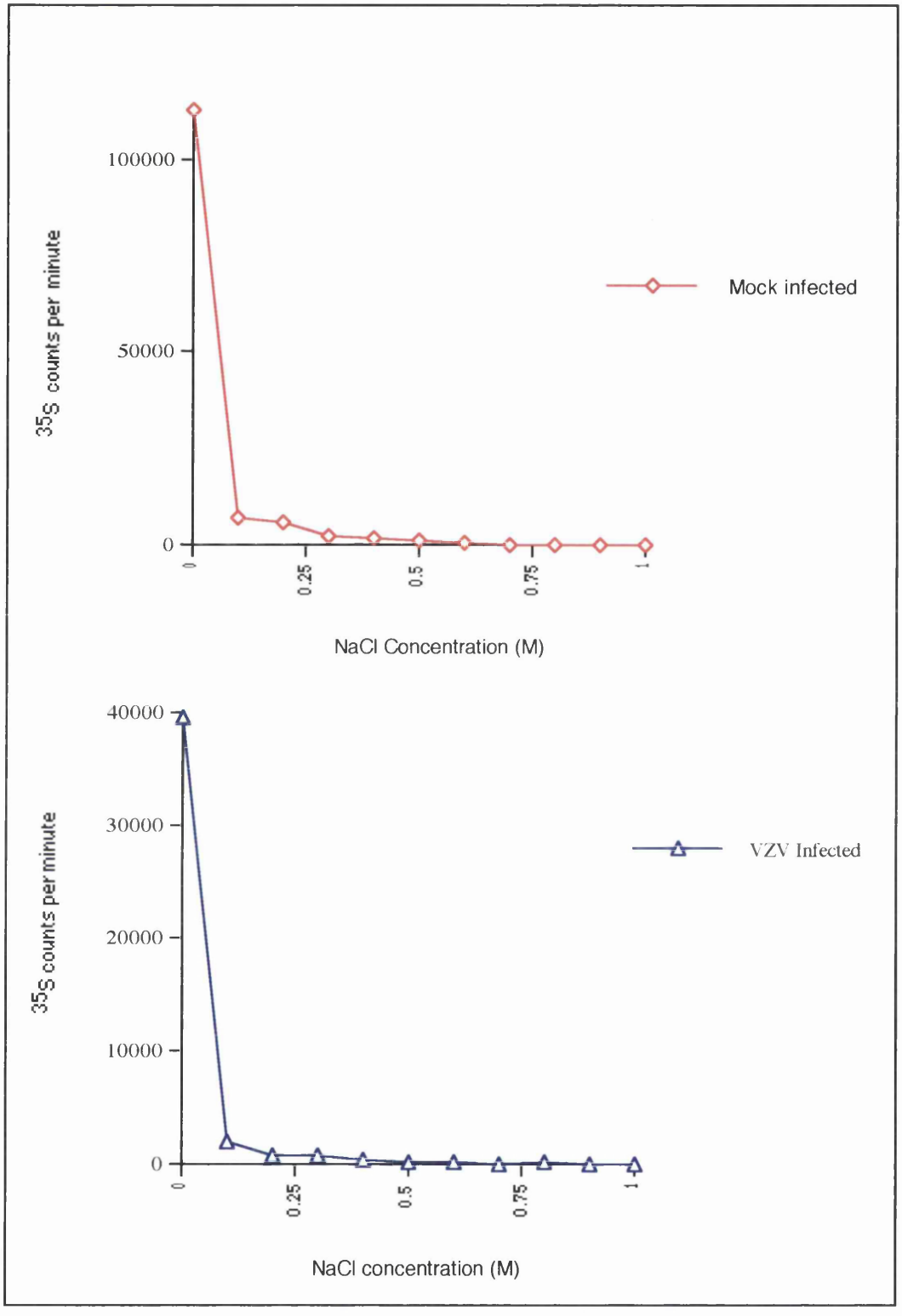


Figure 4.1. Radioactivity present in proteins eluted from HiTrap column.

^{35}S labelled Mock infected and VZV infected cells were loaded onto a HiTrap affinity column which had TS covalently coupled to it. Proteins that were found bound to the column were eluted by a salt gradient and radioactivity in samples were analysed to give an indication of binding.

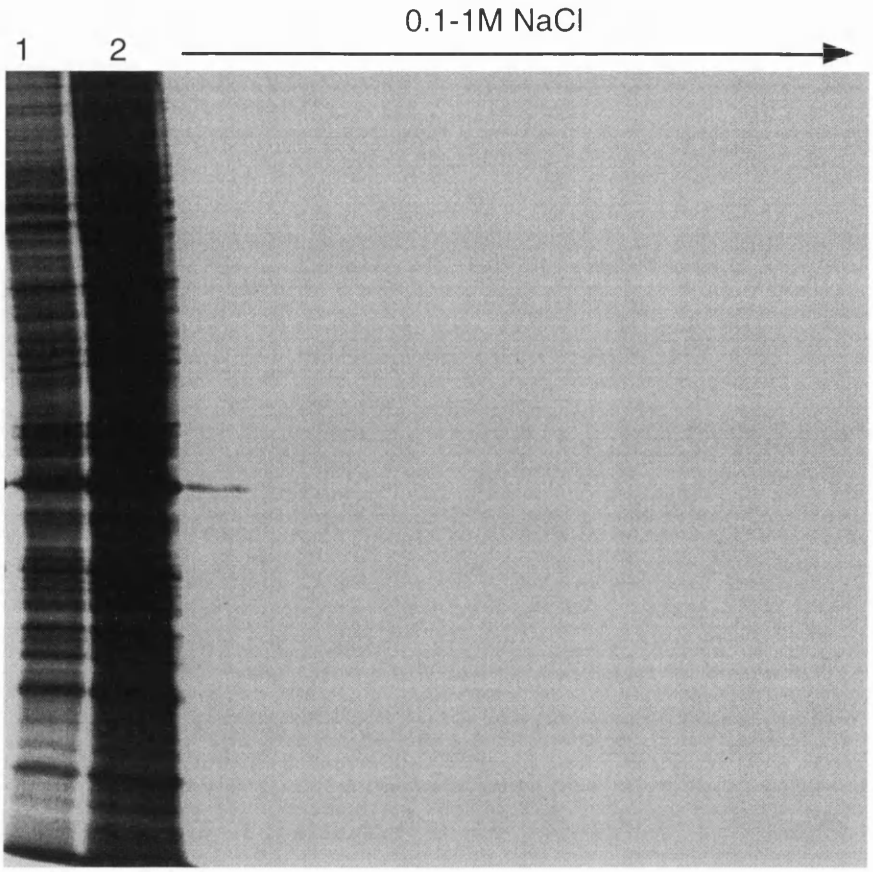


Figure 4.2. Autoradiograph of fractions eluted from a Hi-Trap affinity column containing bound TS.

^{35}S-methionine labelled mock infected cells were applied to an NHS-activated Hi-Trap affinity column containing covalently bound TS. Following elution from the column in an increasing salt gradient, fractions were precipitated with BSA and resolved by SDS-PAGE. Lane 1 shows a 10µl sample of the protein extract that was loaded onto the column and the proteins coming through the column in the buffer wash are shown in lane 2. Apart from one protein band the subsequent lanes showed that no labelled proteins were eluted.

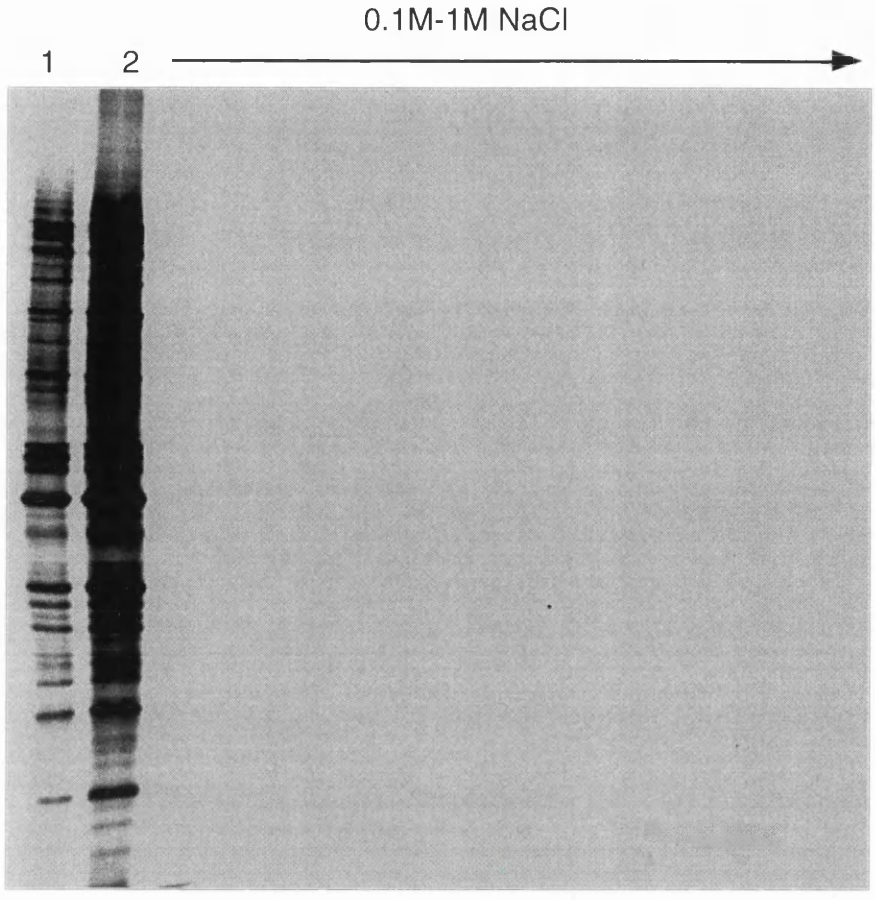


Figure 4.3. Autoradiograph of fractions eluted from a Hi-trap affinity column containing bound TS.

^{35}S-methionine labelled VZV infected cells were applied to an NHS-activated Hi-Trap affinity column containing covalently bound TS. Following elution from the column in an increasing salt gradient, fractions were precipitated with BSA and resolved by SDS-PAGE. Lane 1 shows a 10µl sample of the protein extract that was loaded onto the column and the proteins coming through the column in the buffer wash are shown in lane 2. The subsequent lanes show that no labelled proteins were eluted.

the column contained 1.2mg/ml of protein. Several buffer washes were performed on the column and the flow through was examined by eye using Bradford reagent which changed from brown to blue in the presence of protein. One fraction was blue in colour and this was assayed in the Bradford reaction. This sample contained 0.1mg/ml protein indicating that 1.1 mg/ml of protein must be coupled to the column.

A second theory was that TS bound to the column was not present in an active conformation. To establish whether TS on the column was active, a tritium release assay was carried out. ^{3}H-dUMP (700,000 cpm) and excess folate were added to the column, which was then incubated at 37°C for 30 minutes and a buffer wash and salt gradient were applied as before. The product from the tritium release assay, $^{3}H_2O$, was expected to come through in the buffer wash but the salt gradient was added to reproduce the experimental procedure that had been used previously. Charcoal was added to the collected fractions to absorb any unreacted ^{3}H-dUMP which was removed by centrifugation and the samples were subjected to scintillation counting. In the presence of charcoal approximately twice as much activity was present in a 200μl sample obtained from the buffer wash than when charcoal was absent. These results indicate that a significant amount of ^{3}H-dUMP had reacted to form tritiated water (Figure 4.4).

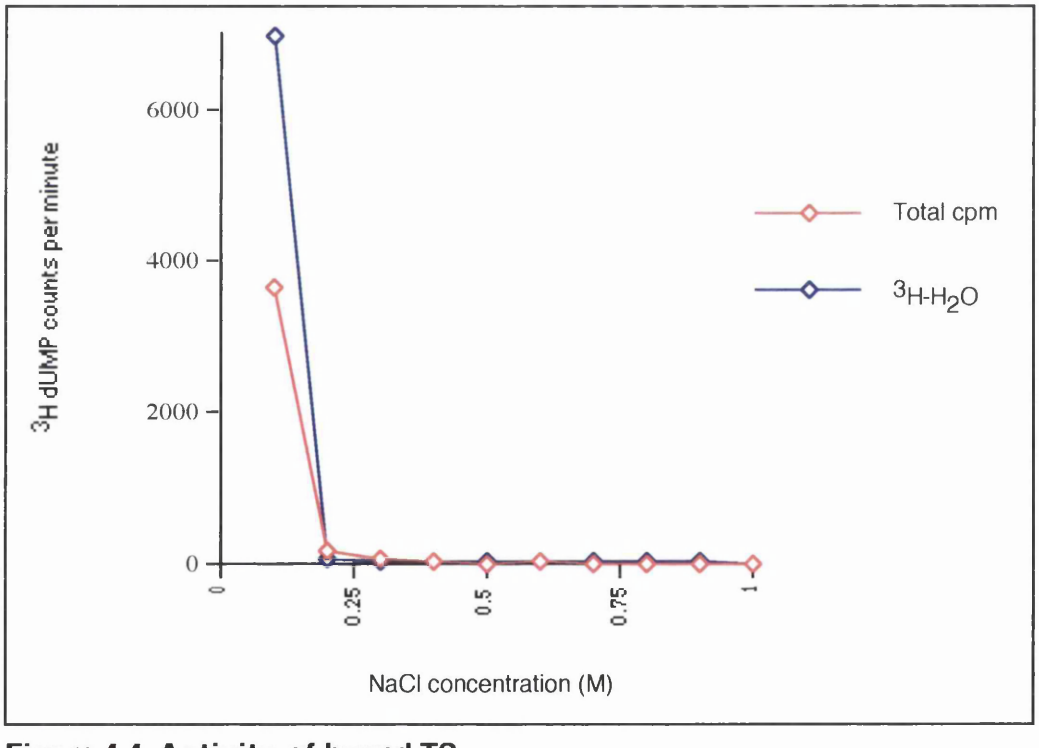


Figure 4.4. Activity of bound TS

700,000 cpm of ^{3}H-dUMP and excess folate were added to TS which was covalently bound onto a HiTrap affinity column and incubated at 37°C for 30 minutes. The column was washed with buffer and fractions were eluted in 0.1-1M NaCl. 200μl fractions from the column were measures for activity in the presence and absence of charcoal. The blue line indicates the amount of reacted dUMP in fractions whereas the red line shows the unreacted dUMP in fractions.

Therefore, having established that TS was efficiently coupled to the column and it was present in the active conformation the experiments were then repeated. Several attempts were made to identify any proteins binding to TS. In addition to infecting the MeWo cells with VZV for varying durations of time (1-3 days), the infected cells were also loaded onto the column which was incubated at various temperatures (room temperature, 37°C). All attempts, however failed to demonstrate that any proteins were binding to TS.

4.3.Discussion

These experiments have shown that no other VZV or cellular proteins could be detected specifically bound to the TS immobilised on the column. These results could be due to the experimental procedure used, it could be that there was a problem with the VZV infected cells or that the protein precipitation steps used were not effective. Alternatively the radiolabelled proteins could have formed a complex with ^{35}S-labelled TS prior to being loaded onto the column and as a result there was no binding to the cold TS on the column. One way of overcoming this problem would be to load purified proteins, which are expected to bind to TS, onto the column one by one.

There are several other techniques available which allow protein interactions to be detected and it may be that one of these methods could be used to detect the interactions of VZV TS. Typically, co-immunoprecipitation experiments have been applied in which antibody to a known protein is used to precipitate associated proteins, at present there is no antibody against VZV TS available. More recently the yeast two hybrid system has been described, this is an *in vivo* method which identifies protein-protein interactions through reconstitution of the GAL4 yeast transcriptional activator protein (Fields & Song, 1989). This protein is a transcriptional activator required for the expression of genes encoding enzymes of galactose utilisation. It consists of two domains: an N-terminal domain which binds to specific DNA sequences and a C-terminal domain which contains acidic regions which are necessary to activate transcription. This procedure involves the construction of two hybrid proteins containing parts of GAL4: the GAL4 DNA-binding domain fused to a protein "X" and a GAL4 activating domain fused to a protein "Y". If "X" and "Y" can form a protein-protein complex and reconstitute proximity of the GAL4 domains then this can lead to transcriptional activation of a reporter gene containing a binding site for GAL4.

A second technique for examining protein-protein interactions is the BIAcore machine (Schuck, 1997). This monitors biomolecular interactions using non-invasive optical detection principle based on surface plasmon resonance (SPR). The reactant is covalently attached to the sensor surface and a mobile second reactant at a constant

concentration is introduced into the buffer flow above the sensor surface, and the progress of complex formation at the sensor surface is monitored. This procedure is followed by a dissociation phase in which mobile reactant is absent from the buffer. The SPR response reflects a change in mass concentration at the detector surface as molecules bind or dissociate. No labelling of interacting components is required and the technology is applicable to a wide range of experimental situations. This technique could be applied to detect interactions in VZV TS. Biotinylated TS could be bound to an avidin-coated chip and fractions from nuclear extracts passed across. Samples showing a detectable interaction could be further fractionated. However this line of investigation was not pursued in order to concentrate on the mutagenesis studies described in the following chapters.

Chapter 5. Purification of TS variants

Purification of TS proteins

In the following chapters several mutants have been produced by region directed mutagenesis. Further characterisation of these mutants requires large amounts of purified protein. This chapter deals with the problems that were encountered in the purification procedure.

5.1. Mutant enzymes tested for solubility

In the purification experiments only three of the mutant enzymes were examined as it was thought that these would give an indication of what would happen in the others (Table 5.1). AZ42 and JB88 are temperature sensitive mutants. AZ42 contains three point mutations in buried regions of the protein and JB88 has a 14 amino acid deletion at the C-terminus. The third enzyme examined contains a point mutation at position 214 and is referred to as N214D. All mutations were introduced into the TS gene in the plasmid pAD768 backbone. Plasmid pAD768 (Harrison, 1992; see Chapter 3 section 3.1) is a pBS$^-$ backbone vector containing VZV TS, this vector confers ampicillin resistance and contains the bacteriophage f1 origin of replication to allow ssDNA isolation. TS is under the control of the *tac* promoter in this plasmid (Figure 5.1).

AZ42 and JB88 contain sufficient enzyme activity to complement Thy$^-$ bacteria and N214D had been found to be Thy$^-$ (refer to Chapters 6, 7 & 8). Protein extracts from all three mutants had shown a significant amount of activity in the tritium release assay (Table 5.1).

Mutant	Amino acid change	Secondary structure	% Wild type activity of crude extract
AZ42	D206Y M207L N214S	Loop Helix J Helix J	29.2
JB88	284-301 14 a.a.deletion	C-terminus	8.8
N214D	N214D	Helix J	0.06*

Table 5.1. Mutants examined for solubility.

This table summarises the mutants which were examined for solubility. The amino acid changes are given and the regions of mutation. The table also provides the activity found in protein extracts of each of the mutants relative to a wild type value of 100%.

* implies that N214D is a purified extract.

5.2. Solubility of TS

To obtain large amounts of variant TS enzymes, the TS genes were subcloned from their original pAD768 backbone (using *NcoI* and *EcoRI)* into the pET-23d expression vector (Figure 5.1).

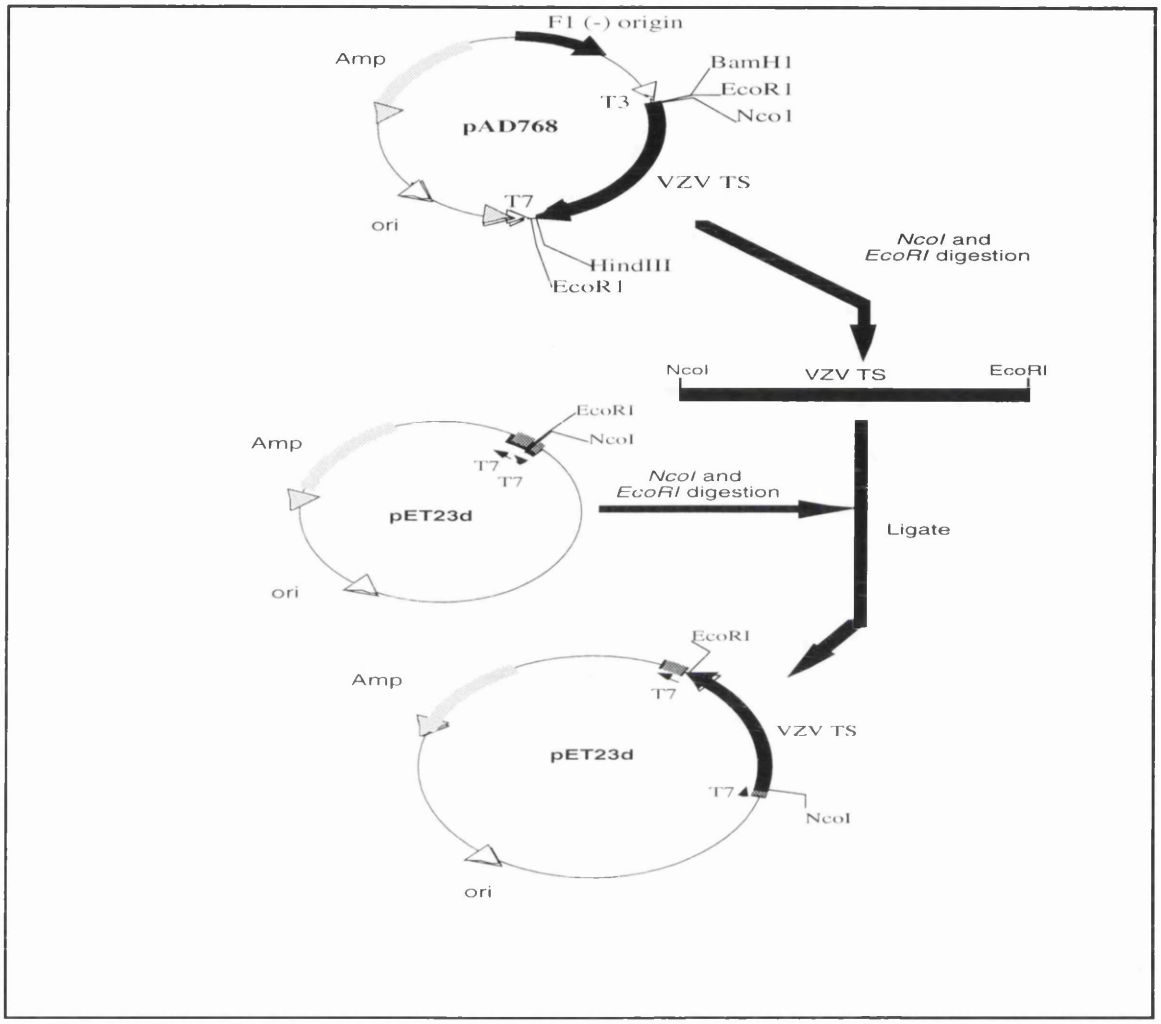


Figure 5.1. Cloning of VZV TS into the pET 23d vector
The TS gene was *EcoRI/NcoI* digested from original pAD768 backbone and cloned into pET23d expression vector under the control of T7 promoter.

The plasmids were initially transformed into an NM522 Thy$^-$ host as these cells are highly transformable. Candidate clones were digested to ensure they had the correct restriction profile. Those containing the correct profile were retransformed into BL21 (DE3) Thy$^-$ cells which contain a copy of the gene for T7 RNA polymerase and should induce expression from the T7 promoter after addition of IPTG. Again the clones produced were analysed by restriction digestion and those containing the correct profile

were test-induced with IPTG. Clones that were induced produced a protein band of around 32KDa.

Initially large cultures of AZ42 were grown (4x700mls) at 30°C and protein expression induced from the T7 promoter by addition of IPTG for 2-3 hours. Samples from the induced and uninduced cultures were removed and analysed by SDS-PAGE. An intense band was seen in the induced samples at around 32K, the position at which purified TS is known to run. This band indicated that high levels of TS protein had been induced. Protein extracts were prepared from the induced culture by sonication and centrifugation and samples were removed from both the supernatant and pellet. Examination of the samples by SDS-PAGE revealed that a significant proportion of TS was present in the pellet in an insoluble form (not shown but refer to Figure 5.2). These are inclusion bodies which are often formed when the protein is overproduced resulting in the aggregation of partially folded proteins in the cytoplasm.

The initial procedure used to try to overcome the problem of protein insolubility was to change the growth temperature and induce the protein at lower temperatures as this had previously been found to overcome certain solubility problems (Lin *et al.*, 1990). 350ml cultures of the C-terminal mutant JB88 and the buried mutant AZ42 were grown at 30°C and the samples split in two, one half of the sample was induced at 26°C for 4 hours and the other half was induced overnight at 15°C. Protein extracts were prepared and fractions from both the supernatant and pellet were analysed by SDS-PAGE. Figure 5.2 shows that both pellet and supernatant samples of the mutants can be seen at the various induction temperatures. From the gel it looks as though 15°C is better than 26°C for JB88 but in all cases the largest proportion of the TS protein was present in the insoluble pellet

A time course was then set up to establish if there was a certain point during induction that the protein started to form insoluble inclusion bodies. A 350ml culture of the buried mutant AZ42 was grown at 30°C and protein expression was induced. Following induction samples were removed at 30 minute intervals from between 1-3 hours. Protein extracts were prepared and samples from the supernatant and the pellet were examined by SDS-PAGE. Figure 5.3 shows a Coomassie stained gel with pellet and supernatant fractions obtained after the varying intervals of induction. The results revealed that even after one hour most of the protein appeared to be present in the insoluble pellet.

A further possible method for the preparation of soluble protein is to express the target gene in a host strain containing a compatible plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Moffatt & Studier, 1987; Studier, 1991). T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layer of the *E.coli* cell wall and it binds to T7 RNA polymerase, inhibiting

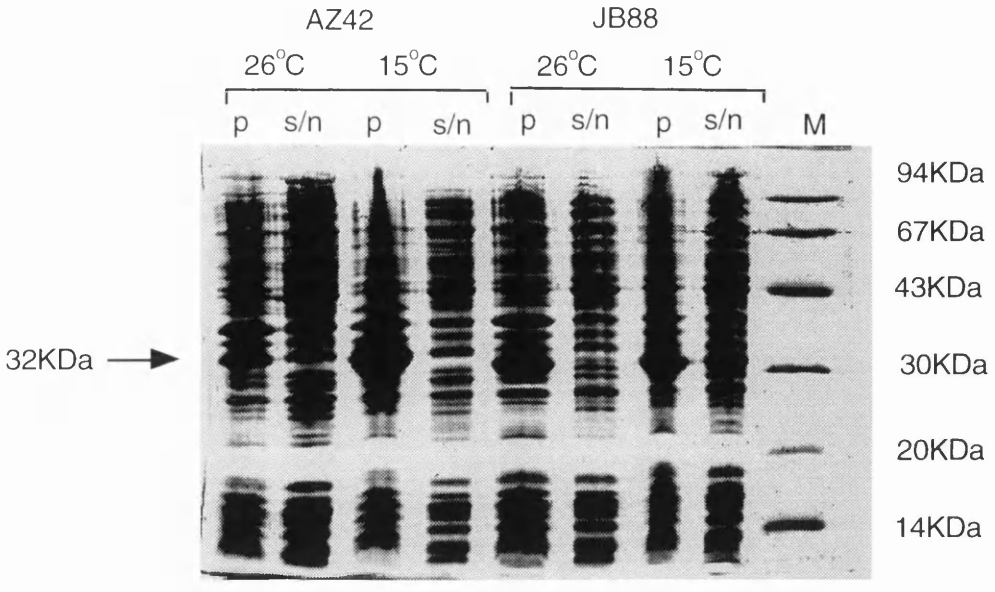


Figure 5.2. Investigation of the induction temperature on the solubility of TS
A Coomassie stained SDS-polyacrylamide gel of protein extracts from the buried mutant AZ42 and the C-terminal mutant JB88. 350 ml cultures of the mutants were grown and TS expression was induced by the addition of IPTG for either 4 hours at 26°C or overnight at 15°C. Protein extracts were prepared and samples from the pellet (p) and supernatant (s/n) were analysed by SDS-PAGE. The position at which purified TS runs is denoted by an arrow. Protein size markers (M) are present on the right. Note that the 14 amino acid deletion causes the JB88 protein to run faster than the multiple point mutant AZ42.

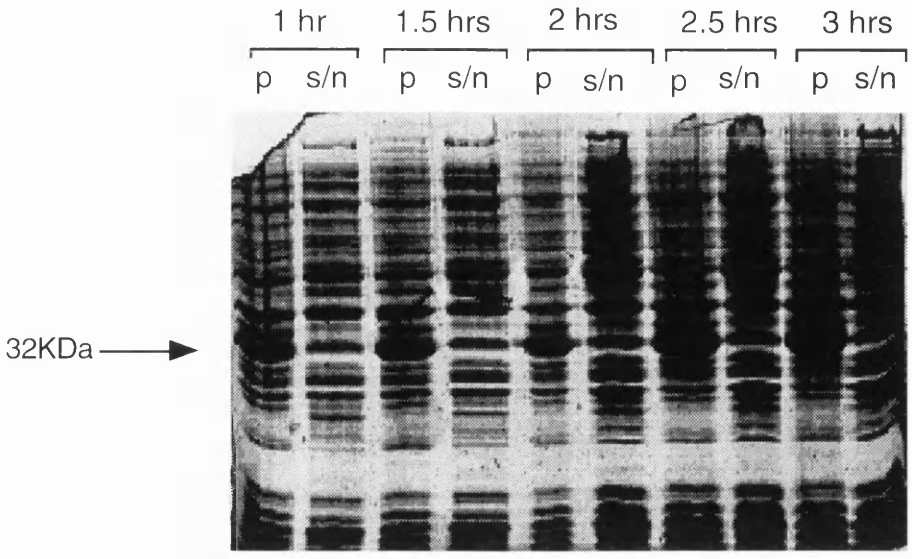


Figure 5.3. Time course of induction

A Coomassie stained SDS-polyacrylamide gel of protein extracts from the buried mutant AZ42. A 350ml culture of AZ42 was grown and TS expression was induced by IPTG. 50ml samples were removed from the culture at 30 minute intervals, 1-3 hours post induction. Protein extracts were prepared and the pellet (p) and supernatant (s/n) fractions were analysed by SDS-PAGE for the presence of TS. The position at which purified TS runs is denoted by an arrow. Protein size markers are not present on the gel.

transcription. The pET vectors containing the mutants were transformed into BL21 pLysS as this strain contains a plasmid that provides a small amount of T7 lysozyme.

The mutants AZ42, JB88 and N214D were grown at 30°C and protein extracts were prepared. Samples from the pellet and supernatant were examined by SDS-PAGE. Figure 5.4 reveals that TS is present in the insoluble pellet for the deletion mutant JB88 and the buried mutant AZ42. An exception was found with the N214D point mutation which appeared to have the protein present in equal amounts in the pellet and supernatant indicating that this mutant produces more soluble TS when it is expressed in the BL21 pLysS cells.

Therefore expression of the TS protein from the pET vector seemed to be producing large amounts of protein very quickly which was resulting in the formation of insoluble protein. Changing the variables such as growth temperature, induction time and host cell did not appear to affect the solubility of the mutant enzymes with the exception of N214D.

5.3. Recovery of insoluble protein from inclusion bodies

Occasionally it is possible to solubilise the inclusion body protein in strong denaturing solutions such as urea or guanidine-HCl and then refold by slowly diluting out the denaturant at low protein concentrations. As the inclusion bodies can be easily isolated by low speed centrifugation this procedure was carried out on the mutants AZ42, JB88 and N214D. 350ml cultures of the mutants were grown up and the cells harvested by centrifugation. The cells were then lysed and the inclusion bodies were spun down (section 3.11.1). The inclusion bodies were solubilised in 8M urea, spun down and the jelly-like pellet was resuspended in urea containing buffer and spun down once again. At each stage the supernatants were retained and these were analysed by SDS-PAGE. Figure 5.5 shows a gel containing samples from the pellet of the solubilised inclusion bodies and also fractions from the supernatants. The results reveal that a significant amount of the protein is still remaining in the pellet but now a larger amount is found in the supernatants. Having established that the protein was present in the supernatant the aim was to refold it to its native form by dialysis. The protein was dialysed, for 1 hour at a time, against 8M urea-containing buffer at room temperature, 4M urea-containing buffer at 4°C and 2M urea-containing buffer at 4°C. Finally the supernatants were dialysed overnight against buffer containing no urea. Following dialysis the protein appeared to have come out of solution and so the samples were spun down and the pellet and supernatant analysed by SDS-PAGE. Figure 5.6 demonstrates that all of the protein was found in the pellet and so the proteins must have aggregated during dialysis and once again formed insoluble structures.

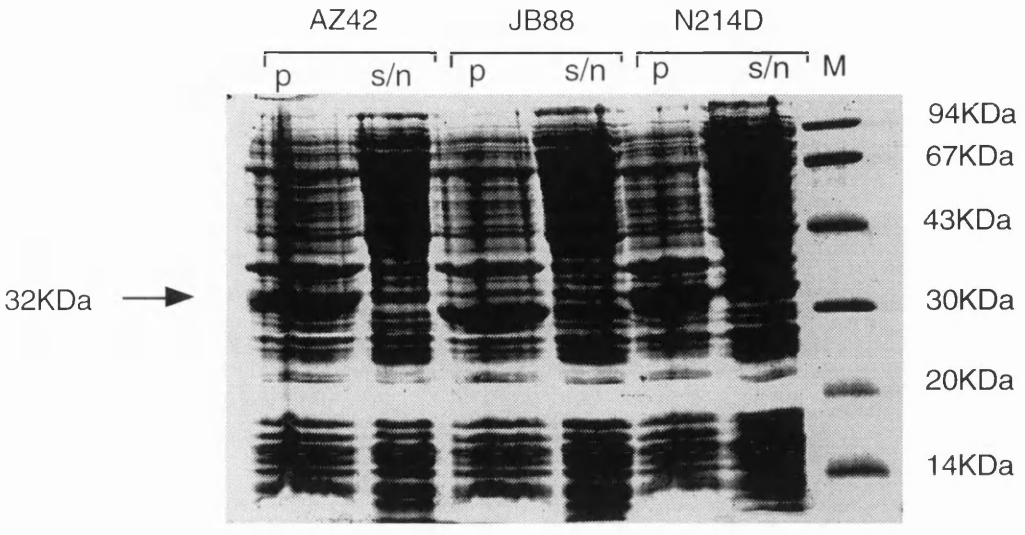


Figure 5.4. Solubility of TS expressed in BL21 (DE3) pLysS

A Coomassie stained SDS-polyacrylamide gel of protein extracts of the buried mutant AZ42, the C-terminal mutant JB88 and the point mutant N214D. 350ml cultures of the mutants were grown and TS expression was induced by the addition of IPTG for 2-3 hours. Protein extracts were prepared and samples from the pellet (p) and supernatant (s/n) were analysed by SDS-PAGE. The position at which purified TS runs is denoted by an arrow. Protein size markers (M) are given on the right. The size difference between JB88 and the point mutants AZ42 and N214D is evident.

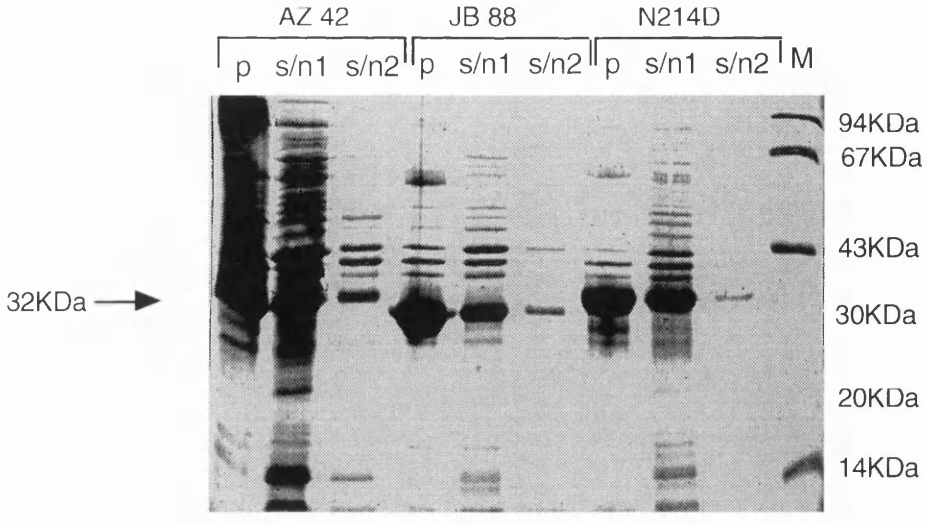


Figure 5.5. Solubilisation of inclusion bodies

A Coomassie stained SDS-polyacrylamide gel showing solubilisation of inclusion bodies from the buried mutant AZ42, the C-terminal deletion mutant JB88 and the point mutant N214D. Inclusion bodies were dissolved in 8M urea, spun down and the supernatant collected. This process was repeated and the pellet (p) and supernatants from each step (s/n 1&2) were analysed by SDS-PAGE. The position at which purified TS runs is denoted by an arrow. Protein size markers (M) are given on the right.

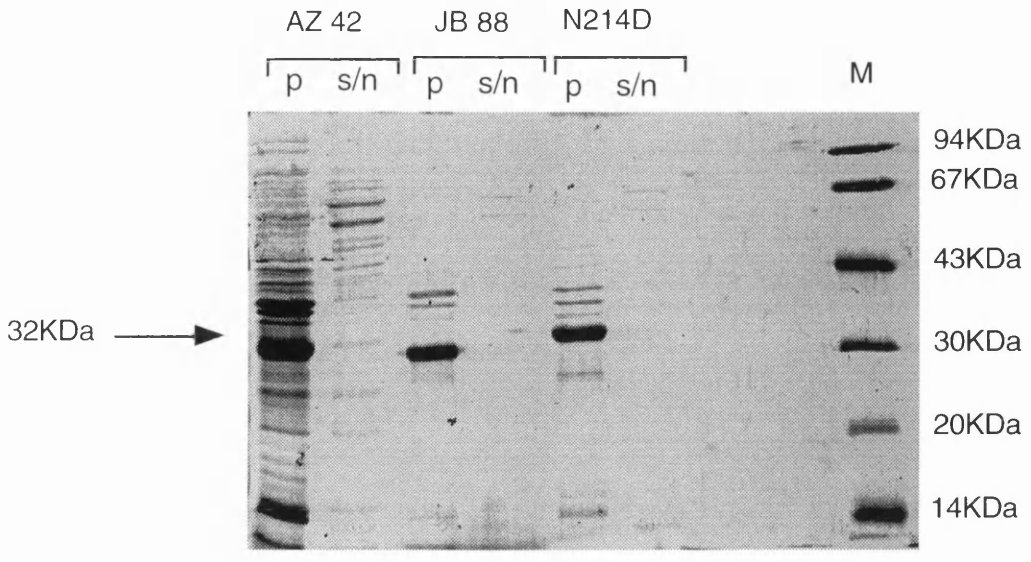


Figure 5.6. Renaturation of TS

Coomassie stained SDS-polyacrylamide gel of protein extracts from the buried mutant AZ42, the C-terminal deletion mutant JB88 and the point mutant N214D. Solubilised inclusion bodies were renatured by dialysing against decreasing concentrations of urea. Following overnight dialysis in phosphate buffer containing no urea, the sample was spun down and protein extracts from the pellet (p) and supernatant (s/n) were prepared and analysed by SDS-PAGE. The position at which purified TS runs is denoted by an arrow. Protein size markers (M) are present on the right.

5.4. Purification of mutant proteins from pAD768 vector

One final variable that could be changed to allow for the production of soluble protein is the expression vector, as the use of strong promoters often results in the formation of inclusion bodies and the pET vector contains the strong T7 promoter. The use of weak promoters sometimes favours the production of lower amounts of protein in a more soluble form. Therefore the mutant genes were used in their original bluescript vector which contains the weaker *tac* promoter, to allow for the production of lower amounts of protein which could be more soluble.

A five litre culture of the deletion mutant JB88, in the original pAD768 backbone, was grown at 30°C. A protein extract was prepared and was loaded onto a hydroxylapatite column. TS probably binds to hydroxylapatite due to its phosphate 'binding' pocket, which co-ordinates the phosphate of dUMP in the active site. This column is disposable and prevents the potential cross contamination of one mutant with another that can occur when columns are re-used.

Once the protein had been loaded onto the column it was washed with 60mls of 50mM phosphate buffer to remove any unbound protein, followed by 100mM phosphate buffer and the TS protein was eluted in 60mls of 300mM phosphate buffer. The 300mM wash was precipitated with ammonium sulphate and dialysed overnight against 1 litre of 50mM phosphate buffer. Each step of the purification was monitored by protein mini-gel analysis of the appropriate collected fractions. The dialysate was then loaded onto an S-sepharose column and the column was washed in 50mM phosphate buffer. TS protein was eluted from the column with an increasing salt gradient and 1ml fractions were collected. A Bradford assay was carried out on the fractions obtained from the column to determine whether they contained protein and the fractions containing protein were analysed by SDS-PAGE. Figure 5.7 shows that a TS band was present at around 32K in fractions 13-18. The TS containing fractions were pooled together and stored at -70°C prior to use.

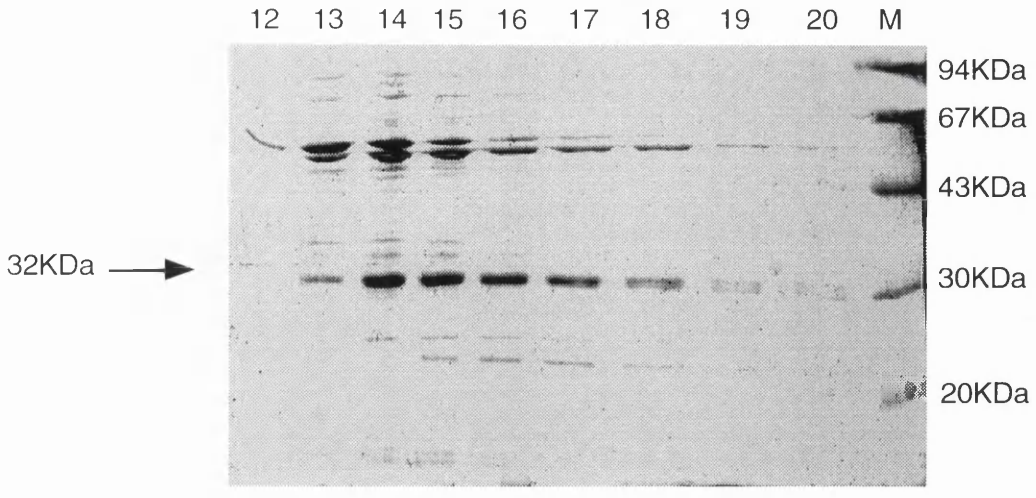


Figure 5.7. Purification of JB88 from S-sepharose column

Coomassie stained gel of fractions collected from purification of JB88 by S-sepharose chromatography. The partially purified protein extract obtained from the hydroxylapatite column was bound to the S-sepharose column, the column was washed with phosphate buffer and the TS protein was eluted from the column with an increasing salt gradient. Lanes 12-20 are fractions eluted from the column which are found to contain TS protein. The position at which purified TS runs is denoted by an arrow. Protein markers (M) are present on the right.

S-sepharose chromatography produced a protein that was partially pure. Two distinct contaminating bands can be seen in Figure 5.7 which migrated more slowly than the TS band. Analysis of the partially purified protein confirmed results that had previously been obtained from crude extracts. The enzyme showed TS activity in the tritium release assay, with the specific activity of the purified extract being 0.10% of activity of the wild type enzyme (see Chapter 7). Although the TS activity of the mutant was low there was sufficient activity present to complement Thy$^-$ bacteria.

Several attempts have been made to purify other C-terminal and buried mutants of VZV TS in their original pAD768 backbones. Purified protein recovered from the column was found to be inactive in the tritium release assay which could be the result of a loss in activity during the purification procedure. A possible solution for cutting down the length of time involved in purification would be to purify the mutants using FPLC chromatography.

5.5. Discussion

Purification of wild type TS is achieved through high level expression in the pET vector followed by hydroxylapatite and S-sepharose chromatography. This procedure produced sufficient amounts of protein on which experimental work could be carried out. The same purification procedure, when applied to the TS variants, proved to be unsuccessful. Low amounts of protein were recovered from the chromatography steps and on examination of the extract which had been applied to the column it was found that a large quantity of the protein was present in the insoluble pellet as inclusion bodies. It may be that a large portion of the wild type enzyme is found in the insoluble pellet but sufficient remains soluble (J.Scott & R. Thompson, personal communication).

The conditions used in the purification procedure were varied and it appeared that the only means of purifying the C-terminal mutant JB88 was to express the TS gene in the original bluescript backbone and purify it from there. The 14 amino acid deletion may expose sufficient underlying hydrophobic amino acids to severely reduce the solubility of the protein. Problems were encountered when this procedure was applied to the other C-terminal mutants and the buried mutant AZ42. In contrast, the point mutant N214D was purified following expression in the pET-23d vector and induction from BL21(DE3) pLysS cells.

Many of the problems with the purification procedure still remain to be resolved however sufficient material was obtained for the kinetic and other characterisation described in the following chapters.

Single amino acid substitutions have been shown to have dramatic effects on the properties of other proteins. The mutation producing sickle cell anaemia results in an adenine to thymine substitution in the sixth codon of the β-globin gene which reduces the

solubility of haemoglobin causing increased destruction of red cells and sickle cell disease (Ingram, 1956). In this sense even single amino acid variants can be considered to be "new" proteins and the problems encountered in purifying the variant TSs while not predictable should not be too surprising.

Chapter 6. Region directed mutagenesis of buried residues in VZV TS

Region directed random mutagenesis ofVZV TS

Mutagenesis studies of TS from *L.casei* and *E.coli* have shown that many of the highly conserved residues can be readily substituted without compromising enzyme activity (reviewed in Carreras & Santi, 1995). This means that conventional site-directed mutagenesis of residues selected on the basis of their conservation is of limited value. These studies have however identified at least four residues that are critically important for enzyme function, including the completely conserved cysteine residue that forms a covalent adduct with the substrate. The high degree of sequence conservation within the TS family makes these enzymes an ideal test bed for the development of species-specific inhibitors.

This section deals with the development of a technique to randomly mutagenise selected regions of the protein. Several different methods have been devised for the random mutagenesis of cloned genes, these include *in vivo* methods such as the use of mutator strains, *in vitro* enzyme dependent approaches and procedures that use chemicals to damage the DNA. Mutagenesis by these procedures can result in an uneven distribution of mutations because of the presence of mutagenic "hot spots" in the DNA. Therefore the spiked oligo approach was developed to introduce single amino acid changes scattered throughout the whole of a targeted region of the gene of interest (section 3.5.2). The oligonucleotides were synthesised by a protocol that gives a defined probability of incorporating one of three "wrong" nucleotide bases at each position along the oligonucleotide (Ner *et al.*, 1988; McNeil & Smith, 1985). The strand selection procedure of Künkel for mutant enrichment was used for random mutagenesis with these spiked oligonucleotides (Künkel, 1985; Künkel *et al.*, 1987). This method uses a single stranded phage M13 DNA (ssDNA) template that contains a number of uracil residues instead of thymine. The uracil-enriched ssDNA (U-ssDNA) is produced in an *E.coli* dut^- ung^- strain which lacks the enzymes dUTPase and uracil N-glycosylase. Lack of dUTPase activity leads to raised levels of dUTP and results in an increased incorporation of uracil into DNA instead of thymine. The absence of uracil N-glycosylase, which normally removes uracil from DNA, means that uracil is stably incorporated. The double stranded molecules produced from the mutagenesis have a parental strand which is uracil rich, whilst the newly synthesised mutant strand contains only thymine. When *E.coli* dut^+ung^+ are transformed with this DNA, there is a strong biological selection against the replication of the parental template strand which is degraded. This results in the production of predominantly mutant double stranded DNA molecules.

The spiked oligo approach was used to generate a series of temperature sensitive mutants as these are an efficient way of identifying amino acid positions which are critical for the correct folding and activity of the protein. Temperature sensitive proteins will only be active at the permissive temperatures. Screening was facilitated by the ability of VZV TS to genetically complement Thy^- bacteria, allowing screening for catalytically active VZV TS at three different temperatures, 31°C, 37°C and 42°C on solid minimal growth media which only permits the growth of Thy^+.

6.1. Construction of buried region mutants

Fifteen spiked oligonucleotides (RO1-15) were synthesised spanning the entire TS gene and were then used as primers in the Künkel method of site-directed mutagenesis to introduce mutations into the cloned VZV TS gene (Figure 6.1). Initially spiked oligos were used that were targetted to buried regions of the protein structure, since it was anticipated that residues involved in stability would be found there.

Following mutagenesis the plasmids were transformed into NM522 cells which had a point mutation in their TS gene, rendering the cells Thy^-. Colonies obtained from the transformation were screened for temperature sensitivity (Table 6.1). Temperature sensitive mutants are capable of complementing Thy^- bacteria on M9 plates at 30°C but not at 37°C or 42°C. At 30°C these mutants are able to form the closed conformation of the enzyme and perform the complete reaction whereas at higher temperatures the enzyme is inactive. Table 6.2 provides a summary of the temperature sensitive mutants which had previously been produced in the laboratory (J. Scott, personal communication).

Random oligo	Colonies screened	Temperature sensitive	Mutant name
RO9	73	12	AZ18-22, 41-47
RO10	100	10	AZ24-27, 48-53
RO11	100	13	AZ28-36, 54-57
RO15	70	14	AZ23, 58-70
RO16	100	10	AZ37-40, 71-76

Table 6.1. Mutations created in the buried region of TS
The table summarises the colonies screened for mutations and the number of temperature sensitive mutants obtained.

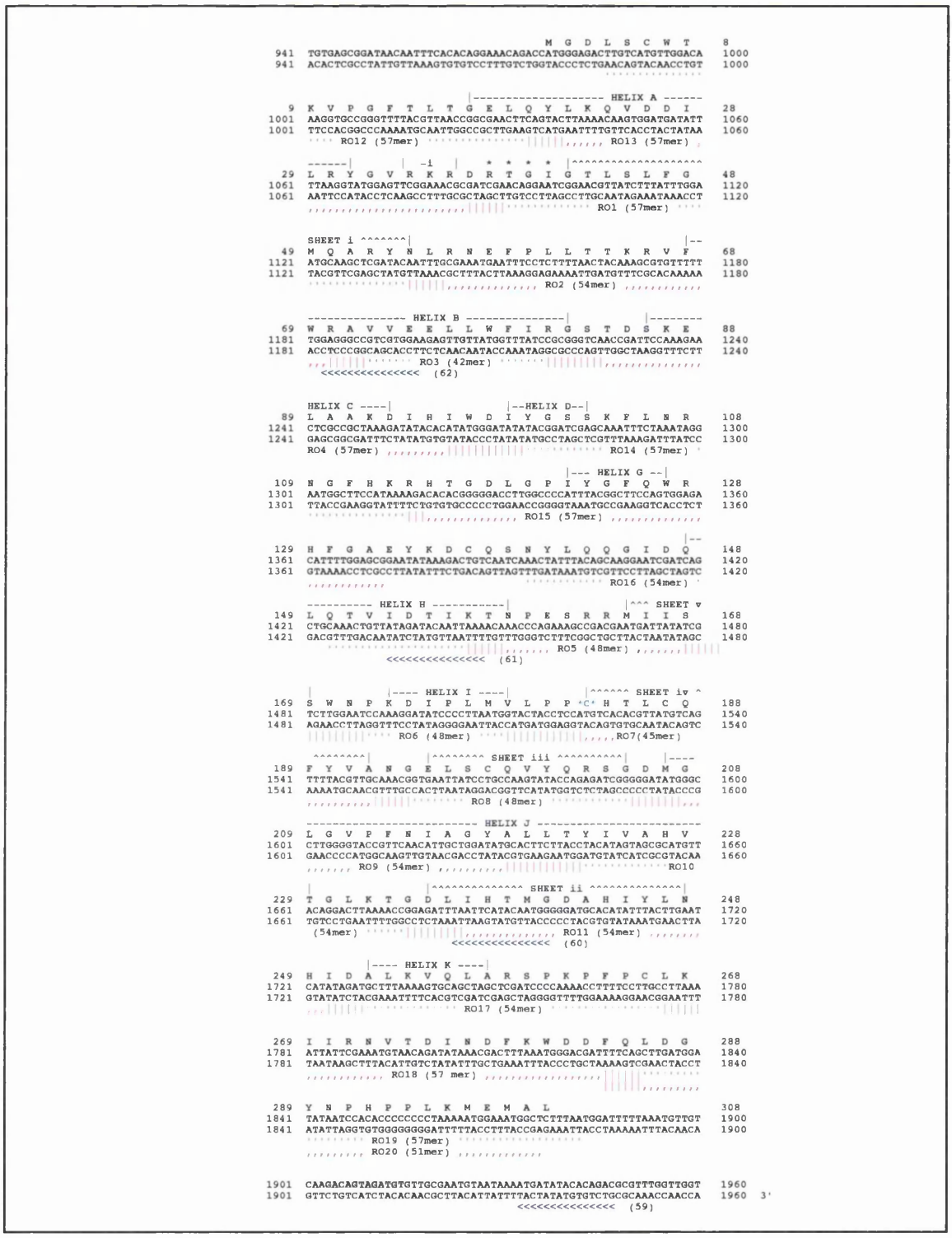


Figure 6.1. Spiked oligonucleotides covering the VZV TS gene

Oligonucleotides used for region directed mutagenesis are indicated in red. Primers used in sequencing are given in blue. The active site cysteine is shown as *C*.

A total of 59 temperature sensitive clones were produced from the random mutagenesis and base changes were identified by sequencing with primers spanning the various regions of the gene (J. Scott, personal communication). 12 of the potential mutants had an unreadable sequence. 47 of the mutants contained a readable sequence, of these 35 had point mutations and 4 had deletions. The spiked oligonucleotide approach should have generated between one and three base changes and from Table 6.2 it can be seen that for the enzymes with point mutations, the most common number of changes is two, which is the expected number.

1 base change	2 base changes	3 base changes	4 base changes	5 base changes
5	19	9	2	0

Table 6.2. Sequenced temperature sensitive mutants from buried regions of TS
The table summarises the number of base changes present in the various temperature sensitive mutants.

6.2. Mutants from buried regions

Eight of the mutants, which had been previously isolated were double checked for temperature sensitivity before they were characterised further. Colonies were again examined for their ability to complement Thy^- cells at 31°C, 37°C and 42°C. All mutants showed growth on M9 plates at 30°C but no growth at 37°C and 42°C .

Table 6.3 provides a summary of the random oligonucleotides used in the region directed mutagenesis procedure and the base changes in these mutants. The results show that base changes were found in various buried regions of the secondary structure. AZ41 contains a silent mutation (G210G) which produced a temperature sensitive phenotype, this suggests that a mutation is present in the TS gene outwith the region targetted by spiked oligo RO9. Figure 6.2 shows the positions in the enzyme structure where these mutations are found.

Mutant	Random oligo	Amino Acids	Amino acid change	Secondary structure
AZ28	RO11	234-251	Y 246 N	β-strand ii
AZ29	RO11	234-251	L 236 I	β-strand ii
AZ41	RO9	205-222	G 210 G	Helix J
AZ42	RO9	205-222	D 206 Y	Loop
			M 207 L	Helix J
			N 214 S	Helix J
AZ47	RO9	205-222	G 210 V	Helix J
AZ65	RO15	114-132	F 125 I	Helix G
			G 124 Y	Helix G
AZ66	RO15	114-132	Y 123 I	Helix G
AZ69	RO15	114-132	G 124 C	Helix G

Table 6.3. Buried region mutants of VZV TS.

The buried mutants were constructed using region-directed mutagenesis with randomly spiked oligonucleotides. Eight of the buried mutants from the 76 produced are given in the table. These mutants contain between one and three base changes.

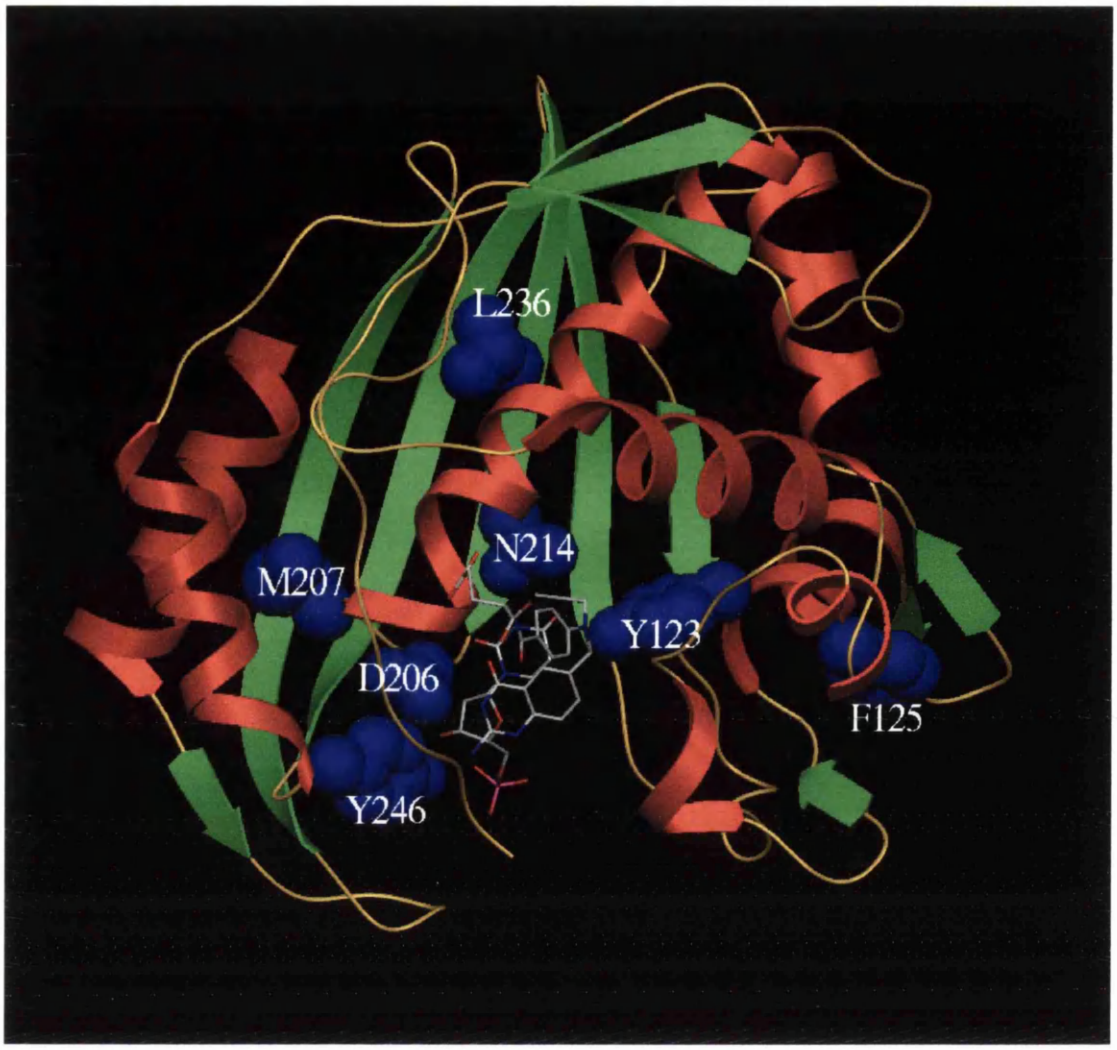


Figure 6.2. Thymidylate synthase monomer

This is a model of the monomer of VZV TS which shows the positions of the buried mutants. In green are β-strands, α-helices are red, turns are yellow and the ligands are grey. The amino acid residues in the mutants are shown in blue space-filling. G124 and G210 are not shown.

AZ28 contains the mutation Y246N in β-strand ii, this is a completely conserved tyrosine and is one of the four residues that are thought to be critical for enzyme activity (refer to Figure 1.13 and pull-out). A G210V mutation is found in AZ47 which is the mobile region of helix J, this is the core helix around which other structural elements fold. It packs diagonally across the β-sheet and is flanked by several other helices. AZ42 contains three base changes, Asp206 is a completely conserved residue and Met207 shows high conservation. Asp206 is in the turn between strand iii and the J helix which begins with Met207. The point mutation of most interest in AZ42 is N214S (refer to Figure 1.13). This asparagine residue is fully conserved and is found in helix J of the protein, it is the only residue that makes direct contact between a protein side chain and

the dUMP pyrimidine ring. Studies on *L.casei* have revealed that N229D (N214 in VZV) mutations decrease the enzymes ability to discriminate between dUMP and dCMP (Liu & Santi, 1992). This topic will be explored further in Chapter 8. AZ65, 66 and 69 contain mutations in helix G, this helix forms part of the hydrophobic protein core (refer to Figure 1.13).

The mutants AZ28, AZ42 and AZ47 were chosen for further examination as these enzymes contain mutations in highly conserved residues (see Figure 1.13 and pull-out). Having established that the mutants were active in the *in vivo* complementation assay, the mutants were examined *in vitro* by the tritium release assay which measures the release of tritiated water from 2'-deoxy[5'^{3}H]uridylate. This assay is performed under conditions of linear kinetics as less than 30% of the ^{3}HdUMP put into the reaction produces soluble counts and it is optimised for the wild type enzyme. The assay is highly sensitive detecting as little as 10^{-8} U/mg TS activity.

Crude protein extracts of the mutants and wild type VZV TS were prepared and assayed at 30°C. The results from the assays are given in Table 6.4 and reveal that AZ47 (G210V) retained 45.8% activity, demonstrating that glycine is important though not essential for catalytic activity. The triple mutant AZ42 (D206Y, M207L, N214S), which contains the important N214S mutation retains significant activity (29.2%). Mutation of the completely conserved tyrosine 246 to asparagine in AZ28 results in a modest 2-fold reduction in activity.

Enzyme	Tritium release pmol dTMP/min/μgprotein	% Wild type
Wild type	0.024	100
AZ28	0.012	50
AZ42	0.007	29.2
AZ47	0.011	45.8

Table 6.4. TS assays

TS activity of crude extracts from the buried region and wild type TS enzymes measured by the tritium release assay at 30°C. All enzymes were expressed in *E.coli* NM522 Thy^{-}.

Having established that the buried mutants were temperature sensitive *in vivo* the mutants were then examined *in vitro*. Crude protein extracts of the mutants were held at 42°C, 5μl aliquots removed at 5 minute intervals and assayed in the tritium release assay at 30°C. Comparison of the temperature profiles of both mutant and wild type enzymes are given in Figure 6.3. When held at 42°C the mutant extracts show a rapid decrease in TS activity. In contrast the wild type extract only produces a decrease to 86% activity revealing that the enzyme is not intrinsically temperature sensitive.

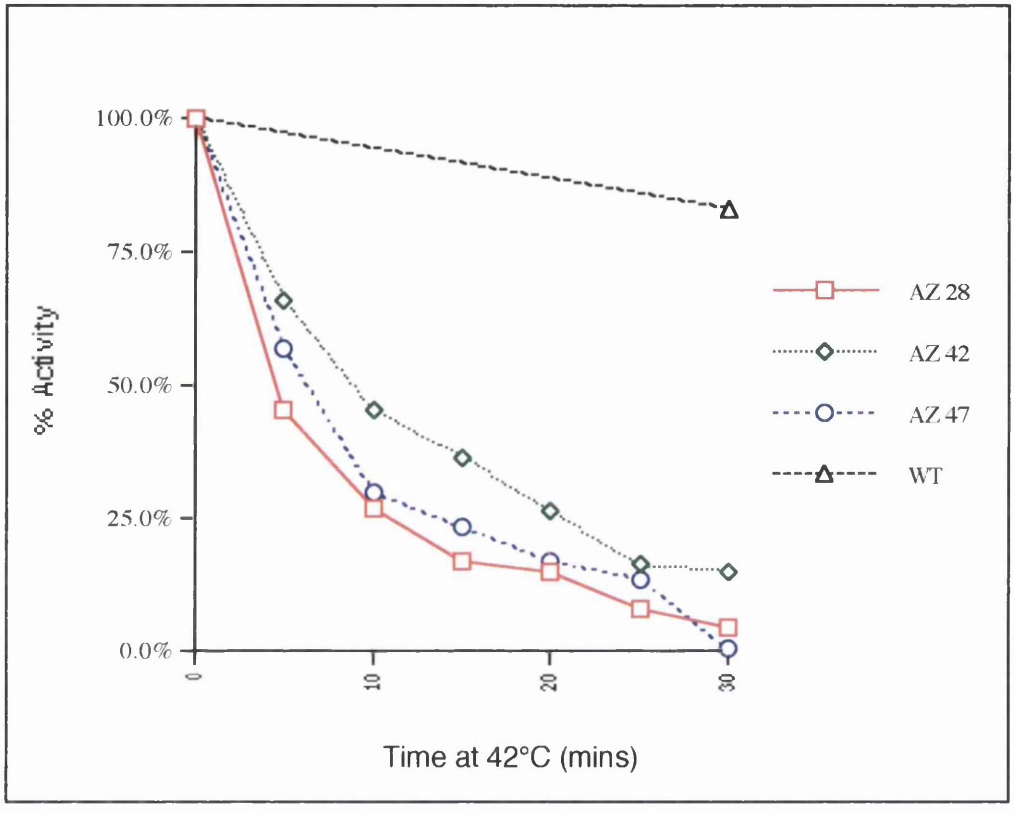


Figure 6.3. Temperature profiles of buried region mutants

Crude protein extracts of the buried mutants were incubated at 42°C and at 5 minute intervals samples were removed and assayed at 30°C in the tritium release assay. The graph shows the decrease in activity of the mutants corresponding to the length of time incubated at 42°C.

6.3. Phenolphthalein inhibition of buried TS mutants

To further characterise this set of mutants phenolphthalein was examined in the tritium release assay. This compound had been identified as an inhibitor of TS by the DOCK computer programme, the programme identifies compounds that fit into the active site of enzymes and so inhibit their reactions (Schoichet *et al.*, 1993). Phenolphthalein has

concentrations of approximately 200μM (J.Scott & R.Thompson, personal communication).

Crude extracts of the wild type enzyme and the three buried mutants have been examined for phenolphthalein inhibition in the tritium release assay at 50, 100 and 500μM concentrations of inhibitor (Figure 6.4).

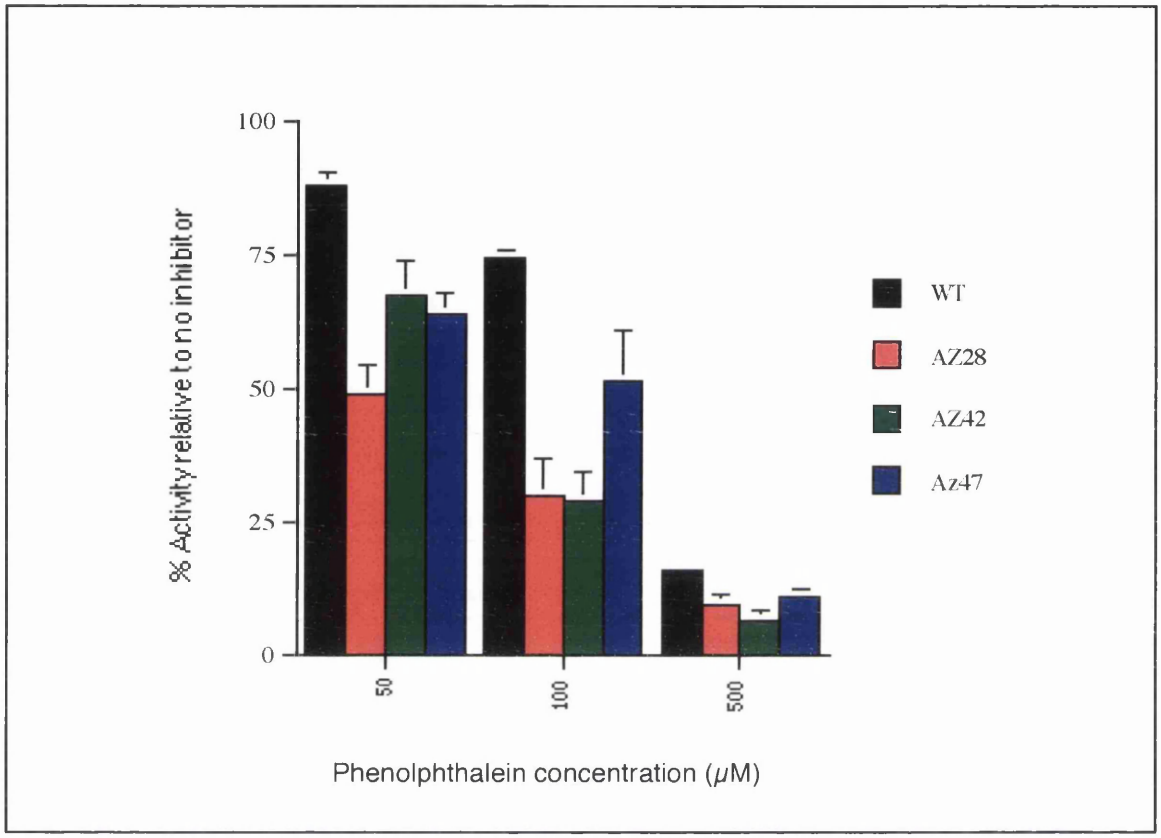


Figure 6.4 Phenolphthalein inhibition

Crude extracts of buried mutants and wild type TS were assayed for phenolphthalein inhibition in the tritium release assay at 30°C. Phenolphthalein was examined at 50, 100 and 500μM concentrations and was added to the reaction prior to the enzyme. The graph shows the activity present in the mutants relative to no inhibitor.

The results indicate that at concentrations of 50μM the wild type enzyme retains 90% activity whereas the mutant enzymes possess around 50-60% activity. Phenolphthalein concentrations of 100μM result in the wild type extract being inhibited to 71% of its initial activity while the buried mutants AZ28, AZ42 and AZ47 have been inhibited to 29.6%, 28.8% and 51.4% activity respectively. Concentrations of 500μM-10mM result in the enzyme becoming almost totally inactive. These results suggest that the mutant enzymes are more sensitive to phenolphthalein than the wild type.

6.4. dCMP inhibition of AZ42

As AZ42 contained the N214S mutation, this enzyme was examined for dCMP inhibition. Ordinarily dCMP is a poor competitive inhibitor of TS and binds to the wild type enzyme with a 1000 fold lower affinity than the substrate dUMP. Replacement of Asn 229 in *L.casei* TS with aspartic acid resulted in the enzyme losing TS activity toward dUMP and becoming a dCMP methylase (Liu & Santi, 1993). Addition of dCMP to the tritium release assay of such a mutant results in the competitive inhibition of the enzyme. AZ42 does not contain an asparagine to aspartic acid mutation but an asparagine to serine mutation. The N214S mutation in *L.casei* has been found to have modest TS activity but dCMP inhibition of this mutation has not been examined. Therefore AZ42 was examined for inhibition by adding 50μM-10mM concentrations of dCMP to the tritium release assay. The activity of the mutant was found to be unaltered by the presence of the dCMP which indicates that an asparagine to serine mutation does not affect the enzyme's dicrimination of dCMP and dUMP whereas a corresponding asparagine to aspartic acid mutation does (see results Chapter 8.).

6.5. Discussion

The initial region directed mutagenesis studies to produce temperature sensitive mutants focused on buried regions of the TS protein as it was expected that residues involved in protein stability would be found there. From the results it can be seen that this idea proved to be correct as a number of mutants were produced in these buried regions which contained between one and three base changes. Although this approach proved to be successful, it had the drawback that many of the clones produced appeared to be temperature sensitive but upon restriction digestion they did not produce the correct restriction pattern. In addition some of the clones which did produce the correct restriction pattern had a wild type sequence within the region of the spiked oligonucleotide. Despite this, the method proved to remarkably efficient at generating temperature sensitive mutants (46 of the 443 colonies screened were temperature sensitive mutants). The mutants selected for further characterisation had all been fully sequenced across the TS region to confirm the absence of additional mutations.

Molecular modelling was performed on the mutants to determine the effects of the amino acid substitutions on enzyme structure (see section 3.14). The substitutions have been illustrated with the mutant residues superimposed on the original residues.

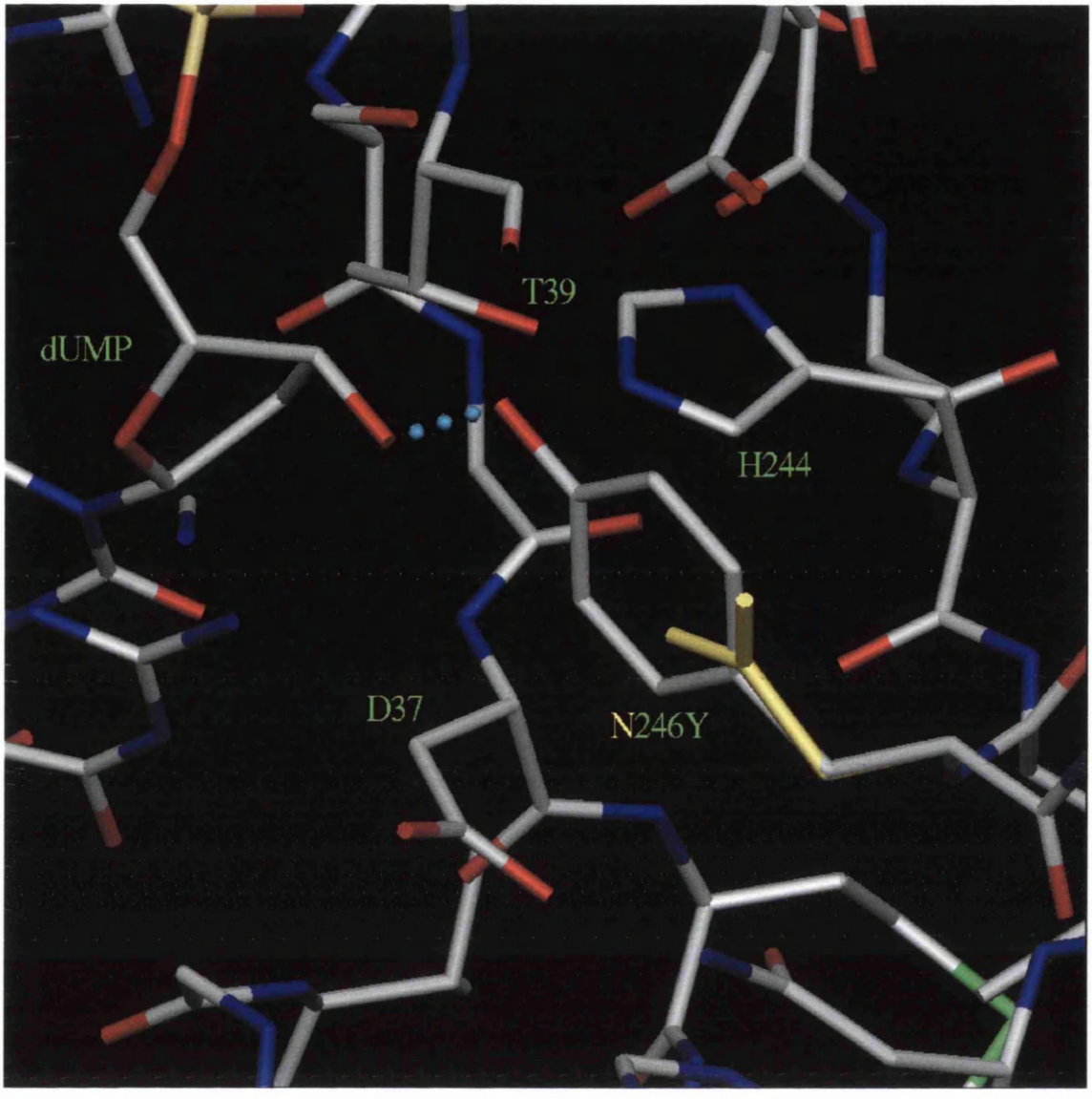


Figure 6.5. Temperature sensitive mutant AZ28 showing the point mutation Y246N

The mutant residue can be seen in yellow superimposed on the original tyrosine residue. Additional residues labelled in green are those in close proximity to the mutated residue. In cyan is a H-bond that is formed between the original tyrosine residue and the substrate dUMP.

AZ28 has the Y246N substitution. Tyr246 is a completely conserved residue and is present in β-strand ii of TS. It is located in a buried pocket surrounded by Thr39, His244 and Asp206. The hydroxyl group of tyrosine makes a hydrogen bond with an oxygen of dUMP with a bond length of 2.35Å. It was thought that if Tyr246 was mutated it would lead to the disruption of the hydrogen bond and affect enzyme activity. This mutant retains sufficient activity to complement NM522 Thy$^-$ bacteria at 30°C. The

mutation in *L.casei* TS, Y261N, does not result in complementation of the bacteria, however if the tyrosine residue is mutated to a methionine then the enzyme is capable of complementing Thy⁻ bacteria (Climie *et al.*, 1990).

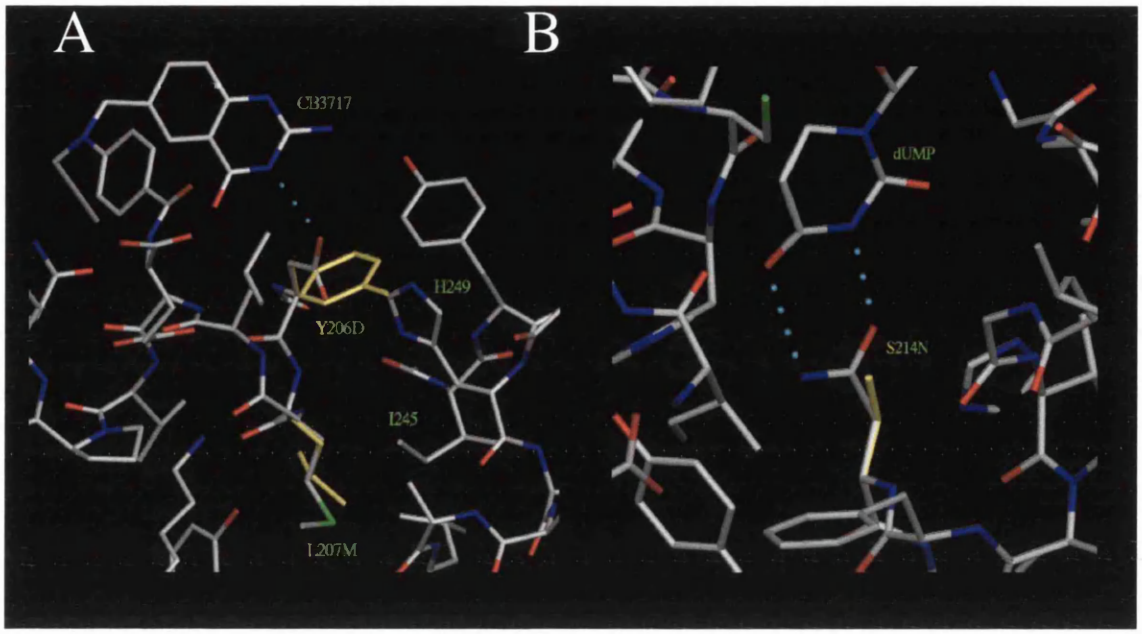


Figure 6.6. Temperature sensitive mutant AZ42 showing the point mutations D206Y, M207L and N214S

Panel A shows D206Y and M207L and panel B shows N214S. The mutant residues can be seen in yellow superimposed on the original residue. Additional residues labelled in green are those in close proximity to the mutated residue. In cyan are H-bonds that are formed between D206 and CB3717 and N214 and dUMP.

Three point mutations are present in **AZ42**; D206Y, M207L and N214S. Asp206 is a fully conserved residue of TS which has been shown to be mutated to a tyrosine residue. This residue makes a H-bond to N3 of CB3717 and substitution of this residue to a tyrosine would result in the loss of this hydrogen bond. It is thought, however that the enzyme could tolerate this change if the histidine residue at 249 moves to accommodate the tyrosine. A similar situation was found in *L.casei*, as the side chain of the corresponding residue (D221) forms a hydrogen-bond network with N-3 of folate. Substitutions at this position in *L.casei*, however generally give enzymes that do not complement Thy⁻ *E.coli* (D. Santi, unpublished reviewed in Carreras & Santi, 1995). The analogous residue in *E.coli* Asp169, could not be restored to Thy⁺ by any of the substitutions made (Michaels *et al.*, 1990).

The second mutation produced in AZ42 was in Met207 which is highly conserved in all but one of the eukaryote enzymes and in the bacterial enzymes it is one of several conserved hydrophobic residues (Figure 1.13 and pull-out). It is present in helix J of the

enzyme in a fairly hydrophobic pocket surrounded by Ile245, Ala252 and Leu253. No mutations have been produced in *L.casei* or *E.coli* which correspond to M207L.

The third mutation present in AZ42 is in the fully conserved Asn214, this residue is found in the highly conserved hydrophobic J-helix which forms the core of TS. It is the only residue that makes direct contact between a protein side chain and the dUMP pyrimidine ring. Hydrogen bonds are formed from Asn214 to N3 and O4 of dUMP, the oxygen of Asn214 forms a hydrogen bond to N3 dUMP with a bond length of 2.86Å and the nitrogen forms a hydrogen bond with a length of 3.01Å. Substitution of this residue for a serine would result in the loss of the hydrogen bonds. This disruption in enzyme structure produces the temperature sensitive phenotype. In *L.casei* it had been noted that N229 (N214 in VZV) mutations decrease the enzyme's ability to discriminate between dUMP and dCMP. The N214S mutation was not affected when dCMP was added to the assay indicating that the mutant resulted in the enzyme still retaining TS specificity for dUMP. It was capable of complementing Thy$^-$ *E.coli* and participating in the tritium release assay. In Chapter 8 an N214D mutation was constructed in VZV TS and this resulted in an increase in sensitivity to dCMP competition for the dUMP binding site although dCMP methylase activity was not directly demonstrated.

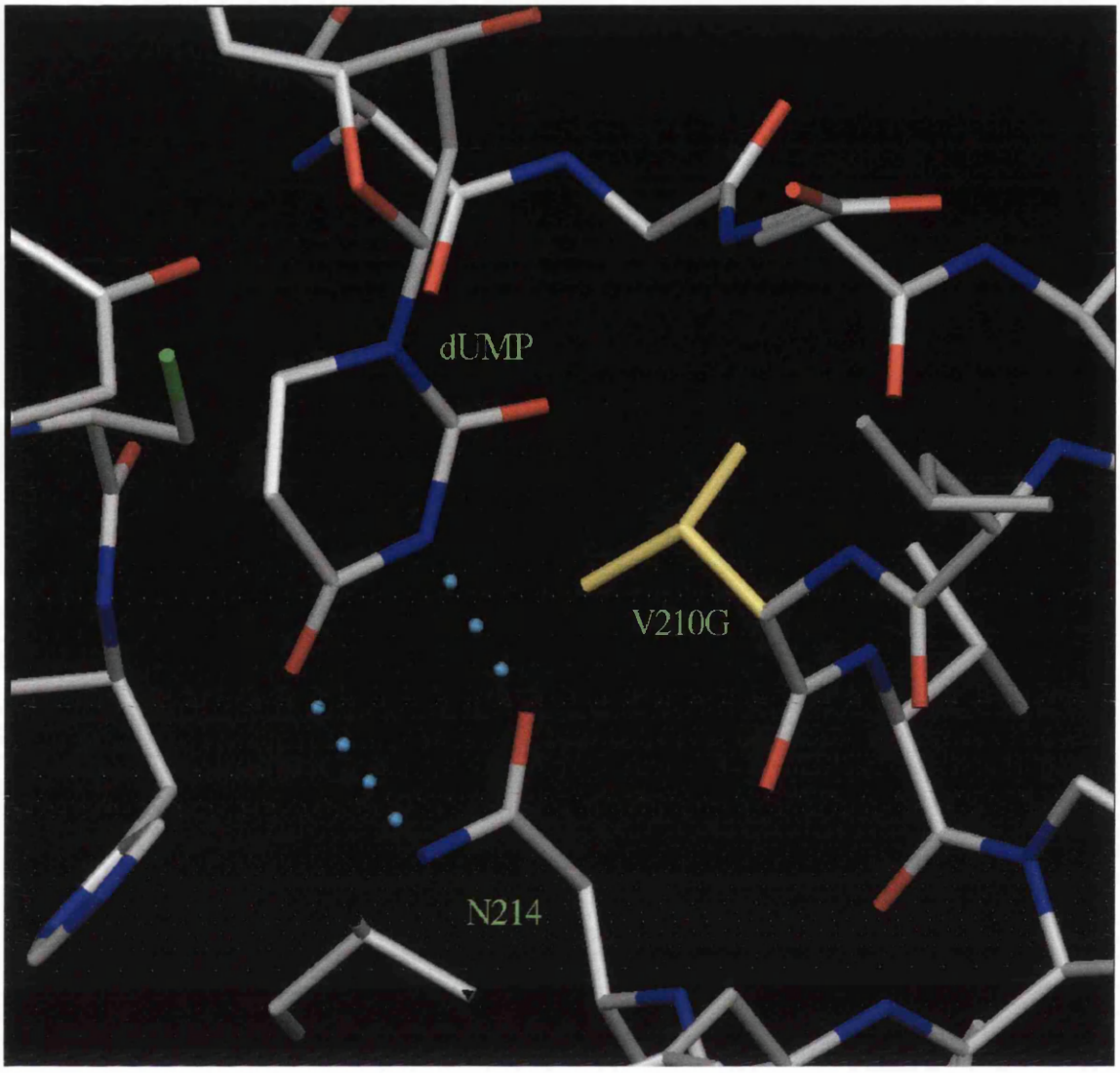


Figure 6.7. Temperature sensitive mutant AZ47 showing the point mutation G210V

The mutant valine residue can be seen in yellow superimposed on the original glycine residue. Additional residues labelled in green are those in close proximity to the mutated residue. In cyan is a H-bond that is formed between asparagine 214 and the substrate dUMP.

AZ47 has the point mutation G210V. Gly210 is fully conserved in all organisms apart from *L.lactis* and is found in helix J (refer to Figure 1.13 and pull-out). Substitution of glycine for a valine resulted in the residue being in Van der Waals contact with dUMP. G210V was capable of complementing Thy⁻ *E.coli* at 30°C and had 42.8% activity in the tritium release assay. No comparison could be made with *L.casei or E.coli* as this mutation had not been produced.

Further examination of the mutants for phenolphthalein inhibition has revealed that all of them appear to be more sensitive to the inhibitor than the wild type enzyme. This suggests that the active site of these enzymes is in a more open conformation than that of the wild type and as a result is more accessible to phenolphthalein.

Chapter 7. Mutagenesis of highly mobile regions of VZV TS

Studies on the highly mobile regions of VZV TS

This section of the results involves examining the effects of mutations on highly mobile regions of the enzyme. Two areas were targeted; the C-terminal tail and the DRTG loop as these are the regions which show most movement upon ligand binding.

The initial studies were focused on the highly exposed DRTG surface loop region which lies between helix A and β-strand i. This region contains four residues; Asp37, Arg38, Thr39 and Gly40 which were thought to be critical for enzyme function (Carreras & Santi,1995). These residues are conserved in 30 of the 35 TS sequences (Figure 1.13). Crystal structures from *E.coli* and *L.casei* have shown that Arg23 (R38 in VZV) is one of four residues that is responsible for the co-ordination of the phosphate of dUMP in the active site. In *L.casei* is was noted that upon substrate binding Arg23 (R38 in VZV) moves to interact with the phosphate moiety of dUMP, this action may be in preparation for the conformational change where Arg23 forms a salt bridge with the C-terminal carboxylate following folate binding (Climie *et al.*, 1990). In *E.coli* three of these loop residues, Asp20 (Asp37 in VZV), Arg21 (Arg38 in VZV) and Thr23 (Thr39 in VZV) have limited tolerance to substitution (Michaels *et al.*, 1990; Kim *et al.*, 1992). At Asp20, Cys and Ser substitutions were tolerated. Asp20 could also tolerate substitution with Glu, a larger but electrochemically similar residue. *In vitro* biochemical assays of the Cys and Ser mutations show that the specific activity of the mutants is reduced by approximately 20 fold which indicates that Asp20 plays a critical role. Arg21, however could not tolerate substitution by any other residue. The long positively charged side chain of Arg21 reaches into the base of the substrate binding pocket to stabilise the negatively charged phosphate moiety of the substrate. The structurally similar lysine residue could not substitute for arginine at this position which is due to the fact that the terminal nitrogen groups of Arg21 also make H-bonds with the carboxyl oxygens of Ile264 at the C-terminus of the protein, as well as indirect H-bonds with the folate cofactor via a water molecule. This interaction with Ile264 is important for stabilising the conformational changes associated with formation of the ternary complex. The interaction is possible only by the presence of multiple nitrogens in the Arg residue. This inability of the Arg21 mutants to complement Thy^- bacteria is best explained by their poor binding to substrate which reduces K_{cat}/K_m below essential levels needed for cell growth. The large increase in folate K_m value likely results from the inability of the mutant to form the salt bridge with the C-terminal carboxylate and effectively close the active site.

7.1. Construction of DRTG loop region mutants

Mutagenesis of the DRTG loop region was performed using two randomly spiked oligonucleotides, RO1 covering amino acids 37-55 and RO13 covering 20-39 (see Figure 6.1). Following mutagenesis the DNA was transformed into NM522 Thy$^-$ bacterial cells and colonies were screened for temperature sensitivity. The plasmids were recovered from the candidate temperature sensitive mutants and *EcoR1* digested to ascertain whether they contained the correct restriction profile. Having established that colonies contained the correct size of TS gene insert, the base changes were identified by sequencing. Table 7.1 summarises the efficiency of the mutagenesis procedure.

Spiked oligo	Colonies screened	Temperature sensitive colonies	Restriction profile	Readable point mutations
RO13	403	53	20	7
RO1	214	34	6	1

Table 7.1. Efficiency of mutagenesis of the DRTG loop region
The table shows the spiked oligonucleotides that were used in the mutagenesis and the results from screening and sequencing.

From Table 7.1 it was noted that a high proportion of the potential mutants screened did not produce the correct restriction profile upon enzyme digestion. EcoRI digestion of potential mutants should result in the TS gene being cleaved from the plasmid backbone to produce two distinct bands on an agarose gel, one band is the plasmid backbone and the other is the TS gene. The agarose gel in Figure 7.1 provides a typical example of temperature sensitive colonies that had been screened by EcoRI digestion. The first two lanes on the gel are control lanes indicating the bands that should appear with uncut and cut plasmid containing the wild type TS gene. Lanes numbered 1-7 contain the potential mutants. Many single bands appeared on the gel which could indicate that one of the EcoRI restriction sites has been affected by the filling in reaction of the mutagenesis procedure. Lane 6 provides an example of the correct digestion pattern for a potential TS mutant.

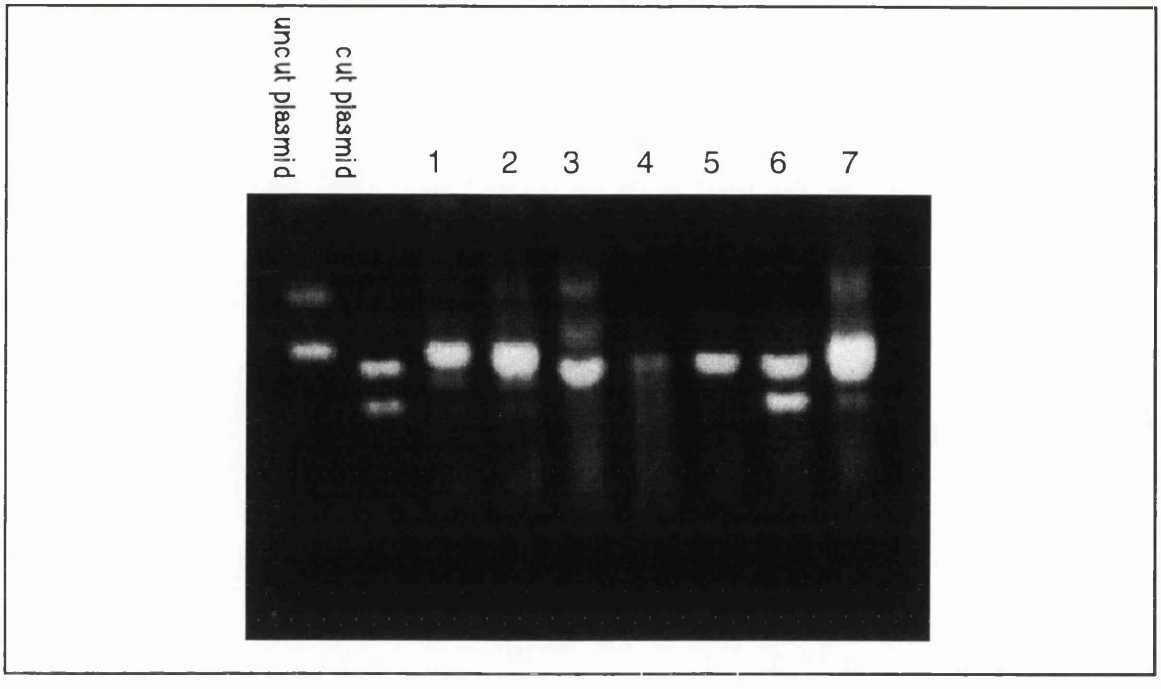


Figure 7.1. Agarose gel showing restriction profiles of potential TS mutants
A 1% agarose gel of an *EcoRI* digest of DNA obtained from potential TS mutants. The first two lanes are controls with uncut and cut pAD768 plasmid containing the wild type TS gene. Lanes numbered 1-7 contain the potential TS mutant. Lane 6 demonstrates a mutant with the correct restriction profile.

Sequencing of the mutants that contained the correct restriction profile has shown that only eight contained a TS sequence with a mutation. The remainder, either had an unreadable sequence or a wild type sequence. The enzymes with the wild type sequence however, had been shown to be temperature sensitive. It appears, therefore that such mutants may contain two copies of the TS gene, one with the wild type sequence and one with a mutation. A dominance effect can occur in such cases which results in the mutant gene producing a temperature sensitive phenotype over that of wild type. When sequenced both wild type and mutant DNA primes and the sequence can look like that of wild type but weaker bands can also be seen which correspond to the mutant sequence.

7.1.1. Characterisation of DRTG loop mutants

Following the mutagenesis procedure eight candidate clones were identified *in vivo* and were then further characterised by examining their catalytic activity *in vitro*. The *in vivo* assay involved the genetic complementation of NM522 cells which have a mutation in their TS gene. This was performed by patching colonies of the mutants onto M9 and M9T plates at 30°C, 37°C and 42°C. All eight mutants appeared to be temperature

sensitive as they could complement the bacterial cells on M9 plates at 30°C but not at 37°C or 42°C (Table 7.2).

Enzyme	M9 plates			M9T plates		
	31°C	37°C	42°C	31°C	37°C	42°C
JB 28	+	-	-	+	+/--	+/-
JB 29	+/-	-	-	+/-	-	+/-
JB 33	+/-	-	-	+	+/-	+/-
JB 35	+	-	-	+	+/--	+/---
JB 36	+/-	-	-	+/--	+/--	+/--
JB 38	+	-	-	+	+	+
JB 47	+	-	-	+	+	+/---
JB 15a	+	-	-	+	+	+/-

Table 7.2. Phenotypes of DRTG loop mutants
Mutants obtained from region directed mutagenesis were screened for temperature sensitivity by patching on minimal media plates with and without thymidine at three temperatures. Growth was scored as + for growth, +/- partial growth, +/-- little growth and +/--- very little growth.

Several of the mutants also showed very weak growth on M9T plates. As the NM522 Thy$^-$ cells used in the genetic complementation assay contain a point mutation and not a deletion in their TS gene they can still produce *E.coli* TS subunits. When a mutant enzyme with a defective VZV TS gene is transformed into the cells then growth of the bacterial strain can slow down as the TS subunits are interacting. This may be why poor growth was seen on M9T plates at varying temperatures. The lack of growth for JB29 on M9T plates could be due to inadequate transfer of cells.

Sequencing of the eight temperature sensitive DRTG loop region mutants revealed that all contained between one and three base changes (Table 7.3). JB28, JB29, JB36 and JB15a are the only mutants which contain point mutations in the DRTG loop residues themselves, the remainder contain mutations in helix A, strand-i and the G loop. In the mutagenesis procedure Tyr31 has been hit four times, Arg34 twice, Asp27 three times and D37 twice. Ochre stop codons (TAA) were found in JB33, JB35, JB36 and JB38.

NM522 cells contain an ochre suppressor which results in the insertion of a tyrosine at the position of the stop codon.

The method of mutant isolation was such that sibling mutants could be found and it is likely that JB13a, JB15a and JB21a, all of which contained the same mutation within the same background could be siblings. Only JB15a was further characterised.

Random oligo	Mutant	Amino acid substitution	Secondary structure
RO13	JB 28	Y 21 S	Helix A
		Y 31 N	Helix A
		R 34 P	Strand -i
RO13	JB 29	R 34 L	Strand -i
		K 35 E	Strand-i
		D 37 A	DRTG loop
RO13	JB 33	L 29 F	Helix A
		R 30 M	Helix A
		Y 31 Oc	Helix A
RO13	JB35	L 22 V	Helix A
		D 27 E	Helix A
		Y 31 Oc	Helix A
RO13	JB 36	L 22 Oc	Helix A
		D 27 G	Helix A
		D 37 N	DRTG loop
RO1	JB 38	D 27 E	Helix A
		Y 31 Oc	Helix A
RO13	JB 47	G 32 E	G loop
RO13	JB 13a JB 15a JB 21a	R 38 Q	DRTG loop

Table 7.3. DRTG loop region mutants

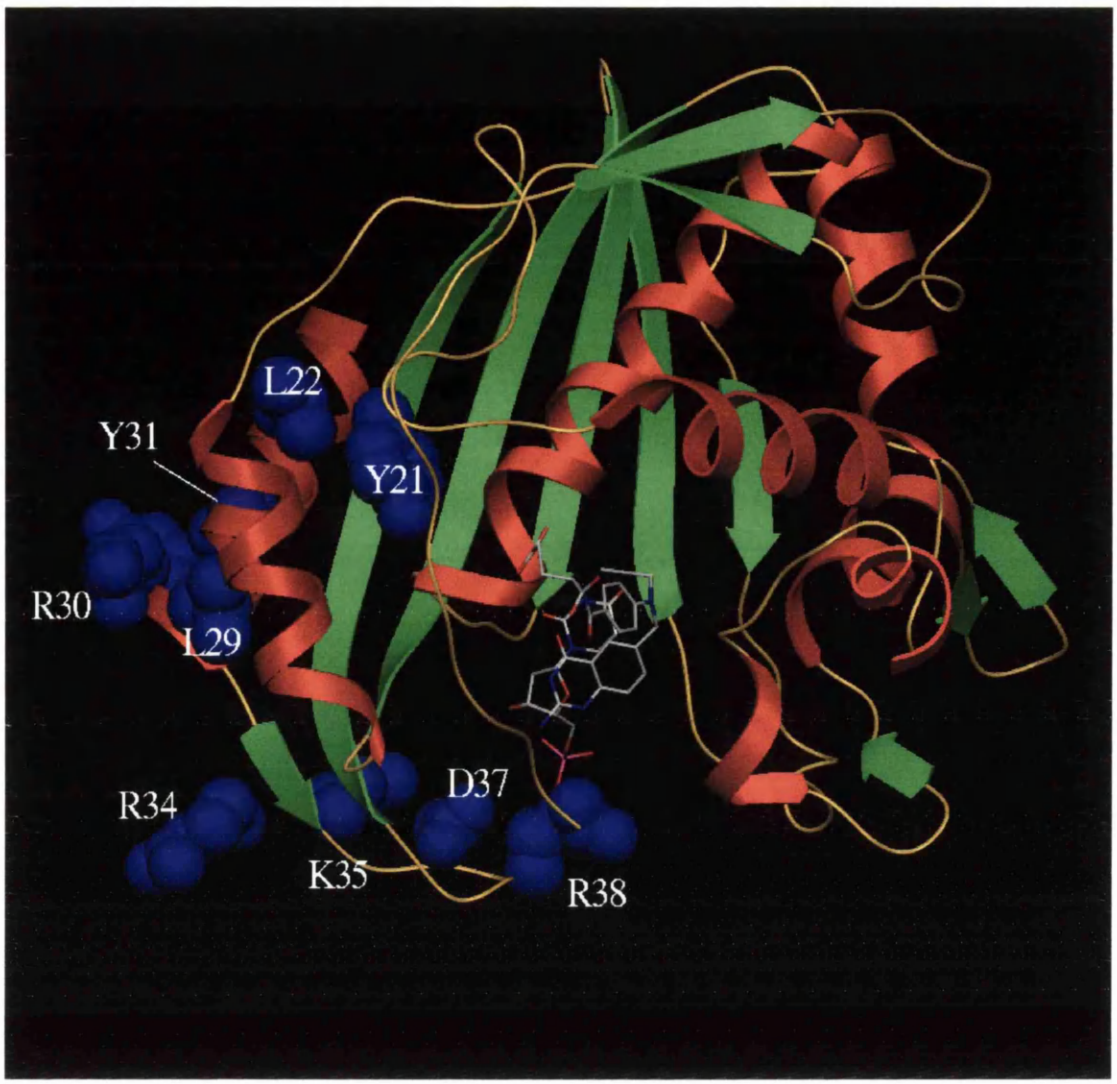


Figure 7.2. Thymidylate synthase monomer
This is a model of the monomer of VZV TS which shows the positions of the DRTG loop region mutants. In green are β-strands, α-helices are red, turns are yellow and the ligands are grey. The amino acid residues in the mutants are shown in blue space-filling. G32 is not shown.

The activity present in the mutants was quantified *in vitro* by the tritium release assay, crude protein extracts were prepared and the assay was performed at 30°C. The results reveal that a significant amount of TS activity was found in all of the mutants with a 2-3 fold reduction from wild type activity (Table 7.4). Previous studies on DRTG loop residues in *E.coli* and *L.casei* had indicated that three of the DRTG loop residues tolerated certain substitutions which resulted in up to 20-fold reduction in activity. Asp20 from *E.coli* (Asp37 in VZV) could be substituted to cysteine, serine or glutamic acid. Thr22 from *E.coli* (Thr39 in VZV) could tolerate substitutions to proline, serine, tyrosine,

glutamine and lysine. Gly23 from *E.coli* (G40 in VZV) could be substituted to any other amino acid. In *E.coli* the fourth DRTG loop residue, Arg21 (R38 in VZV) could not withstand any kind of substitution (Michaels *et al.*, 1990; Kim *et al.*, 1992). Crucially, however these studies had been performed at 37°C.

Enzyme	Tritium release pmol dTMP/min/ug protein	% Wild Type
Wild type	0.0240	100
JB 28	0.0080	33.3
JB 29	0.0110	45.8
JB 33	0.0150	62.5
JB 35	0.0087	36.3
JB 36	0.0093	38.8
JB 38	0.0067	27.9
JB 47	0.0092	38.3
JB 15	0.0071	29.6

Table 7.4. Enzyme activity of DRTG loop mutants
Crude extracts of the loop mutants and the wild type enzyme were prepared and TS activity was measured by the tritium release assay at 30°C. All enzymes were expressed in *E.coli* NM522 Thy$^-$ cells.

Temperature profiles of the loop mutants were examined *in vitro* as it had already been established that the mutants were temperature sensitive *in vivo*. Fractions of the crude extracts were held at 42°C and at 5 minute intervals aliquots were removed and assayed at 30°C by the tritium release assay. The loop mutants all produced a steady decrease in activity over time indicating that the longer the extracts were held at 42°C the less TS activity is present (Figure 7.3). By comparison the wild type extract only showed a slight decrease in activity verifying that this enzyme is not affected significantly by temperature.

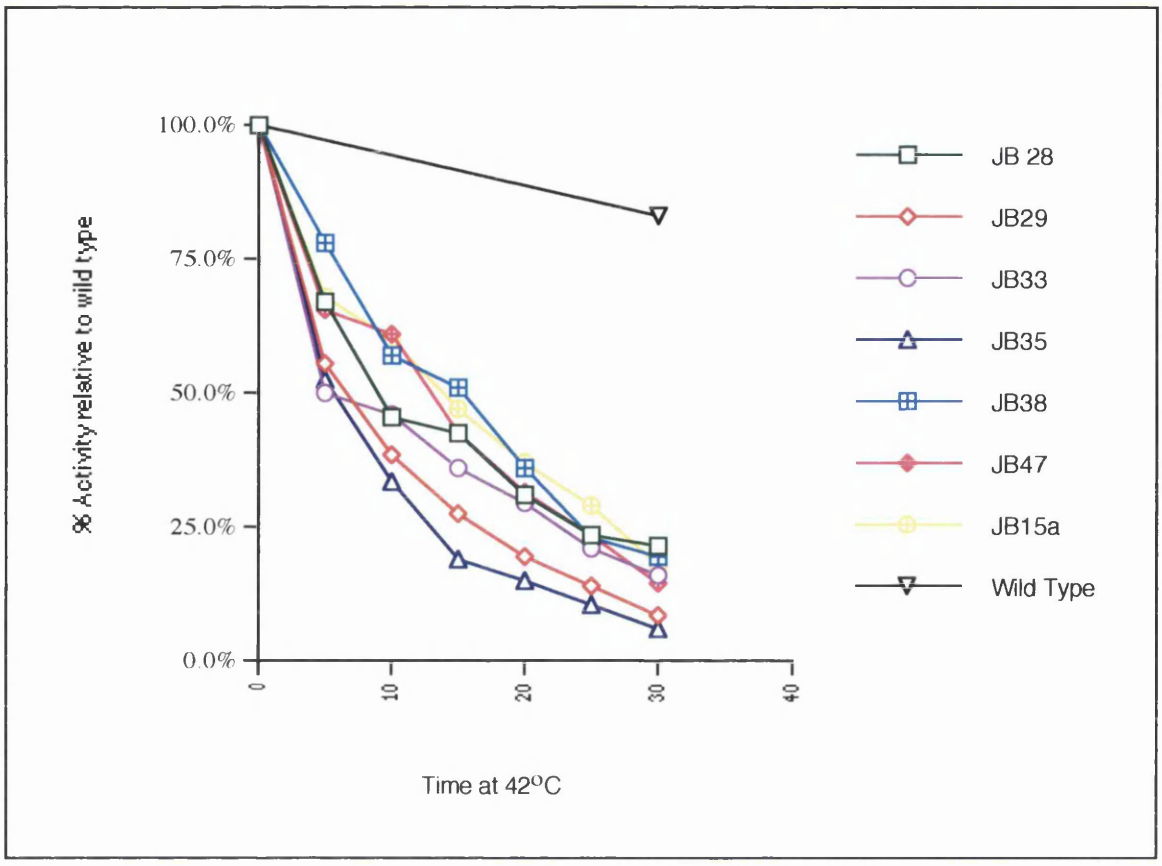


Figure 7.3. Temperature profiles of DRTG loop mutants

Crude protein extracts were held at 42°C and at 5 minute intervals aliquots were removed and assayed for TS activity at 30°C in the tritium release assay.

7.1.2. Discussion

Having established that the spiked oligonucleotide approach could produce a variety of temperature sensitive mutants targeted to various buried regions of TS, the same approach was used to successfully generate eight temperature sensitive mutant in the DRTG loop region.

Examination of the VZV TS ternary complex model reveals several things about the DRTG loop region. Firstly the loop is held in place by a number of hydrogen bonds which are formed between amino acids 32-35 from β-strand -i and residues 42-54 in β-strand i. In addition there are a number of residues which can tolerate mutations as they are surface exposed residues, these are Leu19, Lys23, Asp26, Asp27, Arg30 and Tyr31. Molecular modelling of the mutants was then used to explain the effects the amino acid substitutions were having on TS structure and function.

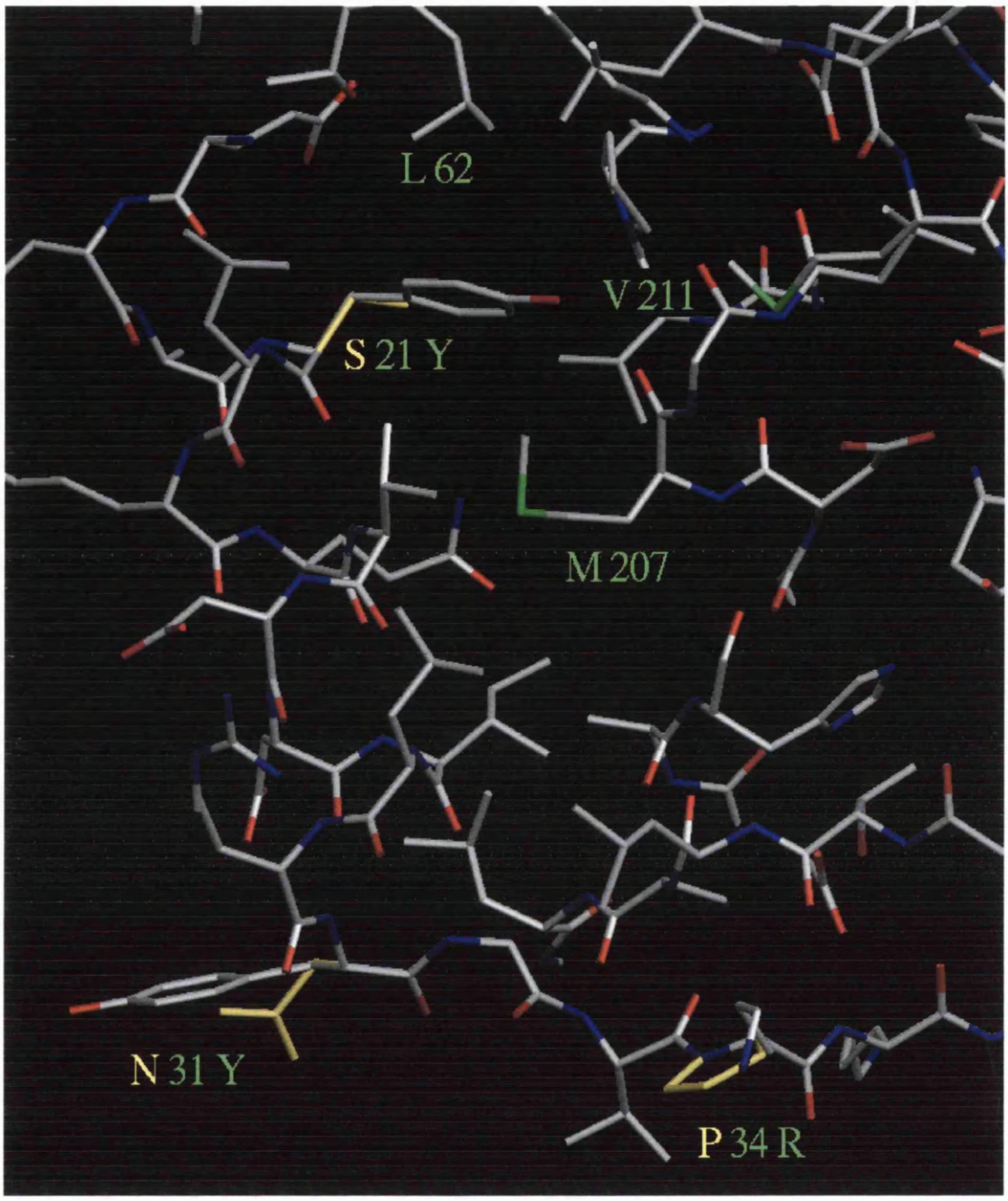


Figure 7.4. Temperature sensitive mutant JB28 showing the point mutations Y21S, Y31N and R34P

The mutant residue can be seen in yellow superimposed on the original residues. Additional residues given in green are those in close proximity to the mutated residue.

Mutant **JB28** has three amino acid substitutions Y21S, Y31N and R34P. Tyr21 is conserved in 34 out of 35 species of TS (see Figure 1.13 and pull-out sheet) the sole exception is *L.lactis* TS. It is found in helix A in a hydrophobic pocket surrounded by Met207, Val211 and Leu62. In JB28 this residue was mutated to a serine which

introduces a cavity into the pocket which would be expected to destabilise the protein. Arg34 is present in strand -i and is a solvent exposed residue and should tolerate amino acid substitutions. It is not very highly conserved and appears in only 4 out of 35 TS sequences (VZV, EHV-2, human and *S.aureus*). Tyr31 is in helix A and is found in only two of the 35 TS species (VZV, HVS). The side chain of this residue is surface exposed and so the position can easily accommodate amino acid substitutions. We conclude that the significant change leading to the temperature sensitive phenotype of JB28 is Y21S.

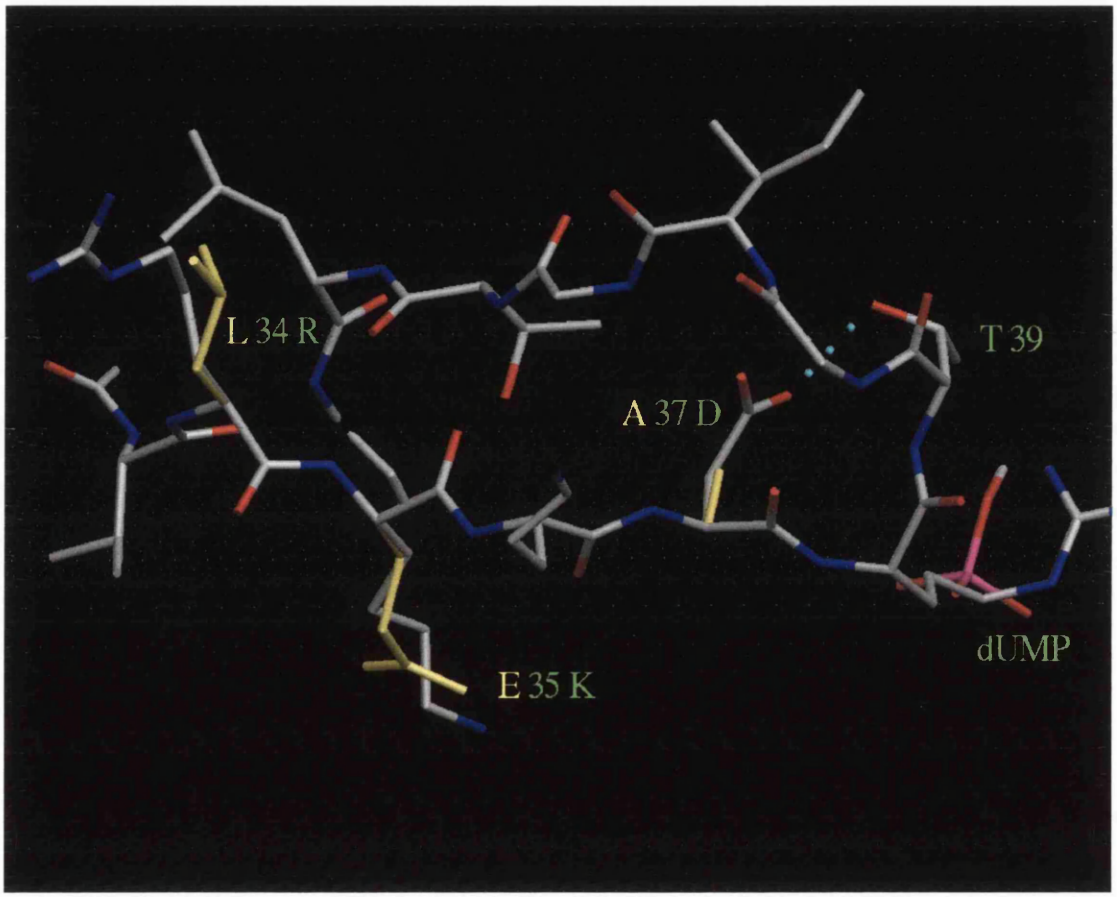


Figure 7.5. Temperature sensitive mutant JB29 showing the point mutations R34L, K35E and D37A

The mutant residues can be seen in yellow superimposed on the original residues. Residues given in green are those in close proximity to the mutated residue. In cyan is a H-bond that is formed between the original aspartic acid residue at position 37 and threonine at position 39.

Three amino acid substitutions are found in **JB29**, R34L, K35E and D37A. Asp37 is conserved in 33 of the 35 TS species and forms part of the DRTG loop. This residue forms a hydrogen bond with Thr39, which maybe important in the structure of the

loop. The O of the aspartate H-bonds to the Oγ of threonine with a favourable bond length of 2.9Å. Mutation of this residue to an alanine results in the loss of the H-bond.

The residues at positions 34 and 35 form part of the short -i β-strand between helix A and β-strand i. Arg34 is not very highly conserved occurring in only 4 out of 35 TS sequences (VZV, EHV-2, human and *S.aureus*). As mentioned above this residue is exposed to solvent and the position should tolerate amino acid substitutions. By contrast Lys35 is more highly conserved being present in 19 sequences across the full range of the TS family. The charge reversal which accompanies the substitution of lysine by glutamic acid might be expected to have more profound affects on the protein structure. It may be that the combination of the loss of the hydrogen bond from Asp37 and the charge reversal at position 35 together contribute to the destabilisation of the protein resulting in the temperature sensitive phenotype. It would require a site-directed mutagenesis approach changing these residues individually to fully resolve this issue.

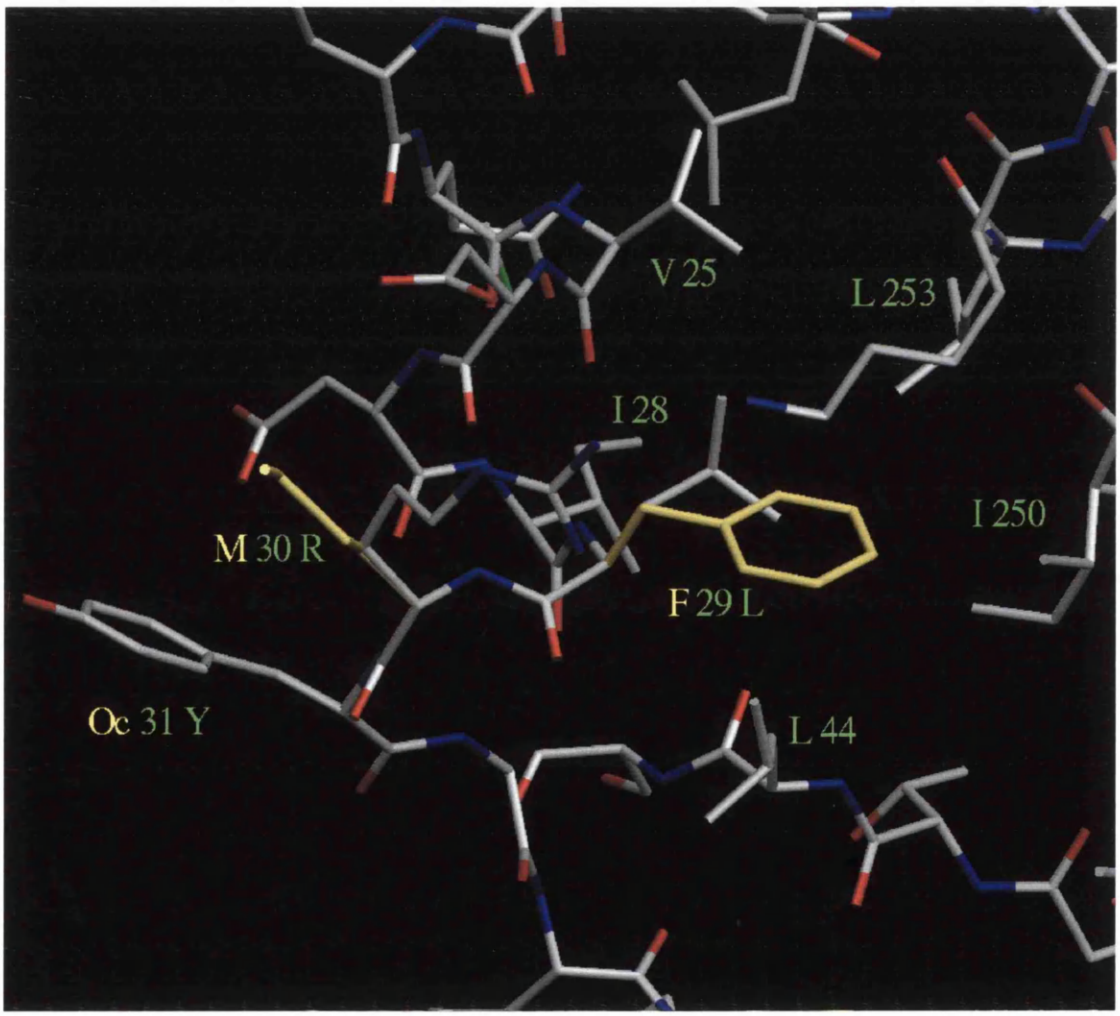


Figure 7.6. Temperature sensitive mutant JB33 showing the point mutation L29F, R30M and Y31Oc

The mutant residues can be seen in yellow superimposed on the original residues. Residues given in green are those in close proximity to the mutated residue.

Mutant **JB33** has three sequence changes two of which result in an amino acid substitution. The changes are L29F, R30M and Y31Oc. Leu29 is in helix A and is present in 15 of the 35 sequences across the full range of the TS family. It is found in a hydrophobic pocket composed of Val25, Leu253, Ile245, Leu44 and Ile28. From the VZV TS model it would appear that phenylalanine, may be accommodated in this pocket but it may be that the increased bulk of the phenylalanine side chain which has a volume of 135Å^3 compared to leucine which is 124Å^3 destabilises the enzyme (Creighton, 1993). Arg30 is in helix A and occurs in only 5 TS sequences from 35, all of which are from eukaryotes. This residue is solvent exposed and so should tolerate the R30M substitution. Tyr31 has been mutated to an ochre stop codon, which is read as a tyrosine by the NM522 bacterial cells and as a result this residue remains the same. These considerations lead to the conclusion that L29F is the change leading to the temperature sensitive phenotype.

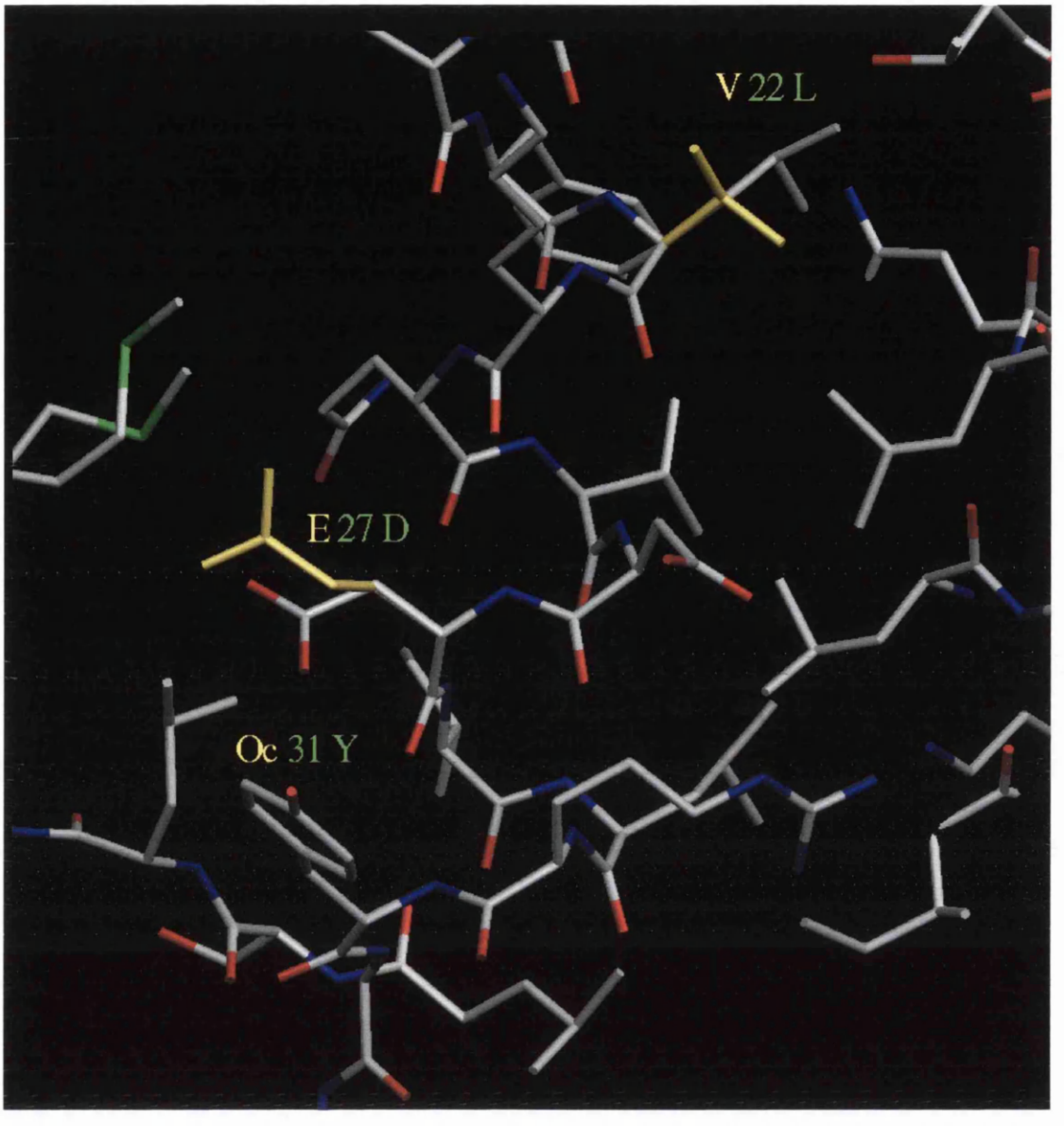


Figure 7.7. Temperature sensitive mutant JB35 showing the point mutation L22V, D27E and Y31Oc

The mutant residues can be seen in yellow superimposed on the original residue. Residues given in green are those in close proximity to the mutated residue.

Mutant **JB35** has three sequence changes two of which results in an amino acid substitutions. The changes are L22V, D27E and Y31Oc. All three amino acids are present in helix A. Asp27 is found in only 8 TS sequences from both prokaryotes and eukaryotes. It is an exposed residue and so can accommodate a change to glutamate. Leu22 is in 28 from the 35 species and is fully conserved amongst the eukaryotic TS enzymes. It is buried in a non-hydrophobic pocket. Mutation of Leu22 to a valine residue results in a decrease in side chain volume from 124Å^3 to 105Å^3, this could produce a hole

in the enzyme and lead to destabilisation. Again Tyr31 has been mutated to an ochre stop codon, which is read as a tyrosine by the NM522 bacterial cells and as a result this residue remains the same and would not affect enzyme structure. These findings lead to the conclusion that L22V is the change which is producing the temperature sensitive phenotype.

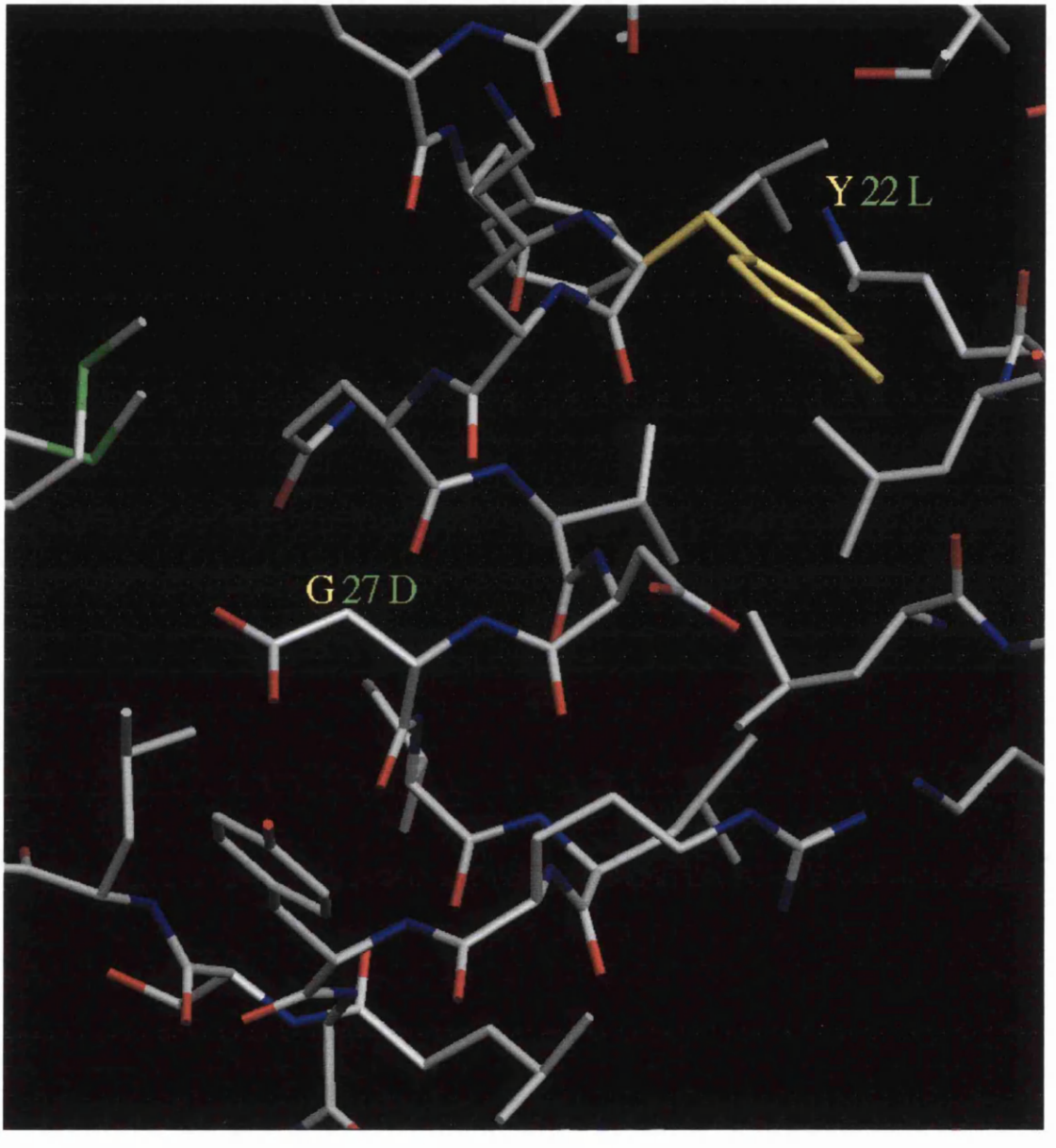


Figure 7.8. Temperature sensitive mutant JB36 showing the point mutations L22Oc and D27G

The mutant residues can be seen in yellow superimposed on the original residues. Residues given in green are those in close proximity to the mutated residue.

L22Oc, D27G and D37N are the three amino acid substitutions found in **JB36**. Asp37 is a highly conserved residue which is present in 35 from 37 TS sequences, the exceptions being *H.influenzae* and *M.genitalium* (both of which are entries from genome sequencing projects). It forms part of the DRTG loop and is found H-bonded to Thr39. Mutation of this residue to an asparagine would result in a change to a residue of similar size which would be a conservative change. The hydrogen bond would be maintained and the substitution would not be expected to affect enzyme structure. For this reason this mutation is not shown on Figure 7.8.

Leu22 is present in 28 from the 35 species and is fully conserved amongst the eukaryotic enzymes. It forms part of helix A and is in a pocket which is not hydrophobic. This residue has been mutated to a tyrosine residue and results in an increase in side chain volume from 124Å^3 to 141Å^3 which would be too big to fit into the pocket occupied by leucine (Creighton, 1993). The tyrosine residue however can adopt a conformation in which it can point out to solvent and this could then be accommodated by the enzyme. Asp27 is present in only 8 TS sequences from both prokaryotes and eukaryotes. This residue is found in helix A and is exposed to solvent. The mutation of aspartic acid to glycine at position 27 would not be expected to cause any problems. This D27G mutation cannot be seen in Figure 7.8 as glycine does not have a side chain. The conformational change required to accommodate tyrosine at position 22 would appear to destabilise the protein.

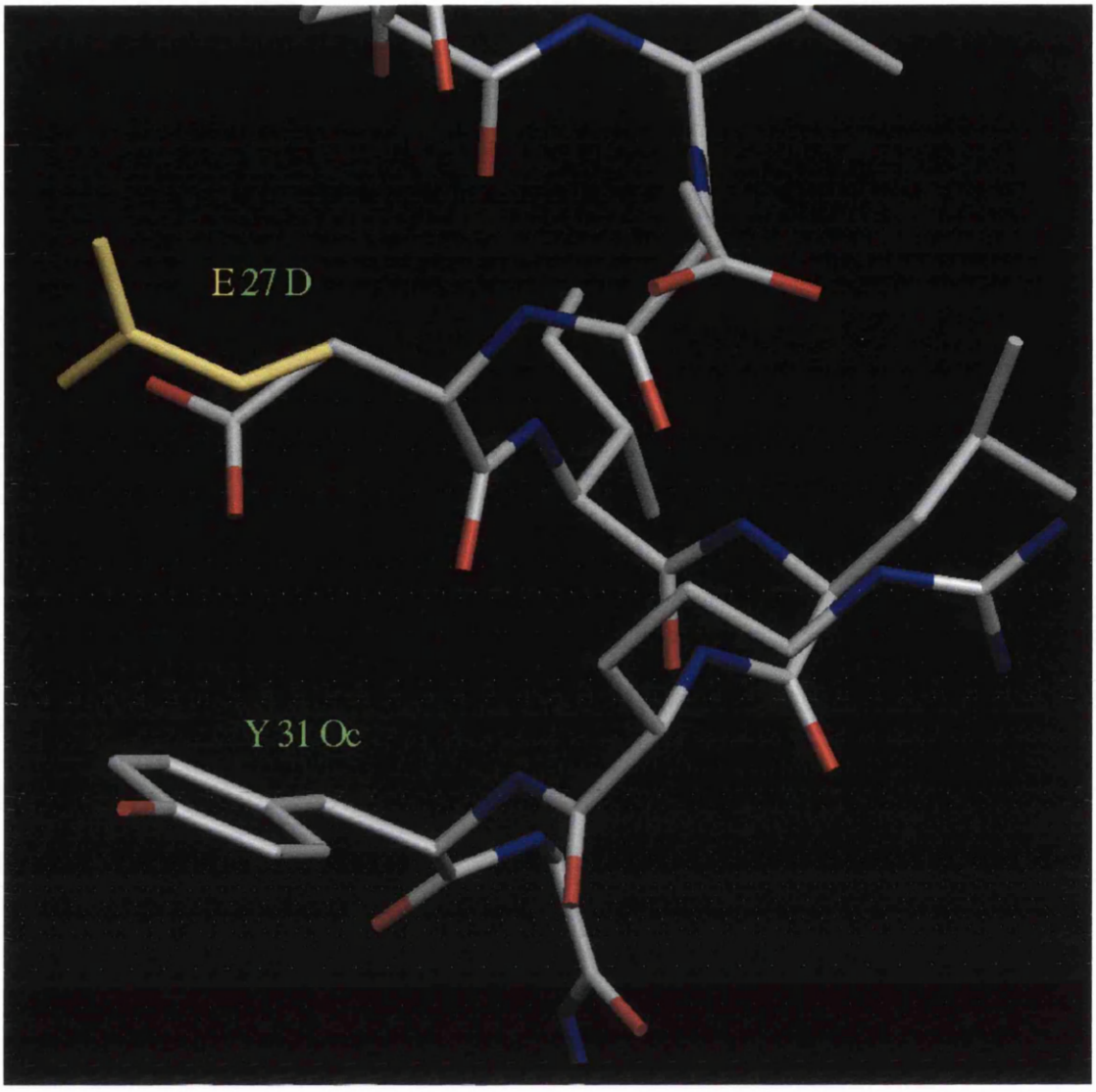


Figure 7.9. Temperature sensitive mutant JB38 showing the point mutation D27E and Y31Oc

The mutant residue can be seen in yellow superimposed on the original tyrosine residue. Residues given in green are those in close proximity to the mutated residue.

Mutant **JB38** has two sequence changes only one of which results in an amino acid substitutions, D27E and Y31Oc. Asp27 and Tyr31 are both surface exposed residues which are present in helix A. As stated previously Asp27 is present in 8 TS species from both prokaryotes and eukaryotes and Tyr31 is present in only 2 eukaryotic sequences. The tyrosine again has been mutated to an ochre stop codon which should have no effect on enzyme structure. The aspartic acid to glutamate change results in an increase in side chain volume from 91Å^3 to 109Å^3. Although Asp27 is a surface exposed residue this change in side chain volume could affect the interactions of aspartic acid with surrounding surface residues and could lead to protein stability.

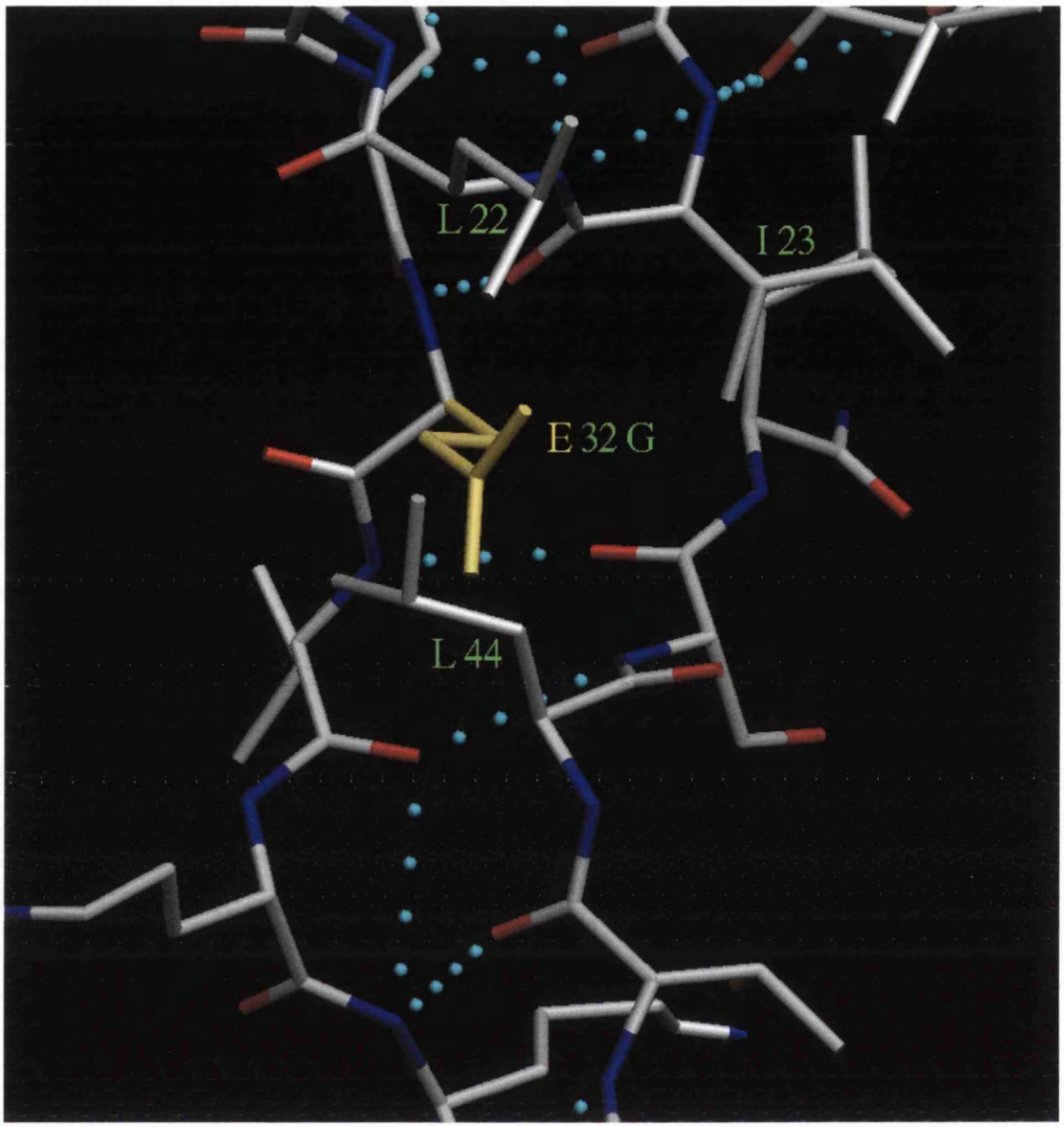


Figure 7.10. Temperature sensitive mutant JB47 showing the point mutation G32E

The mutant glycine residue can be seen in yellow superimposed on the original glutamate. Residues given in green are those in close proximity to the mutated residue. In cyan are the hydrogen bonds formed.

Mutant **JB47** has the amino acid substitution G32E. Gly32 is a fully conserved residue of TS present in what could be called the G loop of the enzyme (refer to Figure 1.13 and pull-out). It has a role in terminating helix A and allowing the enzyme flexibility to switch into strand -i and the DRTG loop. The single proton side chain of glycine points into a hydrophobic region composed of Leu22 and Ile23. In addition Leu44 makes close Van der Waals contacts with Gly32. This glycine residue is one of several that form

hydrogen bond interactions with β-strand i and hold the DRTG loop in place. From these considerations it is unlikely that Gly32 can tolerate any kind of substitution. It is interesting to note therefore that G32E is active at 30°C and this mutant deserves further study.

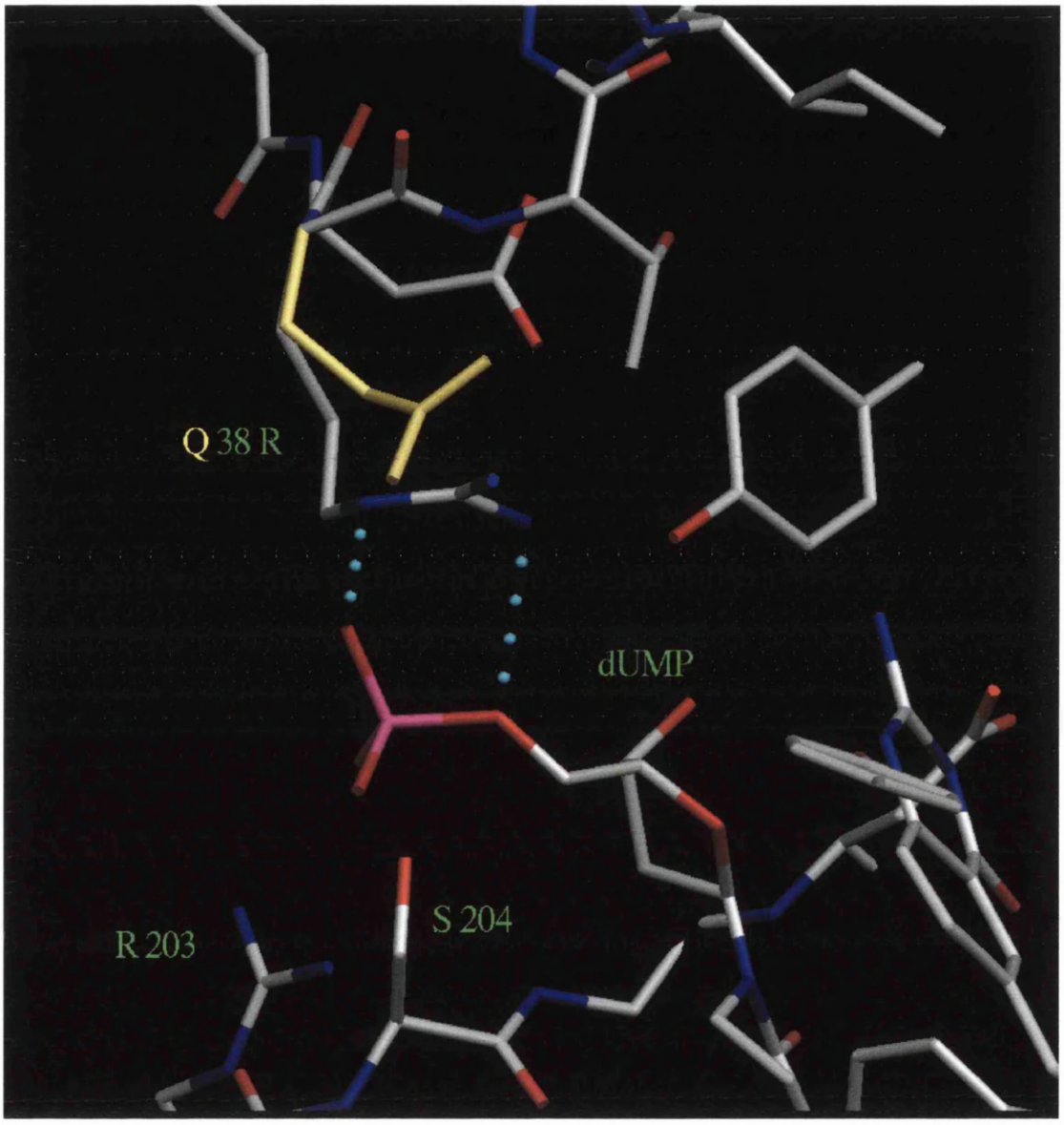


Figure 7.11. Temperature sensitive mutant JB15a showing the point mutation R38Q

The mutant glutamine residue can be seen in yellow superimposed on the original arginine residue. Residues given in green are those in close proximity to the mutated residue. There are two hydrogen bonds formed between Arg38 and the substrate dUMP, these are shown in cyan.

JB15a has the amino acid substitution R38Q. Arg38 is found in 32 of the 35 TS species and is present in the DRTG loop structure. Upon ligand binding, the C-terminus and DRTG loop move to form a lid over the active site and Arg38 forms a direct hydrogen bond with the C-terminal carboxylate (Leu301). This hydrogen bond is thought to stabilise the packing of the loop and C-terminus after ternary complex formation.

In addition Arg38 is one of four highly conserved arginines that form a hydrogen bond network around the 5'-phosphate of dUMP, the other residues involved in forming this network are Arg164, Arg165, Arg203 and Ser204. It is the NH2 of Arg38 that hydrogen bonds to the oxygen of dUMP with a bond length of 3.3Å and the nitrogen group hydrogen bonds to a second oxygen of dUMP with a bond length of 2.9Å. The H-bonds formed by Arg203 to the phosphate of dUMP and the position of Ser204 are shown Figure 7.11. Mutating Arg38 to glutamine would allow the nitrogen group of glutamine to form a hydrogen bond to one of the oxygens of dUMP and so only one of the hydrogen bonds would be destroyed. The decrease in enzyme activity found in this mutant can perhaps better be explained more by the loss of the hydrogen bond to the carboxylate group of the C-terminal leucine residue.

7.2. The C-terminus of VZV TS

Crystallographic studies show that, upon ligand binding, the C-terminus undergoes a large conformational change to form an active-site lid and part of the folate binding site (Montfort *et al.*, 1990). Carboxypeptidase cleavage of the terminal valine from one subunit of *L.casei* leads to complete loss of activity, although dUMP still binds with wild type affinity (Galivan *et al.*, 1976). Likewise, removal of the C-terminal residue from both subunits of *L.casei* or *E.coli* TS also renders the enzyme unable to complement TS-deficient *E.coli* hosts (Climie *et al.*, 1990, Michaels *et al.*, 1990). This has been presumed to be due to the disruption of H-bonds between the C-terminal carboxyl group and the side chains of Trp85, Arg23 and Thr24, and to PABA ring of the cofactor (Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990; Matthews *et al*, 1990b).

The *L.casei* TS mutants were inactive in the complete TS reaction but they were still able to participate in the partial reaction, catalysis of the dehalogenation of BrdUMP (Carreras *et al.*, 1992). This suggests that the C-terminal interactions are independent of the first stage of the covalent binding of dUMP, and the activation of the C5-H.

VZV TS appears to be unique in that following removal of the C-terminal amino acid to produce the mutant L301Δ it has slighly elevated debromination activity and 3% of the wild type activity when measured in the tritium release assay at 37°C (Harrison *et al.*, 1995).

Unique interactions occurring at C-terminus of VZV

Harrison *et al.*, (1995) compared the C-terminal tail of VZV to that of *E.coli* and *L.casei* to establish whether the differences in activity in the C-terminal deletion mutants could lie in the C-terminal tail itself, a region with which C-terminal tail interacts or a combination of both. Investigation of the final 6 amino acids from the various enzymes revealed that VZV has 4 amino acid substitutions (refer to Figure 1.13). The most significant is Glu298 in VZV which is replaced by a proline in *L.casei* and *E.coli.* Construction of a VZV TS mutant containing a proline at this position resulted in an enzyme with a 15-20 fold decrease in tritium release activity. Removal of the C-terminal residue from the E298P protein did not affect activity indicating that neither mutant acts as an accurate mimic of the *L.casei* TS C-terminal tail. Structural differences between VZV and *L.casei* were therefore not thought to reside solely in the C-terminal tail.

Further studies on the C-terminus involved examination of models from VZV and *L.casei* TS. In *L.casei* TS four hydrogen bonds are formed between the C-terminal residue and Arg23, Thr24, Trp85 and the PABA ring of the cofactor (Matthews *et al.*, 1990; Finer-Moore *et al.*, 1990; Montfort *et al.*, 1990). Four identical interactions were found in the *E.coli* enzyme but only two equivalent bonds are formed in VZV between the C-terminal residue and Arg38 and Thr39 (equivalent to Arg23 and Thr24). The third interaction in *L.casei* which involves Trp85 cannot occur in VZV TS as it has a tyrosine residue (Tyr100 in VZV) in place of the tryptophan. This residue cannot be positioned in the VZV enzyme such that its OH group could form a H-bond with the carboxylate group of the C-terminal residue without steric clashes. Removal of the C-terminal residue from *L.casei* (V316Δ) results in the disruption of the hydrogen bond network and leads to the inactivation of the enzyme.

As VZV retains a significant amount of activity when the C-terminus is removed it has been predicted that a further interaction must occur which closes down the enzyme sufficiently to allow catalysis. It has been suggested (Harrison *et al.*, 1995) that hydrophobic interactions may occur between the side chains of Ile99/Tyr100, the C-terminal residue and Met299 (Figure 7.12). This would result in VZV being stabilised more by hydrophobic contacts across the face of the enzyme between the C-terminus and helix D rather than H-bond contacts observed in *L.casei* and *E.coli.* In addition the aromatic ring structure of Tyr100 may also help stabilise the binding of the cofactor in an analogous manner seen by tryptophan. The small amount of activity observed in the C-terminal deletion mutant of VZV (L301Δ) is due to the decrease but not the abolition of these hydrophobic contacts, as the three remaining residues can still interact to hold the two sides of the active site together.

TS from *E.coli* and *L.casei* have a valine residue at the antepenultimate position in their C-terminus compared to methionine in VZV. They also contain the hydrophilic amino acid glutamate at the position equivalent to Ile99 of VZV. These residues may be

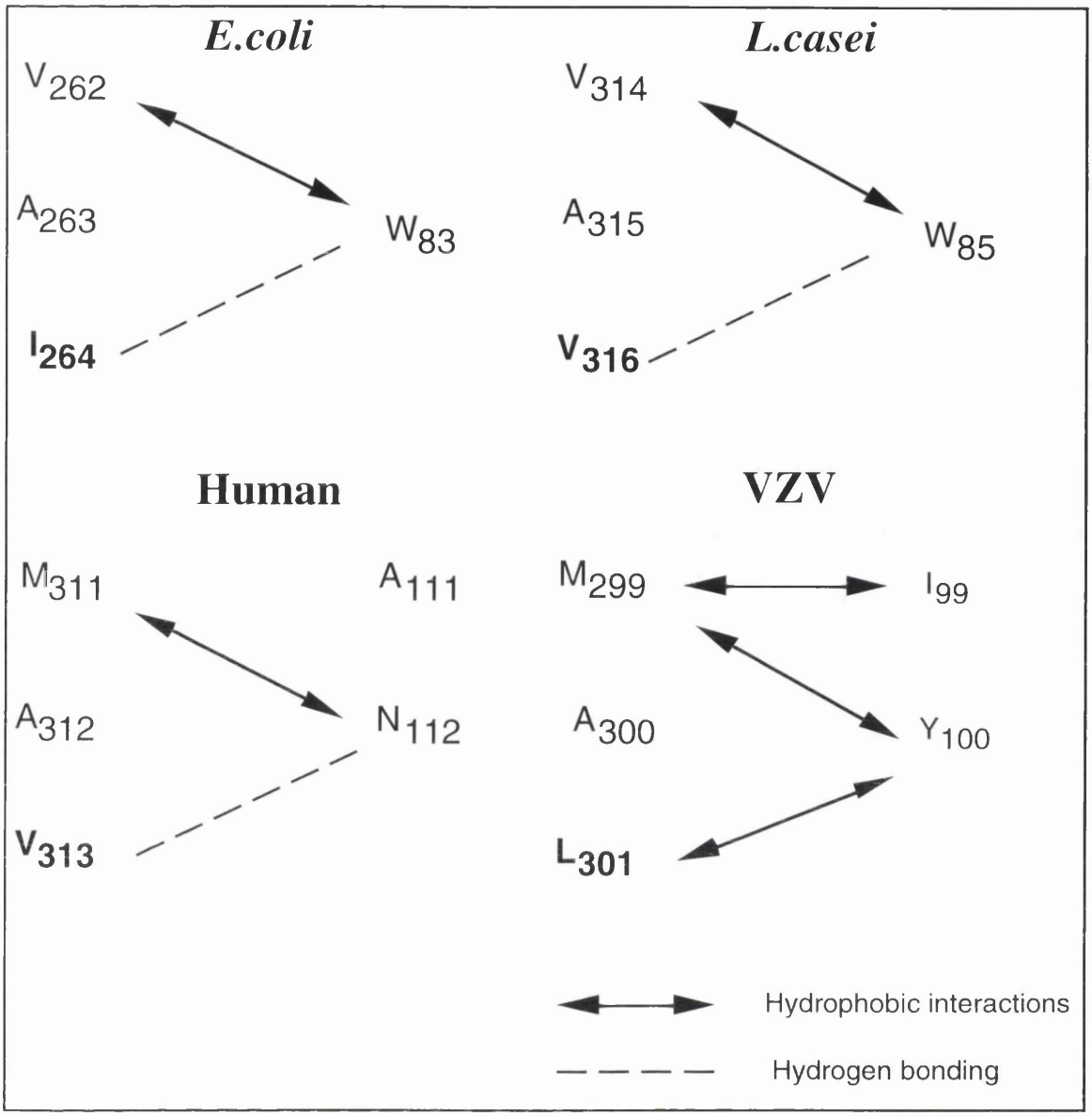


Figure 7.12. Interactions occurring at the C-terminus of thymidylate synthase
The C-terminal interactions of *L.casei*, *E.coli*, *VZV* and human TSs. Hydrophobic interaction are given as solid black lines and hydrogen bonds are dashed lines. The C-terminal residues are in bold.

sufficient to prevent significant hydrophobic interactions in *L.casei* and *E.coli*. These observations further support the importance of the hydrophobic interactions in VZV TS.

Examination of other eukaryotic TS sequences shows that they do not contain a tyrosine residue at the position equivalent to Tyr100 but an asparagine residue (refer to Figure 1.13). An asparagine has been modelled in place of tryptophan in *E.coli* TS such that its N-H group could form a H-bond to the carboxylate group of the C-terminal residue. This suggests that the asparagine residue in eukaryotes may play an analogous

role to the conserved tryptophan in TS from prokaryotes and these TSs could also be inactive if their C-terminal residue is removed (P.T. Harrison, PhD thesis, Glasgow University 1992). Therefore it appears that it is the tyrosine residue in VZV TS, which is uniquely found in place of a conserved tryptophan or asparagine residue, that is in part responsible for the residual activity in VZV L301Δ.

7.2.1. Mutagenesis of the C-terminal region

Region-directed mutagenesis was performed on the C-terminus of VZV TS using spiked oligonucleotides RO19 and RO20 which covered amino acids 284-301 and 284-300 respectively. The efficiency of the mutagenesis procedure is given in Table 7.5.

Spiked oligo	Colonies screened	Temperature sensitive colonies	Restriction profile	Readable point mutations
RO19	716	65	24	0
RO20	230	56	9	4

Table 7.5. Efficiency of mutagenesis procedure
The table shows the spiked oligonucleotides that were used in the mutagenesis and the results from screening and sequencing.

The results demonstrated that a large number of the potential temperature sensitive mutants did not produce the correct restriction profiles and had similar profiles to those seen in Figure 7.1. Single stranded DNA was extracted from the mutants that did contain the correct restriction pattern and these were sequenced. Mutations were initially identified by sequencing across the C-terminal region using primer 59. As before the absence of further changes introduced during the mutagenisis step was confirmed by sequencing the complete TS gene with primers 59-62. The mutants are shown in Table 7.6.

Proteins	C-terminal sequence
Wild type	**283** D F Q L D G Y N P H P P L K M E M A L **301**
JB 73	**283** D F Q L M D I I H T P P **294**
JB 79	**283** D F Q H H D G Y N P H P P L K M E M A L **301**
JB 88	**283** D F Q L D **287**
JB102	**283** D F Q L D G Y N P H P P L K E W L F N G F L N V V K T V D V L R M **316**

Table 7.6. C-Terminal mutants

C-terminal mutants produced by region-directed mutagenesis using spiked oligonucleotide RO20 which covered amino acids 284-300. Mutated residues are shown in red.

JB79 contained a L286H point mutation and the other three mutants produced major reconstructions of the C-terminus. JB73 has a deletion in Leu286 which changes the reading frame of the remaining amino acids and produces an amber stop codon at position 284, resulting in a seven amino acid deletion. JB88 contains a point mutation in Gly288 which produces an amber stop codon at position 288 resulting in a 14 amino acid deletion. In contrast JB102 carries a deletion in Glu298 which changes the frame of the amino acids and results in an amber stop codon being present at position 316 rather than 301, resulting in a 15 amino acid extension. The C-terminus of VZV TS with the positions of mutations and deletion are shown in Figure 7.13.

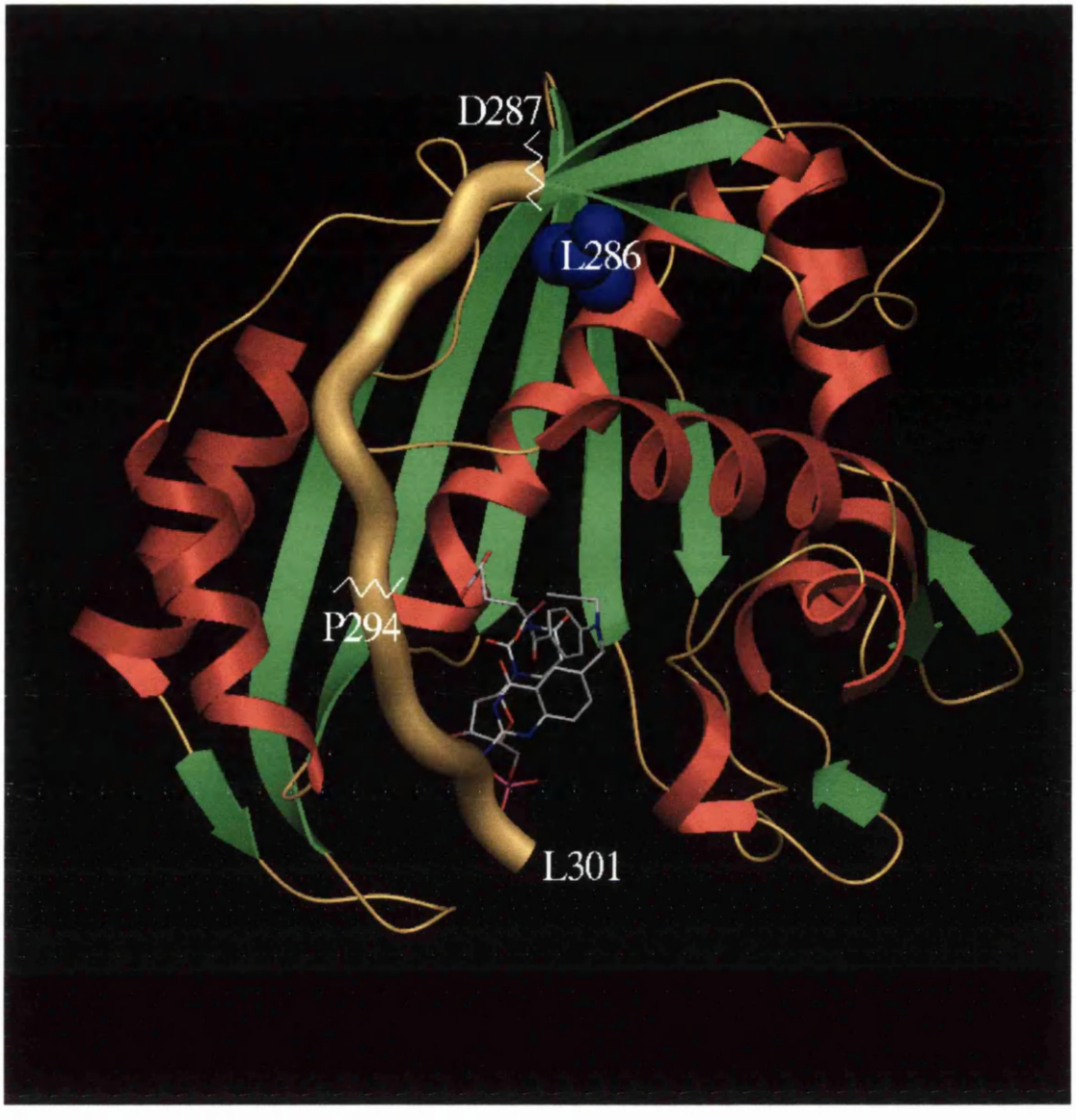


Figure 7.13. Thymidylate synthase monomer

A model of the monomer of VZV TS showing the positions of the C-terminal mutants. In green are β-strands, α-helices are red, turns are yellow and the ligands are grey. The amino acid residues in the mutants are shown in blue space-filling. The C-terminus is present as a thick yellow strand with the positions of the deletions shown as white zig zag lines.

As C-terminal mutants from other TS enzymes had shown no activity in the complete reaction, the four C-terminal mutants produced were assayed for activity. All four mutants had been shown to be catalytically active *in vivo* as they could complement the growth of NM522 Thy$^-$ cells at 30°C. Crude protein extracts were prepared of the mutants and the tritium release assay was performed at 30°C to quantify the TS activity of the mutant enzymes. The specific activity of the crude extracts are given in Table 7.7.

Enzyme	Tritium release pmol/min/ug protein	% Wild type
Wild type	0.0205	100
JB 73	0.0021	10.2
JB 79	0.0013	6.3
JB 88	0.0018	8.8
JB 102	0.0037	18.0

Table 7.7. TS activity of C-terminal mutants

Crude extracts of the C-terminal mutants and wild type enzyme were prepared and the activity was measured by the tritium release assay. All enzymes were expressed in *E.coli* NM522 Thy$^-$ at 30°C

Analysis of the crude extracts from the C-terminal mutants show that they contain significant activity. Although these experiments were performed on crude extracts at 30°C they complement previous studies on the C-terminus of VZV TS which revealed that removal of the C-terminal residue L301 resulted in a significant decrease in enzyme activity but not complete inactivation (Harrison *et al.*, 1995). In contrast, similar studies performed on *L.casei* TS revealed that deletion of 4 or 8 amino acids from the C-terminus of this enzyme resulted in complete inactivation of the enzyme as the enzyme could not form a covalent complex with the substrate and cofactor (unpublished data cited in Carreras & Santi, 1995).

From the crude extracts temperature profiles of the mutants were determined to establish whether the mutants were temperature sensitive *in vitro* as it had already been established that they were temperature sensitive *in vivo* (Figure 7.14).

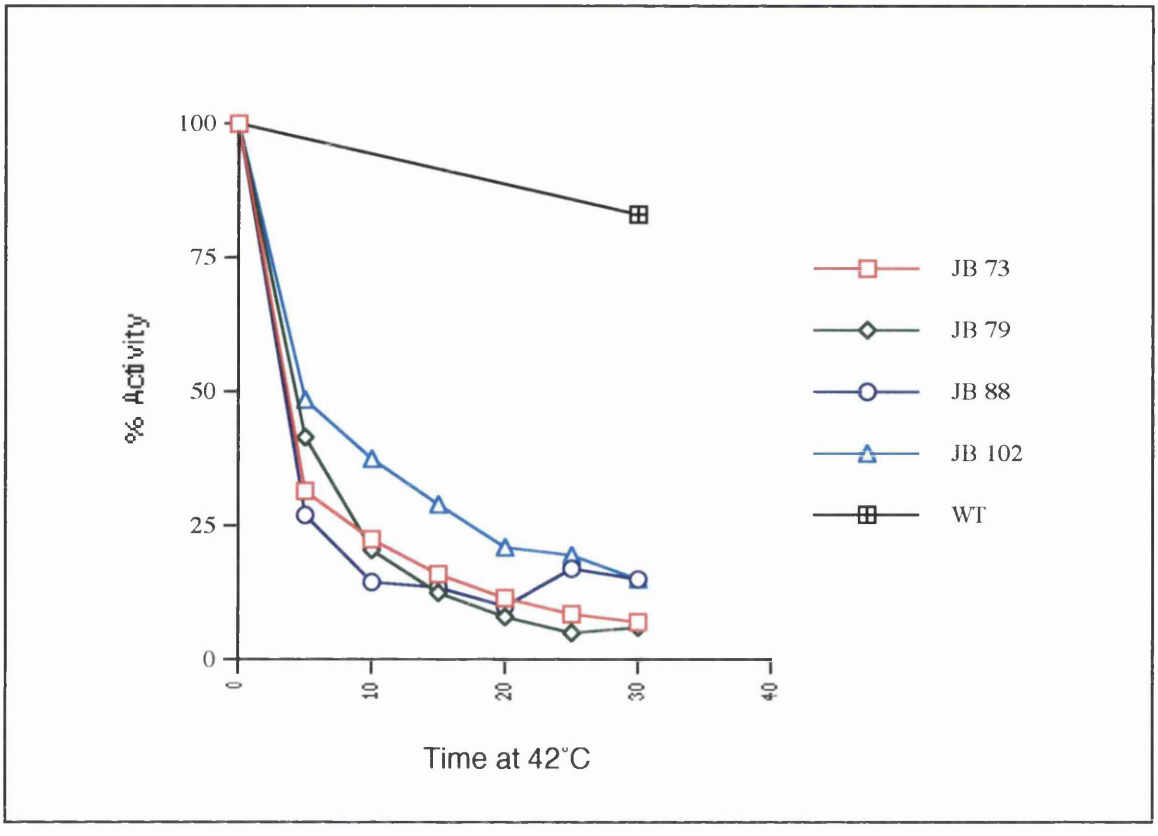


Figure 7.14. Temperature profiles of the C-terminal mutants
Crude protein extracts of the mutants were prepared and samples from the extracts were held at 42°C. At 5 min intervals 5μl aliquots were removed and assayed for TS activity in the tritium release assay at 30°C. The graph shows the decrease in activity of the mutants in response to the length of time incubated at 42°C.

All of the mutants displayed a sharp decrease in activity the longer they were held at 42°C, in contrast the wild type extract shows only a slight decrease in activity. Therefore the mutants were temperature sensitive *in vitro* as well as *in vivo*.

7.2.2. Characterisation of purified JB88

In order to purify the C-terminal deletion mutant JB88, the TS gene was digested from its original pAD768 backbone and ligated into the pET23d vector which is a high expression vector under the control of the T7 promoter (refer to Figure 5.1). The protein was insoluble when produced in such large amounts and so it was recloned into the original backbone under the control of the *tac* promoter (refer to Chapter 5). Following expression of JB88 from the pAD768 vector, the enzyme was purified by hydroxylapatite and S-sepharose chromatography. Figure 7.15 is a Coomassie stained polyacrylamide gel demonstrating the purity of the mutant following S-sepharose chromatography.

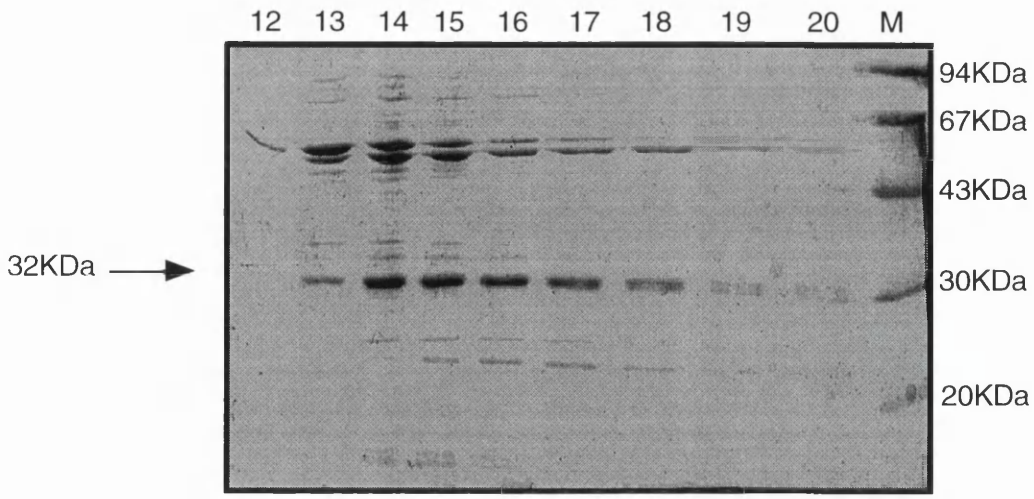


Figure 7.15. Purification of JB88 on an S-sepharose column

Coomassie stained gel of fractions collected from purification of JB88 by S-sepharose chromatography. The partially purified protein extract obtained from the hydroxylapatite column was bound to the S-sepharose column, the column was washed with phosphate buffer and the TS protein was eluted from the column with an increasing salt gradient. Lanes 12-20 are fractions eluted from the column which are found to contain TS protein. TS is present at 32KDa and is denoted by an arrow. Protein markers (M) are present on the right.

JB88 was found to have 0.10% of wild type activity (Table 7.8). This is very low but is sufficient to complement Thy$^-$ NM522 cells. In contrast the crude extract had 8.8% activity, this may reflect loss of activity during the purification process.

Enzyme	Tritium release assay pmol/min/ug protein	% Wild type
Wild type	8.34	100
JB 88 14 a.a. deletion	0.0084	0.10

Table7.8. TS activity of the deletion mutant JB88

The C-terminal mutant JB88 and the wild type enzyme were purified and the activities were measured by the tritium release assay. All enzymes were expressed in *E.coli* NM522 Thy$^-$.

Further characterisation of the deletion mutant required kinetic analysis. Spectrophotometric assays, which measure the increase in absorbance at 340nm concomitant with dihydrofolate production were carried out on purified JB88. The protein extract was added to a microtitre plate containing spectro assay buffer and either varying concentrations of substrate or cofactor (S). Graphs were generated of the increase in absorbance over time at the varying concentrations of substrate and from these the velocity (V) of the enzyme at the different concentrations could be calculated. This allowed a graph to be drawn of 1/V against 1/S from which a K_m value could then be obtained. This procedure was performed several times for both dUMP and folate and the mean K_m values are given in Table 7.9

Enzyme	**dUMP K_m (μM)**	**Folate K_m (μM)**
Wild type	5.00 ± 0.31	25.77 ± 2.20
JB 88 14 a.a. deletion	1.41 ± 0.34	523 ± 87.2

Table 7.9. Kinetic analysis of purified TS enzymes
Spectrophototmetric assays were performed on purified wild type enzyme and the C-terminal deletion mutant JB88. The assays measure the increase in absorbance at 340nm concomitant with dihydrofolate production and allow K_m values for dUMP and folate to be evaluated. The K_m results are given as the mean K_m value ± the standard error.

The K_m value for dUMP was found to be 3 fold lower than the wild type enzyme which would suggest that the active site of the mutant enzyme is in a more open conformation allowing the substrate to enter more readily (Table 7.9). In contrast the folate K_m value for the C-terminal mutant has dramatically increased by around 25 fold which suggests that reduced folate binding must play a part in the diminished activity of the mutant enzyme. This very high folate value could possibly be the result of the failure of the active site to close down upon ligand binding. Therefore it appears that in the C-terminal mutant JB88, removal of the 14 amino acids from the C-terminus results in an enzyme which has difficulty forming a closed complex. These data are consistent with that obtained from the C-terminal deletion mutant L301Δ which had a dUMP K_m value of

0.65 ± 0.09μM and a folate K_m value of 514 ± 83.12μM at room temperature (Harrison *et al.*, 1995).

7.2.3. Kinetic analysis of C-terminal extracts

The remaining three C-terminal mutants; JB73, JB79 and JB102 could not be purified which resulted in them being characterised more fully from crude extracts. Fresh crude extracts of the C-terminal mutants were prepared, dialysed and assayed for enzyme activity (Table 7.10). Having established that the extracts were active, spectrophotometric assays were performed to obtain a K_m value for dUMP.

Enzyme	dUMP K_m (μM)
Wild type	-
JB 73	1.79 ± 0.29
JB 79	1.27 ± 0.17
JB 88	-
JB102	1.53 ± 0.32

Table 7.10. Kinetic analysis of C-terminal mutants
Crude extracts of C-terminal mutants were prepared and analysed specrophotometrically to initially obtain a K_m value for dUMP. Spectrophotometric analysis involves the examination of the increase in absorbance at 340nm concomitant with dihydrofolate production. The results for the K_m for dUMP are given as the mean K_m value ± the standard error.

The C-terminal mutants JB73, JB79 and JB102 produced low dUMP K_m values but inconsistent results were obtained from the crude extracts of the wild type enzyme and JB88 and so no conclusive result could be drawn. Determination of the folate K_m also presented a few problems. It appeared that when the concentration of folate in the assay was increased then the TS response appeared to get lower and when folate was omitted from the assay the extracts produced the highest response. These results indicated that the crude extracts must contain an agent that interferes with absorbance.

7.2.4. Inhibition of C-terminal mutant enzymes

Phenolphthalein inhibition

Phenolphthalein inhibition was examined initially on crude preparations of all four C-terminal mutants by the tritium release assay. Phenolphthalein was added to the assay at 50, 100 and 500μM concentrations. Inhibition of the enzymes can be seen in Figure 7.16.

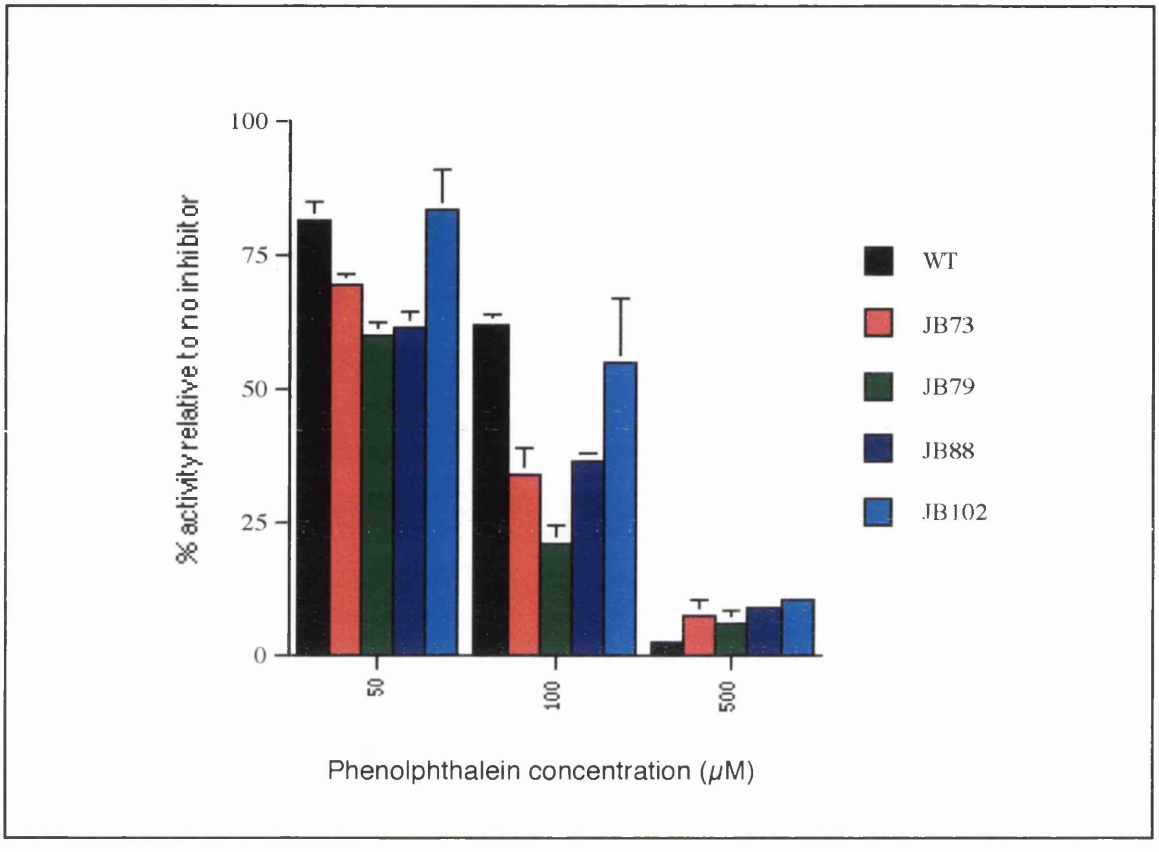


Figure 7.16. Phenolphthalein inhibition of C-terminal mutants

Crude extracts of C-terminal mutants and wild type TS were assayed for phenolphthalein inhibition in the tritium release assay. Phenolphthalein was examined at 50, 100 and 500μM concentrations. The graph shows the activity present in the mutants relative to no inhibitor.

The results from the assay are shown in Figure 7.16 and reveal that at concentrations of 500μM, phenolphthalein drives down TS activity in all of the mutants as well as the wild type enzyme, although the mutant enzymes show slightly more resistance. JB73, JB79 and JB88 were much more sensitive to phenolphthalein than the wild type extract at 50 and 100μM concentrations. JB102, however showed more resistant to phenolphthalein than the wild type enzyme at 50μM and 500μM and was only slightly more sensitive at 100μM.

The tritium release assay was then repeated with purifed JB88, again using 50, 100 and 500μM concentrations of the inhibitor. The results are shown graphically in Figure 7.17.

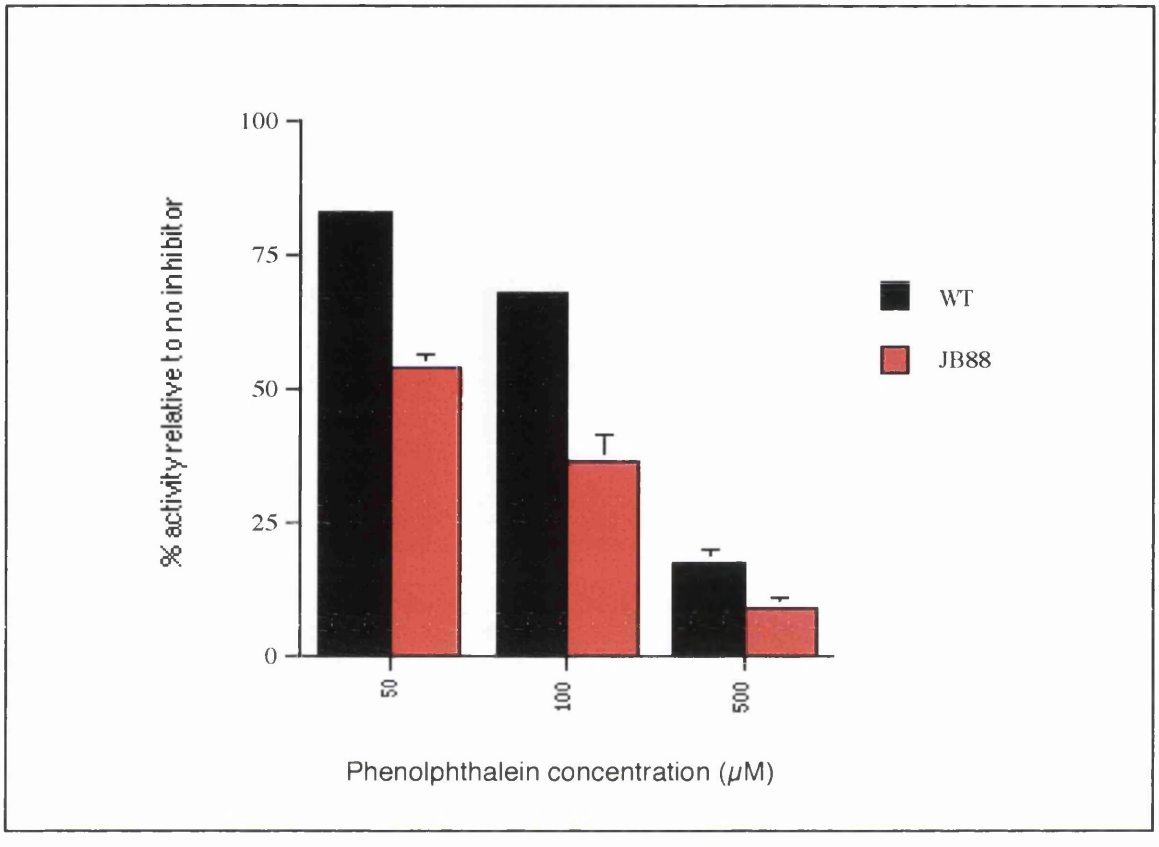


Figure 7.17 Phenolphthalein inhibition of JB88
A purified extract of the C-terminal deletion mutant JB88 was examined for inhibition by phenolphthalein at 50, 100 and 500μM concentrations. The graph shows the results from an experiment of the activity present in the mutant enzyme relative to no inhibitor.

Therefore, JB88 is more sensitive than the wild type enzyme to phenolphthalein irrespective of whether it is present as a crude or purified extract. These findings support the results obtained from the spectrophotometric assays on this mutant where a low dUMP and high folate K_m values were produced which indicated the more open conformation of the enzyme. The greater sensitivity to phenolphthalein also demonstrates that the mutant enzyme is in a more open configuration and so is more accessible to phenolphthalein.

Folate analogue inhibition

Folate analogues that inhibit TS were developed modelled on the structure of 5,10 methylenetetrahydrofolate. The folate analogues ICI 1-8 were developed based on the lead compound CB3717. This compound allows the formation of a covalent bond between TS and dUMP. However, CB3717 cannot form a covalent bond with dUMP and so methylene transfer cannot occur. The folate analogues ICI 1, ICI 4 and ICI 5 were examined in the tritium release assay to determine whether they could inhibit purified JB88 (Table 7.11)

Inhibitor concentration	**ICI 1**		**ICI 4**		**ICI 5**	
	WT	**JB88**	**WT**	**JB88**	**WT**	**JB88**
0.5ng/µl	73.2%	62.5%	96.2%	100%	86.5%	67.2%
5ng/µl	29.5%	51.1%	86.7%	100%	80.1%	79.9%
50ng/µl	8.2%	17.6%	36.0%	100%	47.1%	89.6%
500ng/µl	3.0%	5.5%	-	-	-	-
5µg/µl	0.5%	2.5%	-	-	-	-

Table 7.11 Folate analogue inhibition

Purified extracts of the C-terminal mutant and wild type enzyme were prepared. The tritium release assay was performed with varying concentrations of the folate analogue compounds ICI 1, 4 and 5 which had been added prior to the addition of the enzyme. The figures are a mean of two values. They are expressed as a percentage of activity in the absence of inhibitor.

JB88 was more resistant to ICI 1 than the wild type enzyme at concentrations of 5ng/µl and above. ICI 4 had no effect on the activity of the mutant enzyme but did affect the wild type. The folate analogue ICI 5 inhibited both the mutant enzyme and wild type at 0.5ng/µl but at higher concentrations it appeared to stimulate the activity of the mutant whereas the wild type was still inhibited. This result requires further investigation.

7.2.5. Discussion

Examination of the 35 TS sequences shows a lack of alignment at the N-terminus and indeed the bifunctional enzymes have an N-terminal DHFR domain. By contrast the C-terminal regions precisely align in all species, indicating the importance of this region. In all cases the five amino acids at the most extreme C-terminal region are small and hydrophobic. 27 of the 37 sequences have a valine as the C-terminal residue, 7 of the remaining 8 sequences have either isoleucine or alanine and VZV TS is unique in that it has a leucine.

Previous studies on *L.casei* and *E.coli* TS have revealed the importance of the C-terminus for enzyme function. Removal of the solvent exposed and highly mobile carboxy-terminal residue results in the complete inactivation of the enzyme in the tritium release assay. In contrast, deletion of Leu301 from VZV TS does not completely inactivate the enzyme, implying structural differences between the viral and bacterial enzymes (Harrison *et al.*, 1995). This thesis has shown that major reconstructions can be produced in the C-terminal region of VZV TS without a complete loss in enzyme activity.

The mutants JB73 and JB88 contain deletions of 7 amino acids and 14 amino acids respectively. Figure 7.13 demonstrates that removal of these amino acids would significantly decrease the length of the C-terminal tail which forms the lid over the active site upon ligand binding. This may result in the enzyme adopting a more open conformation. The deletion mutants have been shown to be functional but they are compromised. The mutant enzymes can complement bacterial cells deficient in TS on minimal media at 30°C and crude extracts have produced ~8-10% wild type activity in the tritium release assay. JB88 which had the 14 amino acid deletion was the only C-terminal mutant to be purified, this was shown to have 0.10% of wild type activity. Kinetic analysis of this mutant revealed a 3 fold reduction in dUMP K_m and 25 fold increase in folate K_m indicating that the mutant enzyme was in a more open conformation and could not form the closed complex as efficiently as that of the wild type enzyme. These findings were supported further by the phenolphthalein results which demonstrated that the mutant enzymes were more sensitive to phenolphthalein than the wild type enzyme and so must be more accessible for the inhibitor which was designed to fit into the active site cavity.

The results from the deletion mutants suggest that the previous model for the C-terminal region may not hold as it was thought that the activity in the Leu301 deletion mutant was due to hydrophobic interactions between Met200, Ile99 and Tyr100. The deletion mutants however do not have Met299 present to form these hydrophobic interactions. The experimental evidence obtained in this thesis suggests that for the deletion mutant JB88 the enzyme does not close down upon ligand binding. In order to determine whether this is the case the crystal structure of the ternary complex of JB88 would have to be solved.

JB79 has a point mutation present in Leu286 which is positioned between a β-strand and the C-terminal tail (refer to Figure 7.13). A leucine to histidine change at this residue produces an enzyme with drastically reduced TS activity (~6% of wild type). This reduction in activity can be explained by the change from an uncharged hydrophobic side chain to a positively charged one which presumably prevents active site closure.

Mutant JB102 has a C-terminal extension resulting from a frame shift. This mutant cannot be modelled in Figure 7.13 as it is impossible to speculate what orientation this extension will take on the surface of the protein. Although this extension is present in an exposed region of the protein and should be readily accommodated it was found to produce a dramatic decrease in enzyme activity. This reduction in enzyme activity can be explained in several ways: if the C-terminal extension docks on the surface of the protein it can pull the C-terminal tail from its normal orientation and this could drastically affect enzyme structure and function. In contrast, the C-terminal extension may not find a position on which to dock and it will move around on the surface, this again will result in the normal orientation of the C-terminal tail being affected. In addition the DRTG loop may also play a role as it packs against the C-terminus upon ligand binding and a hydrogen bond is formed between Arg38 and the C-terminal carboxylate. This bond may limit the orientation the additional residues can adopt.

Phenolphthalein data from JB102 suggests that this enzyme shows more resistance to the inhibitor than the other three C-terminal mutants and the wild type extract. This could suggest that the tail is covering the active site and so phenolphthalein cannot enter as readily. The results on this mutant are from crude protein extracts and the result have to be corroborated with purifed enzyme.

The C-terminal residue of human TS is a valine. In addition this enzyme has an asparagine at position 112 which forms a hydrogen bond with the carboxylate of valine. Asn112 was proposed to play a role similar to Trp85 in *E.coli* as an asparagine can be modelled in place of tryptophan in *E.coli* TS such that its N-H group could form a H-bond to the carboxylate group of the C-terminal residue. This suggests that the asparagine residue in eukaryotes may play an analogous role to the conserved tryptophan in TS from prokaryotes and these TSs could also be inactive if their C-terminal residue is removed (Harrison *et al.*, 1995). It was predicted that removal of this C-terminal valine residue would completely inactivate the enzyme but subsequent work in our laboratory has shown that the removal of this residue produces an enzyme (~50% pure) which can perform the partial debromination reaction at wild type levels but has 0.042% wild type activity in the tritium release assay (J. Scott, R. Thompson, personal communication). This level of activity is 100 fold lower than that found in VZV L301Δ and is not sufficient to complement Thy^- bacteria. Thus there remains a significant difference between the human and viral enzymes.

Chapter 8. Pyrimidine discrimination by VZV TS

Analysis of the completely conserved residue asparagine 214

As an approach to understanding the structure function relationship of TS, the properties of "replacement sets" of key amino acid residues of the enzyme by all or most possible substitutions were investigated (Michaels *et al.*, 1990, Kim *et al.*, 1992). One such key residue was Asn229 (*L.casei* numbering), this residue is conserved in all sequenced TSs (Asn214 in VZV) and provides the only side chain that directly hydrogen bonds with the pyrimidine ring of the substrate dUMP. The carboxamide moiety forms a cyclic hydrogen bond network with the NH-3 and O-4 of the base and is a prime candidate for assisting proton transfer reactions that occur at O-4 of the pyrimidine ring of dUMP (Figure 8.1)

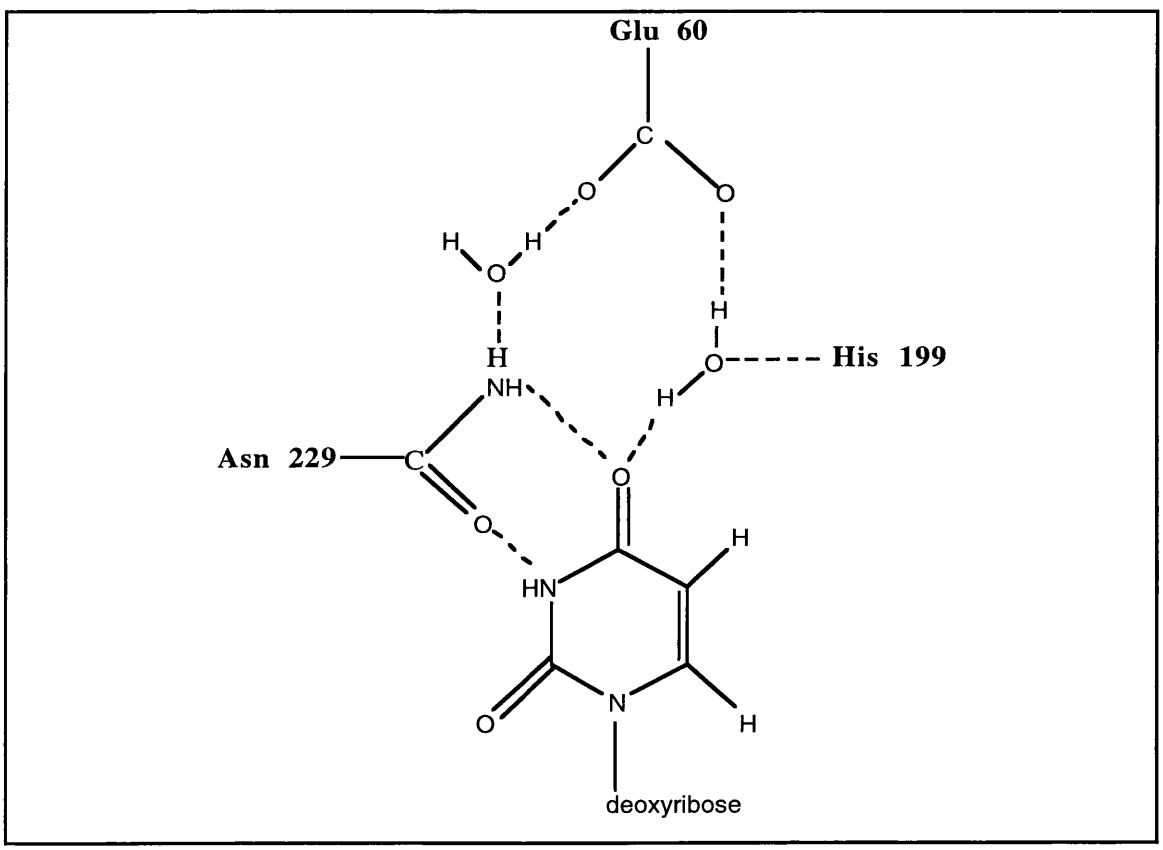


Figure 8.1. Schematic diagram of the hydrogen bond interactions among the pyrimidine ring of the substrate dUMP and the amino acid residues at the active site (Montfort *et al.*, 1990; Montfort *et al.*, 1993). The numbering is from *L.casei* TS.

It has recently been proposed that the substrate specificity of TS for dUMP versus dCMP resides in the single amino acid residue Asn229 (Asn214 in VZV TS). The

complete replacement set of *L.casei* TS mutants were catalytically active indicating that that Asn229 assists but is not essential for dUMP binding and catalysis. In addition the results demonstrated that the N229D mutant altered the pyrimidine specificity of the enzyme and resulted in it becoming a dCMP methylase (Liu & Santi, 1993a). The wild type enzyme can effectively discriminate between the related deoxyribonucleotides dUMP and dCMP as substrates. dUMP is a relatively tight binding substrate with a K_m of ~5μM, in contrast dCMP is not a substrate but is a poor competitive inhibitor with a K_i of 3000μM (Liu & Santi, 1993b). With the exception of TS N229Q, most of the mutants bind dCMP as well as or tighter than dUMP. In particular the N229D mutant has a dUMP K_m value of 50μM and a dCMP K_i value of 150μM. The mutants were assayed to identify those which could catalyse dCMP methylation and it was found that in addition to N229D, N229E also catalyses the reaction (Liu & Santi, 1993b). The aim of the experiments described in this section was to produce a corresponding mutation in asparagine 214 of VZV TS. The mutant enzyme was then examined for sensitivity to dCMP which would indicate a change in pyrimidine recognition.

8.1. Construction of N214D

Previously an asparagine to serine mutation (AZ42) had been produced in residue 214 by region directed mutagenesis (Chapter 6), however in addition this mutant also contained D206Y and M207L mutations. Therefore a single asparagine to aspartic acid change was produced in residue 214. Construction of the N214D mutation in VZV TS required an oligonucleotide to be synthesised which contained the corresponding asparagine to aspartic acid mutation at residue 214 (Figure 8.2). Site directed mutagenesis was performed using the Künkel strand selection procedure and the mutation was initially identified by sequencing using primer 62. Sequencing the whole gene with primers 57-62 revealed that there were no nucleotide changes apart from that introduced by mutagenesis. The N214D mutant was initially characterised by genetic complementation using a Thy^- strain of *E.coli* and was shown to be unable to complement NM522 Thy^-.

Amino acid	Val	Pro	Phe	Asn	Ile	Ala	Gly	Tyr
	211	212	213	**214**	215	216	217	218
Wild type sequence	GTA	CCG	TTC	**A**AC	ATT	GCT	GGA	TAT
Mutated sequence	GTA	CCG	TTC	**G**AC	ATT	GCT	GGA	TAT
	Val	Pro	Phe	Asp	Ile	Ala	Gly	Tyr

Figure 8.2. A point mutation constructed in asparagine 214 of VZV TS by site directed mutagenesis to produce N214D. In red is the mutated residue.

8.2. Expression of N214D

Detailed studies of the N214D mutant required large amounts of the enzyme which were obtained by cloning the mutant into the pET-23d high level expression vector. The mutant gene was digested from the original bluescript backbone with *Eco*R1 and *Nco*1, the digest was run on an agarose gel and the TS fragment was extracted. The pET-23d plasmid was digested with the same enzymes and a ligation reaction was set up to ligate the TS construct into the expression vector. The target gene was initially cloned into NM522 Thy$^-$ cells that do not contain T7 RNA polymerase gene, once established in this non-expression host the plasmid was transferred into a BL21 pLysS expression host and protein expression was induced by the addition of IPTG. Purification was achieved by hydroxylapatite and S-sepharose chromatography (Figure 8.3).

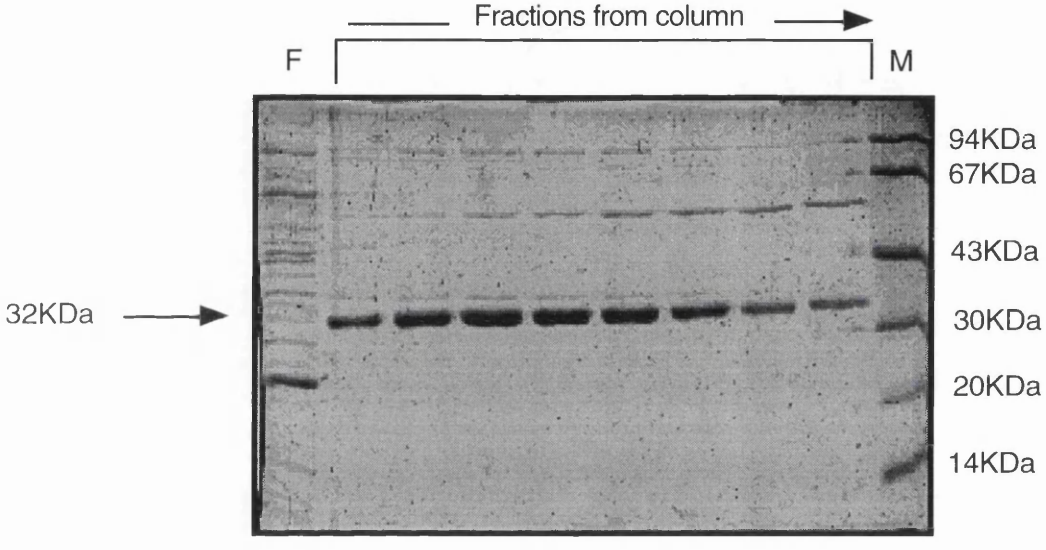


Figure 8.3. Purification of TS from S-sepharose column

Coomassie stained gel of fractions collected from purification of N214D by S-sepharose chromatography. The partially purified protein extract obtained from the hydroxylapatite column was bound to the S-sepharose column, the column was washed with phosphate buffer (F) and the TS protein was eluted from the column with an increasing salt gradient. The position at which purified TS runs is denoted by an arrow. Protein size markers(M) are present on the right.

A tritium release assay was carried on the purified extract to quantify the TS activity present. The specific activity of the mutant protein was found to be 0.06% of that of the wild type enzyme, this very low specific activity confirmed the Thy$^-$ phenotype of the mutant which had been shown by an inability to complement Thy$^-$ bacteria (Table 8.1). Previous studies (Liu & Santi, 1993) have shown that an N229D mutation in *L.casei* produces a Thy$^-$ phenotype with similar TS activity to the VZV N214D mutant.

Enzyme	Tritium release pmol/min/ug protein	% Wild type
Wild type	8.34	100
N214D	0.0047	0.06

Table 8.1. Enzyme activity of N214D mutant
TS activity of purified extracts of the N214D mutant and wild type TS enzymes measured by the tritium release assay. All enzymes were expressed in *E.coli* BL21 pLysS.

8.3. dCMP inhibition of N214D

Ordinarily dCMP is a poor competitive inhibitor of TS and binds to the wild type enzyme with a 1000 fold lower affinity than the substrate dUMP. Replacement of Asn229 in *L.casei* TS with aspartic acid resulted in the enzyme losing TS activity toward dUMP and becoming a dCMP methylase (Liu & Santi, 1993).

The purified N214D enzyme of VZV was assayed for dCMP inhibition in a tritium release assay in which dCMP was present at concentrations of 500μM-10mM. The results are presented graphically (Figure 8.4) and reveal that high concentrations of dCMP have no effect on the wild type enzyme. However the mutant enzyme shows a large decrease in enzyme activity at concentrations of 1mM dCMP and above indicating that the dCMP is inhibiting the enzyme by competing with the substrate dUMP.

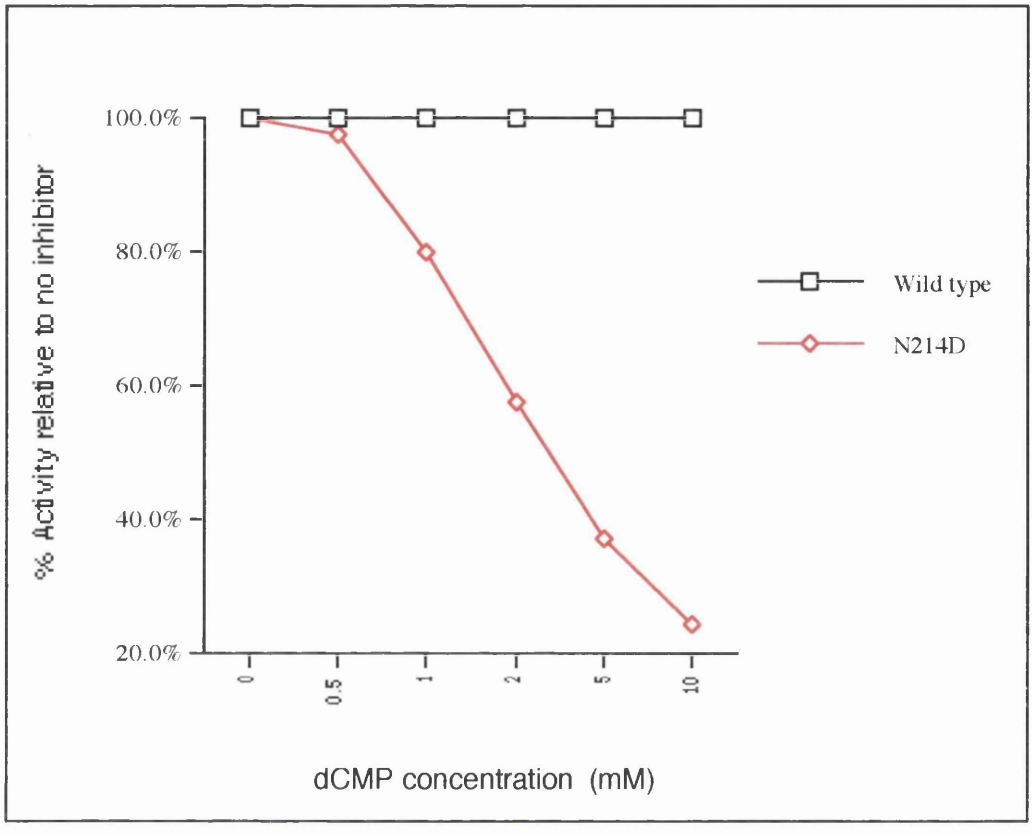


Figure 8.4. dCMP inhibition of N214D

TS activity of N214D and the wild type enzyme examined in the presence of increasing concentrations of dCMP (500μM-10mM) with a fixed dUMP concentration of 23.4pmoles.

8.4. Kinetic studies

It did not prove possible to measure the kinetic parameters of N214D. Alteration of the substrate concentration in the spectrophotometric assays did not appear to affect the velocity of the reaction. It may be that the low sensitivity of the spectrophotometric assay and the low activity of the N214D extracts combined to frustrate accurate measurements. Studies in *L.casei* TS have shown that an asparagine to aspartic acid mutation at residue 229 resulted in a ten fold increase in dUMP K_m value implying that there is a decrease in affinity for dUMP. With the exception of N229Q, the complete replacement set of mutants of *L.casei* bind dCMP as well as or tighter than dUMP and bind dCMP 300-3,000 fold tighter than wild type TS (Liu & Santi, 1993).

8.5. Discussion

Previous studies on Asn229 of *L.casei* has shown that mutations in this residue result in the enzyme having an increased affinity for dCMP, in particular the N229D mutation resulted in the enzyme converting to a dCMP methylase. The corresponding

mutation produced in VZV TS could not complement *E.coli* NM522 Thy$^-$ cells *in vivo*, similar results had been noted in *L.casei*. The N214D mutant enzyme did produce a low but measurable amount of activity in the tritium release assay which is comparable with the activity present in the N229D mutant of *L.casei* TS(Liu & Santi, 1993a). When dCMP inhibition was examined in this assay, the wild type enzyme did not appear to be inhibited by the presence of dCMP whereas the mutant enzyme showed inhibition at concentrations of 1mM and above. These results suggest that the substrate specificity of the mutant enzyme has been altered and the dCMP is now competing with the dUMP. K_m values were not obtained for the VZV mutant enzyme but similar studies of the *L.casei* enzyme revealed that in the N229D mutant the K_m for dUMP was increased by 10 fold from that of the wild type enzyme. Further characterisation of the N214D mutant from VZV would require the analysis of both dUMP and dCMP as substrates in the tritium release assay. The *E.coli* mutant N177D gave an initial velocity with dCMP which was 35 fold faster than that obtained with dUMP indicating that the mutant enzyme is more active on dCMP than dUMP (Hardy & Nalivaika, 1992).

Therefore the wild type TS enzymes discriminate binding of dUMP versus dCMP by the exclusion of dCMP from the active site. This exclusion is thought to be the result of untoward interactions between the carboxamide group of Asn or Gln at position 214 and dCMP. If dUMP was replaced by dCMP in the active site of either of the two enzymes, this would place the carboxamide oxygen of the side chain adjacent to the heterocycle nitrogen of dCMP and the amide nitrogen adjacent. This close proximity of the relatively negative groups of the enzyme and dCMP would lead to a repulsion of the ligand from the enzyme and account for poor binding. Rotation of the Asn or Gln carboxamide group at 214 to better accommodate the dCMP binding would require disruption of the H-bond network with other amino acids.

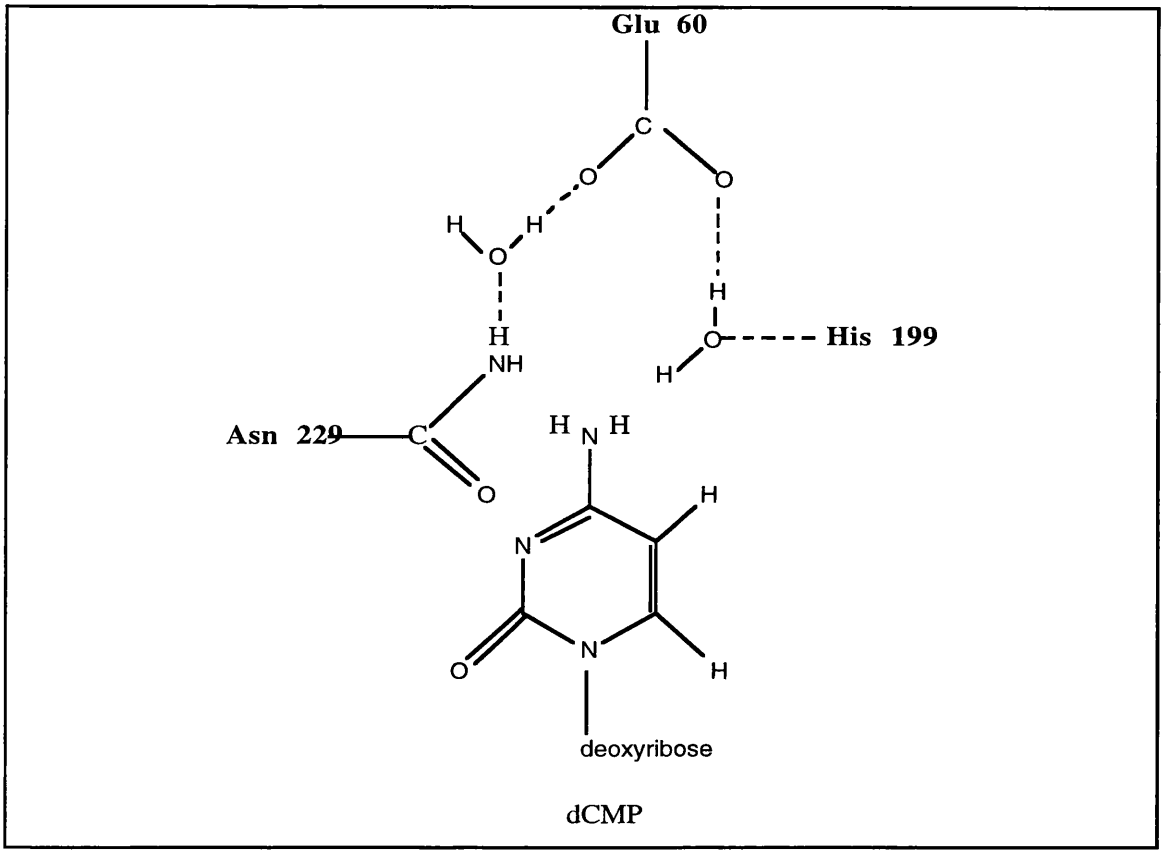


Figure 8.5. Schematic diagram showing that the hydrogen bonding once formed between dUMP and N229 is disrupted between dCMP and N229 (Liu & Santi, 1993b). The numbering is from *L.casei* TS.

These findings have important ramifications as there are a large number of enzymes that bind uracil nucleotides in preference to cytosine nucleotiodes and these enzymes may employ a similar mechanism for discrimination. One such enzyme which recognises uracil is deoxyuridine 5'-triphosphate nucleotidehydrolase (dUTPase), this enzyme catalyses the hydrolysis of dUTP to dUMP and pyrophosphate. The enzyme serves two biological functions: it contributes to the pool of dUMP an essential precursor in the biosynthesis of thymidylate nucleotides via the TS reaction and it maintains the intracellular concentration of dUTP at a low level thereby reducing the incorporation of uracil into DNA. *E.coli* dUTPase is highly specific for its substrate and discriminates not only different bases and sugar moieties but also the phosphate group. NH-3 of the pyrimidine ring makes a H-bond with the main chain of the enzyme, while the carbonyl group at C-4 makes a weak H-bond with Asn84 (Larsson *et al.*, 1996). It is thought that this asparagine group may contribute to discrimination of cytosine in a similar manner to TS. This asparagine residue however, is not an evolutionary conserved residue and is only found in *E.coli* dUTPase. Crystallographic studies of human dUTPase (Mol *et al.*,

1996) have demonstrated that a hairpin loop is responsible for specific recognition: uracil is inserted into the distorted antiparallel hairpin and dUTPase main-chain hydrogen bonds recognise O2, N3 and O4 atoms. These interactions mimic DNA base pairing, selecting uracil over cytosine and sterically precluding thymine and ribose binding.

A second class of enzyme which discriminate against cytosine are uracil DNA glycosylases (UDGases), which are a ubiquitous, highly conserved and extremely specific class of DNA repair enzymes. Their biological function is the specific removal from DNA of the RNA base uracil. In the human UDG-6 amino uracil complex, uracil binds at the base of the groove within a rigid preformed pocket that confers selectivity for uracil over other bases by the shape complementarity and by main chain and Asn204 side chain hydrogen bonds (Mol *et al.*, 1995). Site-directed mutagenesis of this residue has identified it as the most critical residue for the recognition of uracil (Mol *at al.*, 1995). This asparagine residue was found to be highly conserved throughout all UDGases and has been identified in HSV-1 (McGeoch *et al.*, 1988) and VZV (Davison & Scott, 1986) and must serve a similar function to that of the human Asn204 residue.

The phenomenon of uracil acceptance and cytosine exclusion by Asn or Gln can also be examined in reverse. In dCMP hydroxymethylase of bacteriophage T4, whose natural substrate is dCMP, an aspartate residue is naturally present in the position of this asparagine. An analogous Asp179 to Asn179 mutation in this enzyme changes the substrate specificity to a dUMP hydroxymethylase (Graves *et al.*, 1992). The alteration in substrate preference is specific to the D179N mutation, since D179A lacks nucleotide specificity, and D179S is completely inactive. The enzyme *HhaI* methyltransferase also discriminates against uracil. It is involved in a variety of biological processes and catalyses the transfer of a methyl group from S-adenosyl-L-methionine to the C-5 position of cytosine. In this enzyme there is a direct interaction between a glutamate residue Glu119 and the N3 and N4 atoms of the cytosine ring again indicating the specificity of either glutamate or aspartic acid for cytosine rather than uracil.

Therefore, it seems likely that the highly conserved nature of Asn229 was not simply to stabilise dUMP in the active site but to prevent the binding of dCMP. These interactions of asparagine or glutamine with uracil, and aspartic acid or glutamate with cytosine may represent a general theme in protein nucleic acid recognition

Chapter 9. Final discussion

9.1. Protein-protein interactions in VZV TS

Affinity chromatography experiments were performed in order to identify proteins interacting with VZV TS. In these studies no other proteins were found to interact with VZV TS. This however, was not a fully exhaustive search and it could be that if the experimental approach used had been improved in several areas then interacting proteins may have been identified. It is likely that such interactions do occur. Co-immunoprecipitation studies have shown that replication complexes exist in the nucleus of HSV-1, all seven of the virally-encoded replication proteins, along with the components of the cellular replication machinery, localise to the nucleus in replication compartments (Olivio *et al.*, 1989; Goodrich *et al.*, 1990; Lukonis & Weller, 1996). To pursue this idea we would require an antibody for VZV TS.

9.2. Mutagenesis studies

The original aim of this study was to examine the structural/functional relationships in VZV TS by producing a series of temperature sensitive mutants targeted to various regions of the protein. A conservation paradox exists for members of the TS family as there are many fully conserved residues which can be substituted without loss of catalytic activity. Previous work has centred on replacing these conserved residues one by one and observing the affect they have on catalysis, these studies however tended to be non-informative. A region directed mutagenesis approach was therefore used to generate temperature sensitive mutants in both buried and solvent exposed region of the enzyme. Unlike other approaches which had specifically targeted conserved residues this approach randomly identified residues that were important for protein stability in targeted regions of the protein. Many of the temperature sensitive mutants had previously been generated in *E.coli* and *L.casei* and had been shown to be inactive when examined for activity at 37°C. We have shown that these mutations can be accommodated at 30°C when there is more order to the enzyme structure. Detailed examination of these mutants on the VZV model have provided some insight into what may be happening at the structural level. The region directed mutagenesis approach can be applied to any protein for which an expression system and *in vivo* assay are available.

9.3. Unique interactions at the C-terminus of VZV TS

Much work has centred on the C-terminus, because it has been implicated heavily in catalysis and closing down the active site. Our studies have shown that VZV TS can retain a small amount of activity even without the C-terminal tail, which moves to form a lid over the active site upon ligand binding. Although the mutants can complement Thy$^-$ bacteria they cannot form a tightly closed ternary complex upon ligand binding as reflected by their high folate K_m values. The mutants have a lower dUMP K_m values which indicate the enzyme is present in a more open conformation allowing the substrate to enter more readily. These findings have been confirmed by the increased sensitivity to phenolphthalein again indicating that the inhibitor can access the active site more readily.

It had previously been proposed that the C-terminus of VZV has hydrophobic interactions rather than the hydrogen bond network which is present in the bacterial enzymes. These hydrophobic interactions were thought to involve Met299, but this residue has been removed in two of the C-terminal deletion mutants. Clearly our understanding of the full range of interactions is incomplete.

9.4. Protein-nucleic acid interactions

The interactions between asparagine or glutamine and aspartic acid or between glutamic acid and cytosine could be a general theme in protein-nucleic acid recognition. We have shown that Asn214 discriminated between dUMP and dCMP as a substrate. When this residue is mutated to an aspartic acid then the enzyme becomes sensitive to dCMP inhibition. Further work on this residue would involve examining dCMP as a substrate for TS in a modified assay. In *E.coli* the N177D mutant has activity on both dUMP and dCMP, with dCMP being a considerably more reactive substrate. In the tritium release assay, the N177D variant has an initial velocity with dCMP which is 35 fold faster then that obtained with dUMP.

A clearer understanding of the results in this thesis can only be obtained when the crystal structure of VZV TS has been solved. This work is now underway in collaboration with Adrian Lapthorne. At present all predictions have been made on the VZV TS model which has been produced from the *E.coli* crystal structure.

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