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Structure and Stability Studies of Shikimate Kinase

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A thesis submitted for the degree of

Master of Science

Biochemistry

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of
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This thesis is dedicated to my parents,
John and Joan

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For the countless others that I have met throughout my time in Glasgow, apologies to anyone I should have mentioned, but haven't.

ABBREVIATIONS

ANS	8-anilino-1-napthalene
CD	Circular dichroism
DEAE	Diethylaminioethyl
DSC	Differential scanning microcalorimetry
EDTA	Ethylene diamine tetra acetate
GdnHCl	Guanidinium chloride
ITC	Isothermal calorimetry
kDa	Kilodalton
M_r	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate
TEMED	N,N,N,N'-Tetramethylethylenediamine
UV	Ultraviolet

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ABSTRACT

This thesis describes a structural and functional study of shikimate kinase, the enzyme which catalyses the fifth step in the biosynthetic shikimate pathway in which shikimate is converted to shikimate-3-phosphate.

Mutagenesis studies were undertaken to discover more about the roles of the assumed catalytic residues in particular the single lysine (K15) within the active site and the neighbouring cysteine (C13). The cysteine remote from the active site (C162) was also mutated to prevent intermolecular disulphide formation which had previously been observed during crystallographic studies.

Biophysical techniques including circular dichroism (CD), fluorescence and microcalorimetry were used to monitor the conformational changes of shikimate kinase accompanying ligand binding and to measure ligand binding.

The single tryptophan (W54) was found to be a very useful fluorophore for monitoring conformational changes. Calculated K_d values from this compared well with the K_m values measured by steady state kinetics. The dissociation constant (K_d) for shikimate was 300 μM and for ATP 700 μM.

When monitored by fluorescence the binding of the ATP to the enzyme showed a degree of co-operativity. The data did not fit a standard Michaelis Menten binding curve but gave a sigmoidal plot with a Hill co-efficient of approximately 2. The binding of ADP gave a Hill co-efficient of approximately 1.5.

Fluorescence quenching studies using the quenching agents of succinimide and sodium iodide allowed the calculation of Stern-Volmer constants. The results provided information about the environment of the Trp-54.

ATP binding to the enzyme was also measured by Isothermal titration calorimetry (ITC) This gave K_d values of only 25-40μM whereas apparent steady state kinetics gave a K_m

value of 700μM. This discrepancy has not been explained but it may reflect the existence of a second ATP binding site.

Thermal stability was measured using differential scanning calorimetry (DSC) which was analysed to give the apparent melting temperature (T_m). Ligand binding (ATP) was found to stabilize the structure of shikimate kinase illustrated by an increase in the T_m from 39 to 47°C. However the shikimic acid provides a lower degree of stabilisation.

The mutant K15M had a higher T_m than the wild type enzyme; this may be due to the formation of an extra ion pair in the structure, formed between the side chains of Asp 32 and Arg 11. Thermal stability was also measured by using CD to monitor the loss of secondary structure at 222nm.

Denaturation and refolding studies were carried out using guanidine hydrochloride (GdnHCl) as the denaturant.

The concentration of GdnHCl needed to bring about 50% of the total change of the native structure in both secondary, measuring the change in CD, and tertiary structure measuring the changes in fluorescence was 1.25M. This was observed as a single transition. Total loss of structure was observed at concentrations of 4M and above.

Refolding of the enzyme was monitored by both fluorescence and CD using both manual mixing and stopped flow techniques. The data indicated that refolding took place in a number of distinctive stages. One of the intermediates appeared to have a high affinity for the fluorescent probe 1-anilino-8-napthalenesulphonate (ANS). An outline model for the refolding of the enzyme could be proposed.

1 Introduction

1.1 The Shikimate Pathway

1.1.1 Background to the Shikimate Pathway

The biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan proceeds via the shikimate pathway. This pathway provides the aromatic amino acids along with other important aromatic compounds required in plants and micro-organisms (Figure 1.1a, 1.1b).

The condensation of erythrose-4-phosphate (a product of the Calvin cycle and/or the pentose phosphate pathway) and phosphoenolpyruvate (derived from glycolysis) is the first step in the pathway and is catalysed by the enzyme 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase. A further six steps lead to chorismate which is the common precursor of all the essential aromatic compounds such as folic acid and vitamin K as well as the major plant cell wall component lignin (Pittard, 1987; Bentley, 1990; Coggins, 1989; Haslam, 1993; Fig 1.1b). It has been estimated that one fifth of the carbon fixed by plants is channelled through the shikimate pathway (Kishore and Shah, 1988).

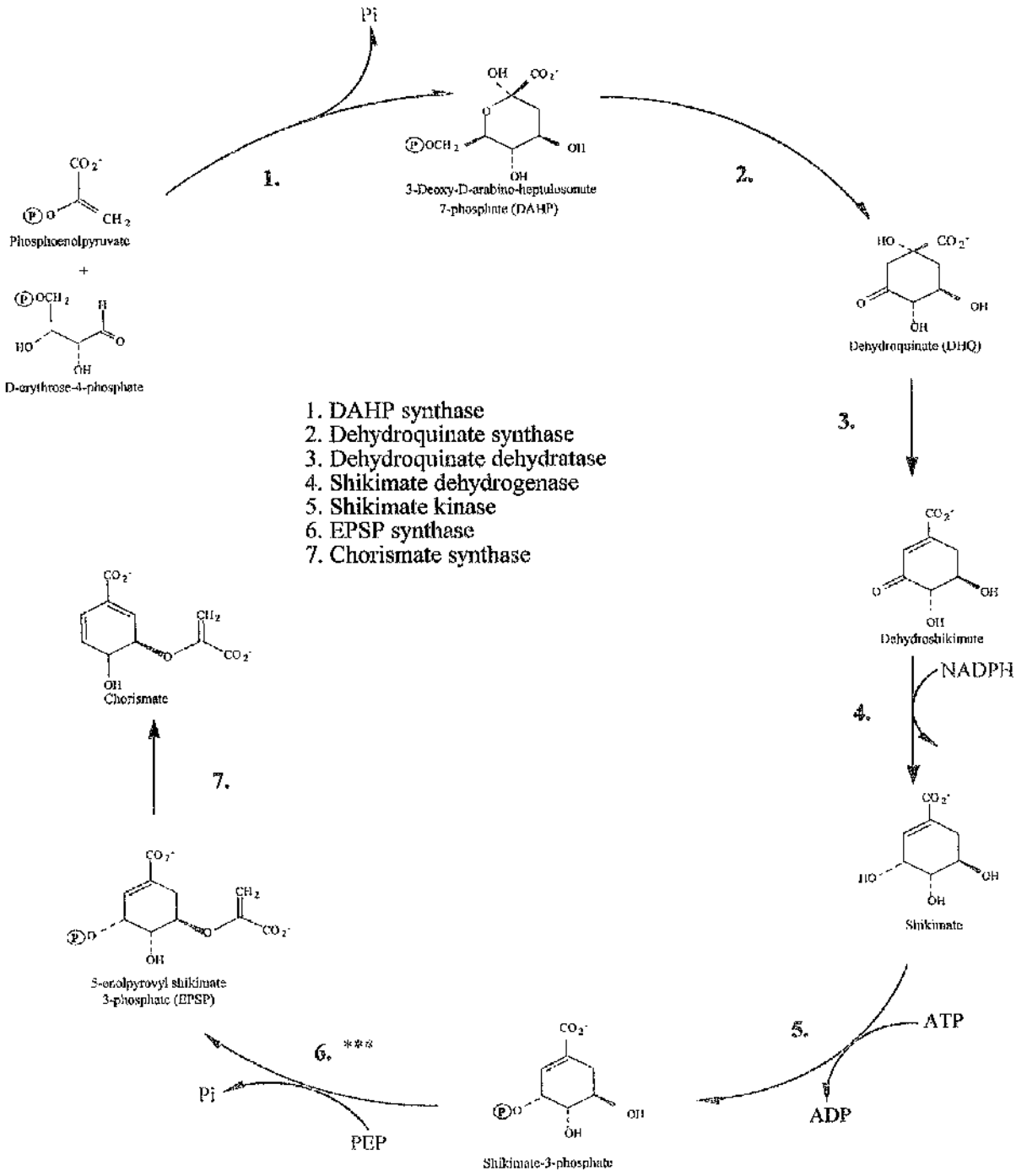
The pathway is named after one of the intermediates, shikimic acid, which was isolated by Eykmann from the fruits of *Illicium religiosum* over a century ago (Eykmann, 1891). The structure of shikimic acid was solved much later by Fischer and Danschat (Fischer, 1932).

The elucidation of the intermediates of the pathway was carried out during work in the 1950's principally by Davis who studied mutants that were auxotrophic for the three aromatic amino acids. These auxotrophs accumulated intermediates preceding the blocked steps in the pathway. This led to the identification of the central pathway intermediates, 3-dehydroquinate, 3-dehydroshikimate and shikimate-3-phosphate (Davis, 1948, 1949,1955). The earlier and later reactions were elucidated with more difficulty. A mutant strain of *Escherichia coli* that accumulated shikimic acid was grown on D-glucose variously labelled

with ^{14}C to determine the distribution of specific carbon atoms from glucose in the shikimate molecule (Gibson and Gibson, 1964). It was determined that of the seven precursor carbon atoms entering the pathway three originated from the glycolytic pathway and four originated from the pentose phosphate pathway. Cell extracts and partially purified enzyme preparations were shown to convert phosphoenolpyruvate and erythrose-4-phosphate to DAHP and then 3-dehydroquinate (DHQate). The position of 5-*enol*pyruvyl shikimate-3-phosphate synthase (EPSPS) in the pathway was determined from the conversion of shikimate 3-phosphate and phosphoenolpyruvate to 5-*enol*pyruvyl shikimate-3-phosphate (EPSP) by cell extracts. Chorismate itself was first characterised by Gibson and Gibson (1964).

1.1.2 The enzymology of the shikimate pathway

The first enzyme of the shikimate pathway is DAHP synthase which catalyses the condensation of the carbohydrate precursors of phosphoenolpyruvate and erythrose-4-phosphate. In a second step catalysed by 3-dehydroquinate synthase, DAHP is cyclised to give 3-dehydroquinate. The third step is carried out by the enzyme 3-dehydroquinate dehydratase (known more commonly as dehydroquinase) and involves the elimination of water from the cyclised DAHP; this introduces the first double bond to produce dehydroshikimate. Subsequent reduction of the keto group by the action of shikimate dehydrogenase produces shikimate in step four. Shikimate is then converted to shikimate 3-phosphate by the phospho-transfer activity of shikimate kinase in step five. The sixth step involves the condensation of phosphoenolpyruvate and shikimate-3-phosphate catalysed by the action of EPSP synthase yielding EPSP, the substrate for the final enzyme of the pathway, chorismate synthase.



*** site of action of Glyphosate

Figure 1.1a : The Shikimate Pathway.

Chorismate synthase, which catalyses the seventh step, introduces the second double bond and produces chorismate which is the branch point intermediate leading through parallel pathways to the aromatic amino acids and the other aromatic compounds (Figure 1.1b).

The pathway intermediates and enzyme reactions that occur within the shikimate pathway are believed, with the single exception of dehydroquinase (Kleanthous *et al.*, 1992; Harris *et al.*, 1993), to be identical in all organisms. Although the catalytic domains are highly conserved at the protein level the genes and the operons responsible for the production of the enzymes are in fact organised differently in different species, probably reflecting contrasting evolutionary histories and the functional and regulatory constraints experienced by each species (Henner and Yanofsky 1993).

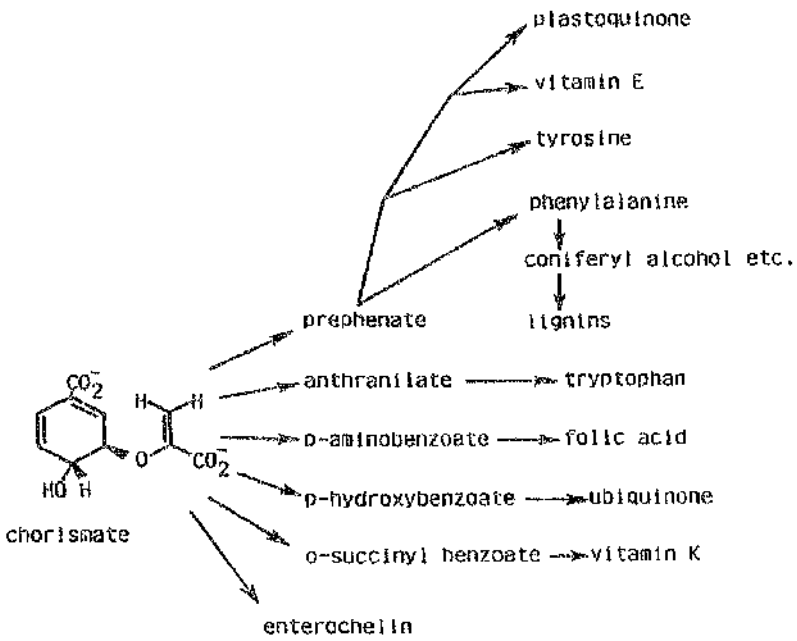


Figure 1.1b : Post-chorismate intermediates and metabolites

1.1.3 The individual enzymes of the shikimate pathway

1.1.3.1 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase

DAHP Synthase (EC 4.1.2.15)

The first committed step in the biosynthesis of aromatic compounds is catalysed by DAHP synthase is the condensation of phosphoenolpyruvate and erythrose-4-phosphate. The flow of metabolites, as within any biosynthetic pathway, is regulated by feedback inhibition brought about by intermediates or end products. In fact DAHP synthase shows a rather complex method of feedback regulation which is different between *E. coli* and *Bacillus subtilis*. In *E. coli* and *Salmonella typhimurium* there are three independent DAHP synthase isoenzymes, phenylalanine sensitive DAHP synthase (phe), tryptophan sensitive DAHP synthase (trp), and tyrosine sensitive DAHP synthase (tyr). Each aromatic amino acid therefore has the ability to affect the flux through the pathway and mutants with varying levels of this resistance to feedback inhibition have been isolated (Pittard, 1966).

However in *B. subtilis* only one DAHP synthase exists which is not inhibited by the aromatic amino acids but rather by prephenate which is an intermediate of phenylalanine and tyrosine synthesis (Jensen and Nasser 1968; Jensen and Nasser, 1968; Jensen, 1976). An excess of phenylalanine and tyrosine results in the feedback inhibition of prephenate dehydratase blocking the further conversion of prephenate. This build-up of prephenate inhibits the DAHP synthase and reduces the flow of metabolites into the shikimate pathway (Jensen and Ahmad, 1988).

This type of sequential feedback strategy could potentially starve the cells of tryptophan, the synthesis of which branches from chorismate. However, chorismate mutase, which catalyses the conversion of chorismate to prephenate is inhibited by the product prephenate and this is suggested to be sufficient to channel chorismate into the tryptophan pathway (Henner and Yanosky, 1993).

1.1.3.2 3-Dehydroquinate synthase (EC 4.6.1.3)

The oxidation, β elimination and intermolecular condensation to convert DAHP to 3-dehydroquinate is carried out by 3-dehydroquinate synthase for which the presence of NAD^+ as cofactor is required.

In *E. coli* this enzyme is a monofunctional polypeptide of molecular mass 40kDa which requires NAD^+ and Co^{2+} or Zn^{2+} for activity (Frost, 1984). The enzyme is expressed constitutively as with the other enzymes of the pathway except shikimate kinase. In *B. subtilis* however 3-dehydroquinate synthase was purified with chorismate synthase and NADPH-dependent flavin reductase (Hasan and Nester, 1978). The enzyme was only active when associated with chorismate synthase whereas the presence or absence of the flavin reductase has no effect on the dehydroquinate synthase activity. The enzyme required NAD^+ and either Co^{2+} or Mn^{2+} for activity.

1.1.3.3 3-Dehydroquinate dehydratase (dehydroquinase) (EC 4.2.1.10)

3-Dehydroquinate dehydratase catalyses the third step in the shikimate pathway and is responsible for initiating the process of introducing the first double bond, thus generating dehydroquinate. There exists two classes of dehydroquinate dehydratase (type I and type II) which catalyse the reactions with opposite stereochemistry (Kleanthous *et al.*,1992; Harris *et al.*, 1993).

The reaction is common to the two metabolic pathways, namely (i) biosynthesis to generate the aromatic amino acids in micro-organisms and plants and (ii) the catabolic quinate pathway in fungi (Figure 1.2) which allows the use of quinate as the sole carbon source (Giles *et al.*, 1985).

Type I DHQase is only involved in the biosynthetic pathway (Chaudhuri *et al.*, 1985,1987a,b) whereas type II enzymes have been found to have either a biosynthetic (White *et al.*, 1990; Garbe *et al.*,1991; Bottomley *et al.*,1996) or catabolic role (Hawkins, 1982).

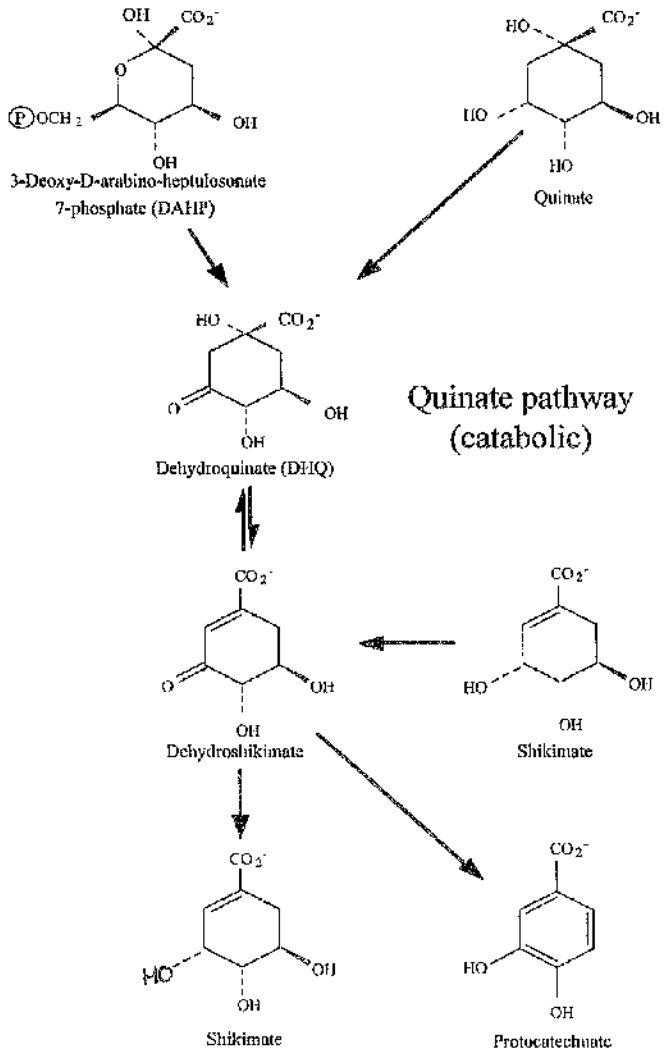


Figure 1.2 : The position of dehydroquinate as the intermediate in the biosynthetic shikimate and catabolic (quinate) pathways.

Both *E. coli* and *S. typhi* possess type I DHQase which generates a Schiff base intermediate (Shneier *et al.*, 1991) while the type II DHQase exemplified by the *Mycobacterium tuberculosis* and *Streptomyces coelicolor* enzyme work via an enolate mechanism (Harris *et al.*, 1996). The crystal structures of both enzymes have now been solved and extensive characterisation has been performed (Gourley *et al.*, 1996 ; Price *et al.*, 1999).

Interestingly *B. subtilis* appears from the genome sequence to have both types of DHQase; this possibility presently being investigated by Alex Herbert, University of Glasgow, (personal communication).

1.1.3.4 Shikimate dehydrogenase (EC 1.1.1.25)

Shikimate dehydrogenase catalyses the reversible reduction of 3-dehydroshikimate to produce shikimate and is an $NADP^+$ specific dehydrogenase. The *E. coli* enzyme has 272 amino acids and exists as a monomer of molecular mass of 29 kDa. The enzyme was found to be strongly selective for NADPH over NADH (Balinsky *et al.*, 1971).

There are two kinds of pyridine nucleotide dependent dehydrogenase enzymes, those with or without catalytically active metal ions such as Zn^{2+} and Fe^{2+} (Aronson *et al.*, 1989). Shikimate dehydrogenase is believed to be a metal independent dehydrogenase similar to lactate dehydrogenase (LDH) and malate dehydrogenase (MDH). The enzymes have an essential active site histidine residue which forms a hydrogen bond with the carbonyl group of the substrate (Adams *et al.*, 1987). This then initiates the hydride ion transfer by polarisation of the carbonyl group. In metal dependent dehydrogenases such as alcohol dehydrogenase such a transfer is initiated by the bound metal ion.

In shikimate dehydrogenase the presence of EDTA does not inhibit activity and the presence of divalent ions has no activating effect on the enzyme activity; thus it belongs to the metal independent category.

1.1.3.5 Shikimate kinase (EC 2.7.1.71)

Shikimate kinase (SK) is the fifth enzyme in the shikimate pathway which catalyses the phospho-transfer from ATP to the C-3 hydroxyl group of shikimate.

This enzyme which is the subject of the thesis is reviewed in section 1.3 below.

1.1.3.6.1 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSP synthase) (EC 2.5.1.19)

5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS) is responsible for the transfer of the enolpyruvyl moiety from phosphoenolpyruvate to shikimate-3-phosphate forming 5-*enol*pyruvylshikimate-3-phosphate and inorganic phosphate.

In *E. coli* the polypeptide has 427 amino acids with a subunit molecular mass of 46 kDa and is monomeric (Duncan *et al.,* 1984). The enzyme from *B. subtilis* has 428 amino acids and is also monomeric (Henner *et al.,* 1986).

This enzyme is targeted by the broad spectrum herbicide glyphosate and is discussed in section 1.6.1.

1.1.3.7 Chorismate synthase (EC 4.6.1.4) (3-Phospho-5-enolpyruvylshikimate phosphate-lyase)

Chorismate synthase catalyses the final step in the shikimate pathway the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate. This involves the abstraction of the 6-pro-R-H and elimination of phosphate which then generates the second double bond within the aromatic ring. For each reaction a reduced flavin co-factor is required as is shown by the *Neurospora crassa* enzyme which possesses an intrinsic NADPH-dependent flavin oxidoreductase (Welch *et al.*,1974; Boocock and Coggins, 1983; Henstrand *et al.*,1995).

The enzymes in *E. coli* (White *et al.*, 1988) and *B. subtilis* (Hasan and Nester, 1978) are monofunctional and must be supplied exogenously with reduced flavin.

The isolation, purification and characterisation of these enzymes has been difficult as they naturally occur within cells at low concentrations. However the advent of recombinant DNA technology has made it possible to construct overproducing strains of *E. coli* which can cope with a 1000 fold increase in the expression of a particular protein which in some cases, can lead up to 50% of the total cellular protein within the cell (Studier and Moffatt, 1986).

With this technology the isolation, expression and characterisation of all the enzymes involved in the pathway has been carried out.

1.2 Organisation of the Shikimate Pathway Enzymes

Although the seven enzymes of the shikimate pathway are structurally and mechanistically similar in all species capable of aromatic amino acid biosynthesis the genetic arrangement is rather different.

The genetic arrangement varies greatly from *E. coli* where the seven enzymes exist as separate monofunctional genes (Berlyn and Giles, 1969; Nakatsukasa, and Nester, 1972, Rothe, 1983; Pittard, 1987) to the multi-functional complex that is found in the fungal species; *Neurospora crassa* (Lumsden and Coggins, 1977; Giles, 1985;), *Aspergillus nidulans* (Kinghorn and Hawkins,1982), *Saccharomyces cerevisiae* (Duncan *et al.*, 1987; Graham, 1993) and other fungal and yeast species such as *Euglena gracilis* (Patel and Giles, 1979) where five of the seven activities are linked on one gene in one single open reading frame and are known as AROM clusters (Duncan *et al.*, 1987; Charles, 1985 and 1986).

In plants however there exists no AROM cluster but a bifunctional arrangement of dehydroquinase and shikimate dehydrogenase, found during extensive studies on photosynthetic organisms by Berlyn (1969;1970,72,73) and more recently in *Pisum sativum* (Deka *et al.,* 1994). Differences within such 'isoenzymes' have led to interest in the evolutionary history and the biochemical pathway origins of these. Enzymes could therefore be used as 'documents of the course of evolution' (Haslam, 1993).

Enzyme	Gene	Map Position (min)	Amino acids	Subunit M_r	Quaternary Structure
DAHP Synthase					
DAHP Synthase (tyr)	*aro F*	57	356	38,804	dimer
DAHPSynthase (phe)	*aroG*	17	350	37,997	tetramer
DAHP Synthase (trp)	*aroH*	37	347	39,000	dimer
3-Dehydroquinate synthase	*aroB*	75	362	38,880	monomer
3-Dehydroquinate dehydratase	*aroD*	37	240	26,377	dimer
Shikimate dehydrogenase	*aroE*	72	272	29,380	monomer
Shikimate kinase I	*aroK*	74	173	19,526	monomer
II	*aroL*	9	173	18,937	monomer
EPSP synthase	*aroA*	20	427	46,112	monomer
Chorismate synthase	*aroC*	51	357	38,183	tetramer

Table 1.1 : Arrangement of the shikimate pathway genes in *E. coli.*

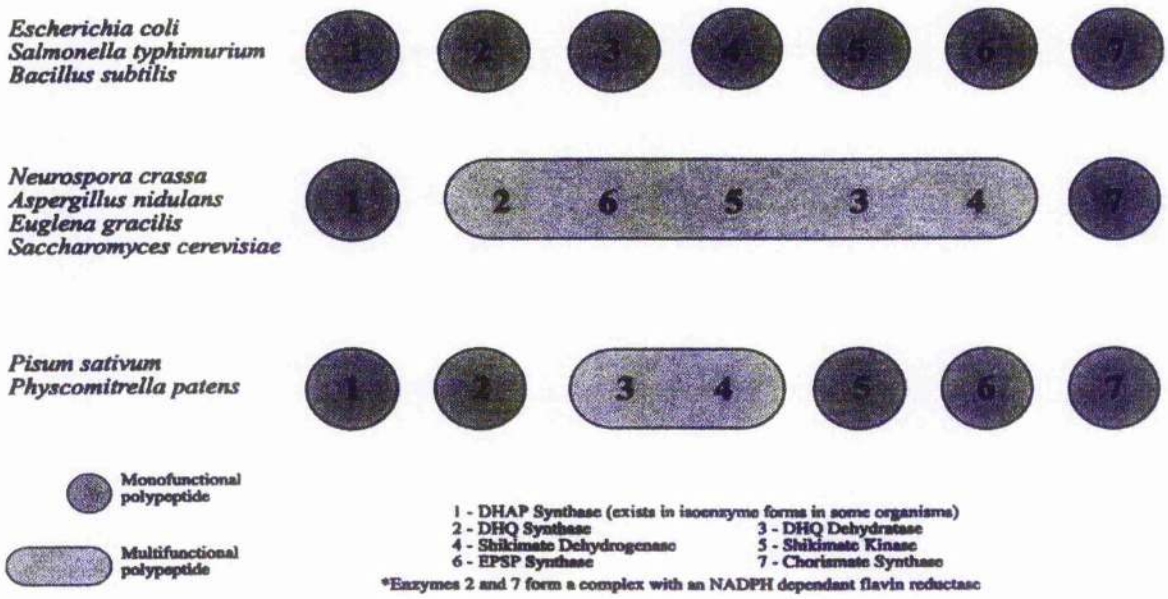


Figure 1.3: Showing the genetic arrangement of the genes for the Shikimate Pathway.

1.3 Shikimate Kinase

1.3.1 Reaction mechanism of shikimate kinase

Shikimate kinase (SK) (E.C. 2.7.1.71) is the fifth enzyme in the shikimate pathway which catalyses the phospho-transfer from ATP to the C-3 hydroxyl group of shikimate.

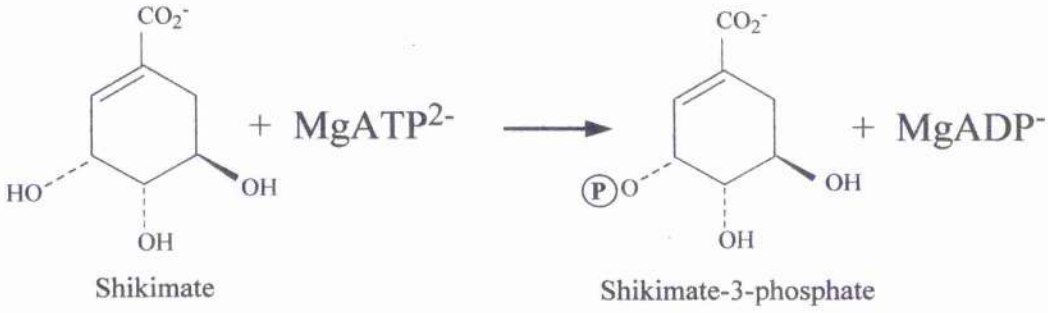


Figure 1.4 : Reaction mechanism of shikimate kinase

1.3.2 Background to the molecular biology of the two isoforms of shikimate kinase

Two isoforms of shikimate kinase (SK) exist, types I and II. This is unusual for enzymes that exist in the middle of a pathway which has led to the proposition that shikimate may be a branch point intermediate (De Feyter *et al.*, 1986).

Both the *aroK* gene encoding SKI and *aroL* encoding SKII are scattered around the chromosome at the positions shown in Table 1.1.

SKII expression is controlled through the *aroL* existing in a two gene operon *aroLM* which is regulated by the *trpR* and *tyrR* regulator gene (Ely and Pittard, 1979; De Feyter *et al.*, 1986). The tyrR protein is of particular interest since it modulates the expression of at least eight unlinked operons. Seven of these operons are regulated in response to co-factors to the tyrR protein and they form an active regulatory system.

Shikimate kinase I (SKI) however is independent of both the amount of extracellular amino acid and the level of the *tyrR* gene product (Ely and Pittard, 1979).

One other *aro* gene which is not subject to the tyrR repression or to end product repression is *aro B* the gene for dehydroquinate synthase the second enzyme of the shikimate pathway. The *aroK* gene encoding the SKI was located upstream of the *aroB* gene (Løbner-Olsen and Marinus, 1992; Whipp and Pittard, 1995). These genes form another two gene operon.

1.3.3 Enzymology of the two isoforms of shikimate kinase

The gene for SKII was cloned and over expressed and found to be a monomer of molecular mass of 18 937 Da (Millar *et al.*, 1986; De Feyter and Pittard, 1986a). The apparent K_m for shikimate was 200 μM and 160μM for ATP (De Feyter and Pittard, 1986b). SKI has been shown to have a much lower affinity for shikimate compared to that of SKII, having a

K_m of 20mM. Between these monomers there is 30% sequence similarity and both are 173 amino acids in length. The role for SKI is still unclear; It has been suggested that SKI has been displaced by the catalytically more efficient and better regulated SKII (DeFeyter and Pittard, 1986c). It is possible that SKI is not involved in shikimate biosynthesis, since it only phosphorylates shikimate fortuitously (DeFeyter and Pittard, 1986b), and that it has a completely different role (or roles) in the cell. SKII may be involved in the phosphorylation of a small molecule rather than a protein (Snell, 1996). The reported sensitivity of SKI to the antibiotic mecillinam is suggestive of an alternative biological role (Vinella *et al.*, 1996). In *B. subtilis* 168 there is a single SK which forms a complex with DAHP synthase; when this complex is dissociated the shikimate kinase becomes inactive.

1.4 Structural comparisons to other known kinases

It has been shown through comparisons of sequence and crystal structure data that there is a degree of sequence conservation in a number of nucleotide binding proteins (Walker, 1982; Moller and Amons, 1985; Fry *et al.*, 1986; Dever *et al.*, 1987; Saraste *et al.*, 1990). The region most conserved is the glycine rich domain which forms a loop between the β-strands and the α- helix (Wierenga and Hol, 1983; Gay and Walker, 1983; Schulz *et al.*,1990; Muller and Schulz, 1992, 1993). Generally this motif is referred to as the A motif or P-loop having the consensus sequence AG-X_4-GKST (Walker *et al.*, 1982). It contains the essential lysine in the motif GK(T/S) which is thought to generate an anionic environment within which the β-phosphate of ATP can be accommodated. Crystal structure analysis of the guanidine nucleotide binding proteins EFTu and p21 and the enzyme adenylate kinase have revealed that this structural motif indeed constitutes a loop which wraps around a phosphate group (Dreusicke and Schulz, 1986,1988).

Shikimate kinase I (SKI) from *Erwinia chrysanthemi* has been shown to have a very similar fold to that of adenylate kinase, yet with only 19% sequence homology (Krell *et al.*, 1998). The structure consists of five β-strands and eight α-helical regions and the sequence contained a Walker type A motif (GXXGXGK(T/S). The 'invariant' lysine together with the glycine rich loop within this motif has been shown to be conserved among 64 sequences of nucleotide binding proteins known. (Hanks *et al.*, 1988).

AROM	*Aspergillus Nidulans*	I G M R G A G K S T A GNWVSKAL
AROM	*Saccharomyces cerevisiae*	I G M R A A G K T T I SKWCASAL
aro L	*Erwinia Chrysanthemi*	V G A R G C G K T T V GRELARAL
aro L	*E. coli*	I G P R G C G K T T V GRELARAL

Figure 1.5: Sequence similarity within the P-loop binding domain of shikimate kinase.

The highly conserved lysine present in this P-loop has been studied extensively both from a crystallographic viewpoint (Pai, 1977; Muller and Schulz, 1993,1996) and with chemical modifying agents and site directed mutagenesis (Tian *et al.*, 1988, 1990). The mutagenesis of the shikimate kinase in this project parallels the mutagenesis work that was carried out on adenylate kinase (Tian *et al.*, 1990) where the two lysines present in adenylate kinase were mutated to methionine. More recently mutagenesis of the lysines seen in human adenylate kinase have been studied (Ayabe, 1997; Bertrand *et al.*, 1997a b).

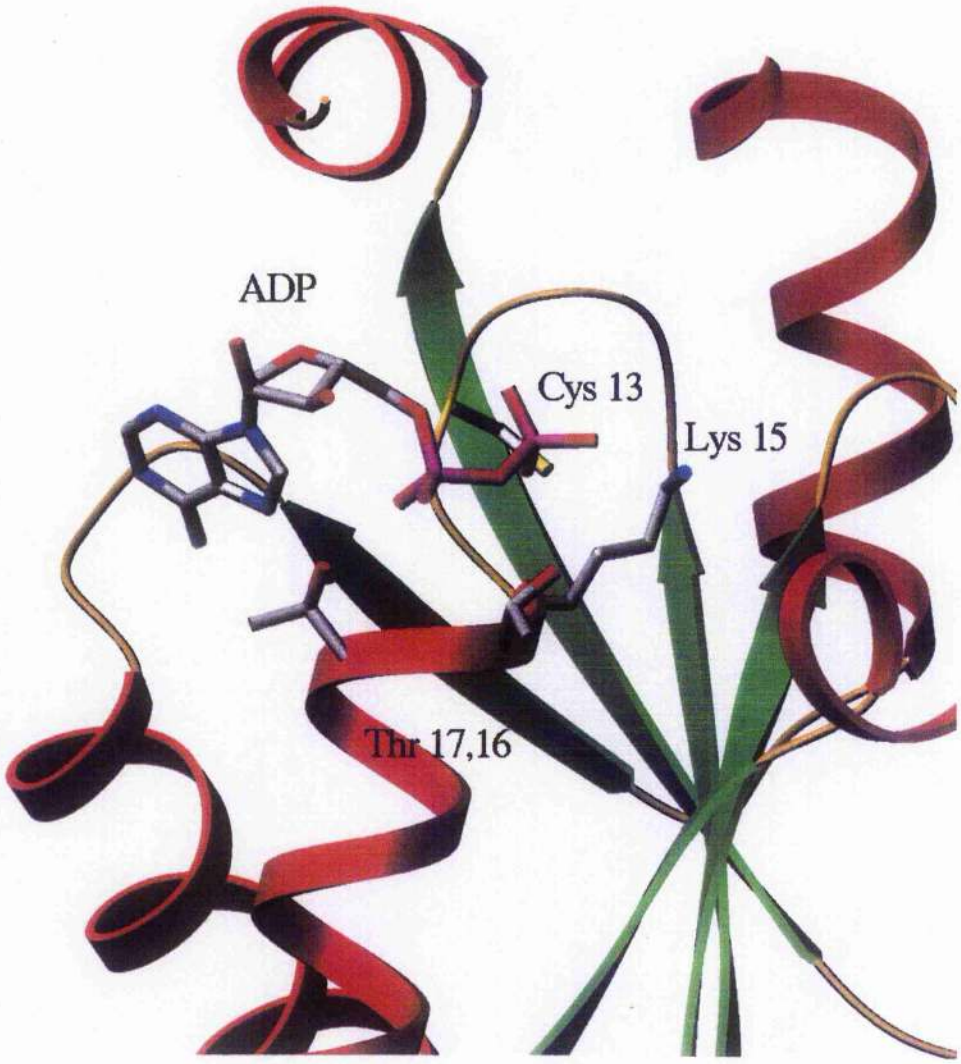


Figure 1.6: The P-loop domain illustrating the active site lysine (K15) and the position of the bound ADP in shikimate kinase II from *Erwinia chrysanthemi.*

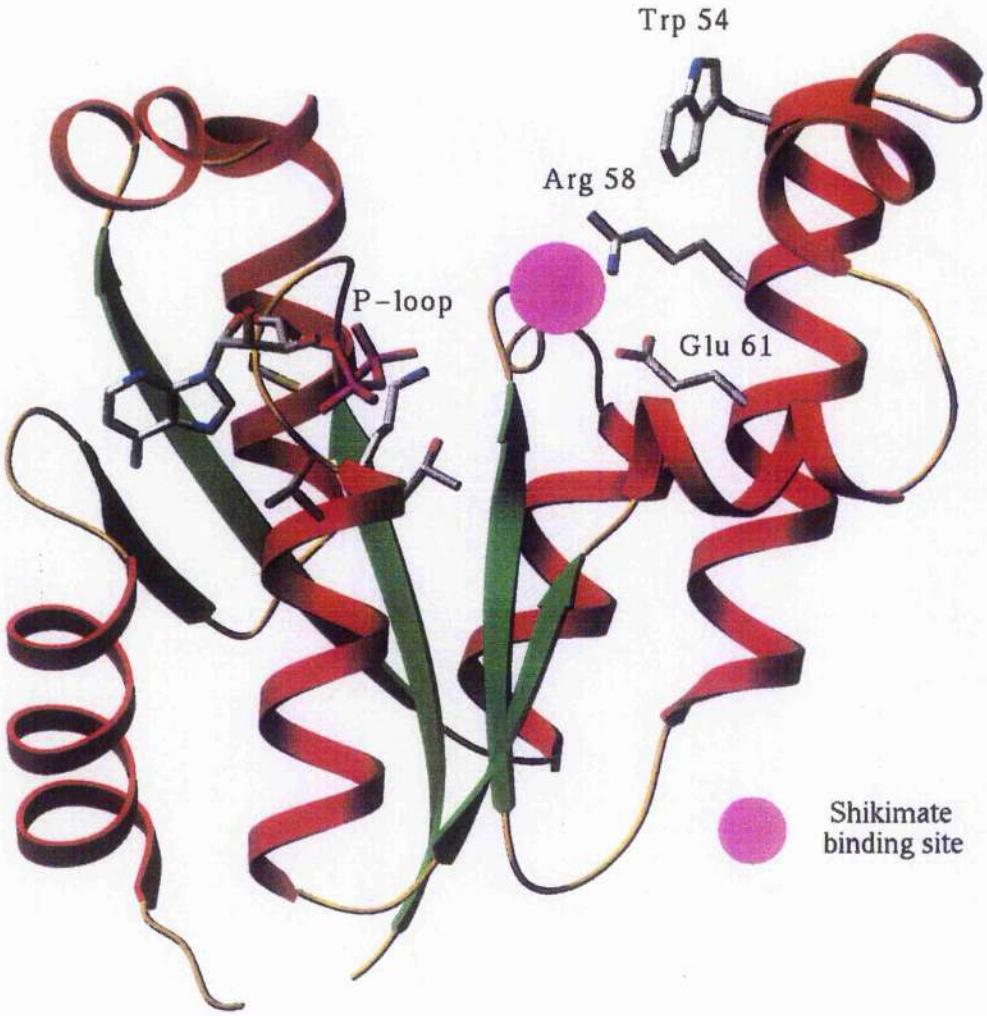


Figure 1.7 : The complete full structure of shikimate kinase indicating regions of the P-Loop and the shikimate binding domain.

1.5 The induced fit mechanism of enzyme action

Large conformational changes are often noticed within NMP kinases during catalysis. Such ligand induced conformational changes which were first described by Koshland for the enzyme phosphoglycerate mutase (Koshland, 1959, Yankeelov and Koshland 1965) and such changes are described by the term 'induced fit'. Induced fit with the NMP kinases occurs within the two flexible regions of the enzyme namely the nucleotide binding domain and the lid domain. This occurs for a number or reasons; to protect the active sites and substrates from the omnipresent water and avoid principally ATP hydrolysis, as well as the saturation of the active sites with substrates and prevention of the escape of the intermediates generated. The actual movement has been described in detail by Schulz and his colleagues who solved a series of crystal structures of adenylate kinase with different bound ligands (Muller *et al.,* 1996; Schulz *et al.,*1990; Gerstain *et al.,*1993 and Sinev, 1996ab; Smirnova *et al.,* 1998). Rotation of the body of the enzyme by 39° and then a lid domain movement of 30° together with a hinge bending rotation of 90° was observed; a 'movie' of the total changes has been produced (Muller *et al.,* 1996).

1.6 Importance of studying the shikimate pathway enzymes.

Mammals do not possess the capacity to synthesise the aromatic amino acids via the shikimate pathway. Much interest therefore, has focused on the opportunity for selectively blocking the shikimate pathway enzymes within potentially pathogenic organisms and thus develop potential herbicidal (Coggins, 1989), antibacterial (Davies *et al.*, 1994) and anti-parasitic agents (Roberts *et al.*, 1998).

1.6.1 Glyphosate

The active agent in the leading herbicide Round-Up is glyphosate which specifically inhibits the sixth enzyme of the pathway 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSP synthase) (Steinrucken and Amrhein, 1980; Amrhein *et al.,* 1983; Boocock and Coggins, 1983). It is a non-selective, broad spectrum and post-emergence herbicide which inhibits the synthesis of the aromatic amino acids through competitive inhibition of the EPSP synthase. The mechanism, however for such inhibition is not yet fully understood. More recent papers (Roberts *et al.,* 1998) have identified the toxic action of glyphosate upon apicomplexan parasites including *Toxoplasma gondii, Plasmodium falciparum* and *Crytosporidium parvum* which have only recently been found to have the shikimate pathway. Genetic engineering techniques have been used to make glyphosate-resistant crop plants (Comai *et al.,* 1985) and they are now widely grown in the United States (Padgette *et al.,* 1995). There are a few examples of plants becoming 'naturally' resistant to the effects of glyphosate but to date the emergence of such resistance has been very rare (Reinbothe *et al.,* 1993). Recently some alternative inhibitors have been reported (Shah *et al.,* 1997). Although there are examples of plants becoming immune to the effects of glyphosate, a range of possible inhibitors based on the structure of glyphosate is being studied intensively.

1.6.2 (6S)-6-Fluoro-shikimic acid

(6S)-6-Fluoro-shikimic acid, an analogue of shikimic acid, was reported as a potential antibacterial agent for use in human medicine (Davies *et al.,* 1994; Ewart *et al.,* 1995). This compound was initially thought to act by inhibition of chorismate synthase (Balasubramanian *et al.,* 1991) but more recently it has been suggested that it acts by inhibiting p-aminobenzoate synthase (Bornemann *et al.,* 1995).

1.7 The principal biophysical methods used in this work

1.7.1. Circular Dichroism

Circular Dichroism (CD) is a spectral technique based on the differential absorbance of left and right circularly polarised components of plane polarised radiation. CD is the result of chromophores that are themselves chiral (optically active) or are placed in an asymmetric environment. Radiation is split into two circularly polarised components, by being passed through a modulator such as a piezoelectric crystal, which is subject to an alternating electric field. If the two components are not absorbed by the sample or are absorbed to the same extent then no CD signal will be produced. If however one of the polarised components is absorbed to a greater extent than the other (e.g. due to intrinsic or environment chirality of a chromophore) then the resulting radiation would be elliptically polarised, for example, instead of a plane an ellipse will be traced out. In practice the two components are not recombined after passage through the sample; the instrument actually measures the change in absorbance.

The term ellipicity (referring to the theoretical recombination) is widely used. From the degree of ellipicity it is possible to relate the signal obtained to structural information. The relative proportions of α-helix and β-sheet in a protein can be determined from signals obtained in the far UV CD arising from the peptide bonds

In the far UV CD (a wavelength below 250nm) the α-helix and β-sheet secondary structures determine the spectral characteristics largely due to the chirality exhibited around the peptide bond. In the near UV CD (250nm-350nm) the spectrum gives a detailed 'finger print' of the tertiary structure of a protein largely as a result of the environments of each aromatic amino acid side chain although there are contributions from disulphide or non-protein co-factors that may be present. CD has been used ever increasingly as a rapid way of studying structural changes brought about upon binding of ligands and can give

information about the folding and stability of the protein in question. For a more detailed review of circular dichroism and its applications see Kelly and Price (1997).

1.7.2 Fluorescence

Protein fluorescence can be used to monitor changes in protein tertiary structure.

In proteins, aromatic amino acids (essentially tryptophan residues) are able to emit fluorescence. This fluorescence occurs when a molecule is excited by radiation. The molecule then decays from its excited state, and the excess energy is released, at a longer wavelength. Protein fluorescence is largely due to the excitation of aromatic amino acid residues particularly tryptophan which has a maximum emission at 356nm (in water) after excitation at 290nm (Price, 1972).

Shikimate kinase has a single tryptophan residue which has proved to be a useful fluorophore for monitoring conformational changes associated with ligand binding. The changes in fluorescence reflect changes in the environment of the tryptophan.

Burial of the fluorophore within a protein or shielding by other amino acid residues leads to a blue shift in the wavelength of the emission maximum. The quenching of protein fluorescence, after ligand binding, is particularly useful for providing information on the conformational changes that occur following ligand binding.

During unfolding/refolding studies the regain of protein fluorescence can be used as an indicator as to the extent of regain of tertiary structure. The denatured, unfolded, protein has an emission maximum of 356nm, indicative of a fully exposed tryptophan. During refolding, the emission maximum will shift to the blue as the tryptophan residues become buried or sheltered within the protein.

1.7.3 Differential Scanning microcalorimetry (DSC)

Differential Scanning microcalorimetry (DSC) is an experimental technique used to measure the heat energy uptake that takes place in a sample during a controlled change in temperature. The simplest level of using DSC is in the determination of the melting temperature for samples in solution although thermodynamic parameters can also be calculated. As the protein is heated at a constant rate in a calorimeter cell an identical reference cell containing buffer is also heated. The differences in apparent heat capacity can be then determined (Cooper *et al.,* 1999) along with the thermodynamic parameters (ΔH_{cal} , ΔH_{vH}).

The whole folded protein is also only marginally more stable than the unfolded form. Thus the conformational stability of almost all naturally occurring globular proteins is between 5 and 15 kcal/mol (21 and 63 kJ/mol) (Arfin and Bradshaw, 1988).

Ligand binding may well have a substantial effect on the conformational stability of a protein which will generate differences in T_m and thermodynamic parameters.

Large binding constants that exist in strong to ultratight protein interactions, which cannot be conveniently measured by conventional equilibrium techniques, have been elucidated by means of DSC (Brandts and Lin, 1989 and 1990). The ionic strength of buffers used during DSC may have an effect on the stabilisation of a protein and this has been investigated by looking at the stabilisation that Zn^{2+} offers carboxypeptidase B (Conejero-Lara *et al.,* 1991). The effect that the scan rate has on the denaturation of yeast phosphoglycerate kinase (Galisteo *et al.,* 1991) has been studied. The formation of enzyme ligand complex via a Schiff base formed in the catalytic mechanism of dehydroquinase type I has been shown to have marked effects on the stability (Kleanthous *et al.,* 1991).

1.7.4 Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a technique which provides a means of measuring affinity constants which can help to provide a complete thermodynamic characterisation of the system under study. ITC provides a great deal of physical information about the binding process including the binding affinity or equilibrium constant (K_{eq}), the molecular ratio or stoichiometry (n) and the heat or enthalpy (ΔH) as well as entropy (ΔS) of binding. The enthalpy of binding, the stoichiometry and the free energy change (ΔG) of the binding process are determined by a non linear fit of the binding isotherm (Cooper *et al.*, 1998). Titration calorimetry is a more practical approach to the routine determination of thermodynamic parameters because nearly all binding interactions are accompanied by a change in enthalpy and thus virtually all reactions of interest will produce a calorimetric signal. Titration calorimetry offers the possibility of directly determining not only the association constant and free energy but also the stoichiometry, enthalpy and entropy in a single experiment.

1.8 Aims of this project ;

1) generation and purification of a number of site directed mutants of shikimate kinase

2) characterisation of the binding of ligands to these mutant proteins.

3) characterisation of the unfolding and refolding of shikimate kinase

4) assessment of the significance of the results in relation to models of both substrate binding and protein folding.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Most reagents were purchased from the Sigma Chemical Company, Dorset, England or Fisher Scientific. These were of ANALAR grade or of the highest grade available. Tryptone, yeast extract and bacto-agar were obtained from OXOID Ltd, Basingstoke Hampshire, England..

Agarose was purchased from Life Technologies Inc. Gaithersburg, MD 20877 U.S.A. and guanidinium choride was purchased from GIBCO BRL Life Technologies, Paisley, Scotland.

2.1.2 Proteins and Enzymes

Restriction enzymes and $Vent_R$ DNA polymerase were obtained from New England Biolabs Incorporated, Hitchin, Hertfordshire, England; Promega Corp, Southampton, England and Boehringer Mannheim, UK.

Bacteriophage T4 DNA ligase and calf intestinal alkaline phosphatase, were purchased from Promega Corp., Southampton, England.

2.2 Bacterial culture strains, plasmids and oligonucleotides

2.2.1 Bacterial strains

Bacterial strain	Genotype
Escherichia coli DH5α	F⁻ ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169
Escherichia coli BL21(DE3)pLysS	F- *ompT hsdS*$_B$ r_B^-,m_B^- *dcm gal* λDE3 Studier and Moffatt (1986)

2.2.2 Bacterial plasmids

Plasmids		Reference
pTB361	Protein Expression Plasmid	Zeneca Patent application no 92301456.8

2.2.3 Synthetic oligonucleotides

The primers were obtained from GENOSYS (Genosys Biotechnologies Europe Ltd.).

Primer	Sequence	Length (bp)
SKPrA	5'GAA GGA GAT ATA CAT ATG ACA GAA CCC 3'	27
SKPrB	5'GCA TTA CAG ATC TCA GGC CGC AGG CAG ACG 3'	30
K15M For	5'GGG TGC GGA **ATG** ACC ACC GTC GGC 3'	24
K15MRev	5' GCC GAC GGT GGT **CAT** TCC GCA CCC 3'	24
C13S For	5'GGC GCC AGA GGG AGC GGA AAA ACC 3'	24
C13S Rev	5'GGT TTT TCC GCT CCC TCT GGC GCC 3'	24
C162S For	5'CCC GCC GCG ATT GTC AGC GAA TTG ATG 3'	27
C162SRev	5'CAT CAA TTC GCT GAC AAT CGC GGC GGG 3'	27
D34N For	5'GGC TAT GAG TTT GTC GAT ACG AAT ATT TTT ATG CAG 3'	36
D34N Rev	5'CTG CAT AAA AAT ATT CGT ATC GAC AAA CTC ATA GCC 3'	36

The bold letters indicate the mutated base

2.3 Growth and storage of micro-organisms

2.3.1 Culture media

Routine bacterial growth was carried out in Luria-Bertani (LB) medium comprising; bacto-tryptone 10g/litre, yeast extract 5g/litre and NaCl 10g/litre.

Solid media was generated by the addition of bacto-agar at a concentration of 1.5%.

All solutions of media were autoclaved at 15psi for 15minutes.

Antibiotics were added to the following concentration value; Tetracycline 12.5μg/ml, chloramphenicol 25μg/ml.

Stock solutions at 12.5mg/ml and 25mg/ml respectively were made up in 70% ethanol and filter sterilised through a 0.22μm millipore filter and stored at -20°C for no longer than 2 weeks.

Antibiotics from the stock solution were added to the pre-autoclaved media when the temperature had cooled to 50-55°C.

Plates were then poured and stored at 4°C for use within 2 weeks.

2.3.2 Growth of strains

Growth was carried out routinely at 37°C. Liquid cultures were grown with shaking at 200 rpm. Agar plates were grown overnight in the 37°C room or in the small bench top incubator.

2.3.3 Storage of strains

Short term storage was achieved by plating out the bacteria and storing plates at 4°C. In the case of BL21 (DE3) pLysS pTB361 strain, re-streaking of plates was necessary every four weeks to maintain the short term storage culture.

Medium term storage of bacteria was accomplished by making glycerol stocks of overnight cultures with the addition of 80% (v/v) sterile glycerol to a final concentration of 15% (v/v) and these were stored at -20 °C.

Long term storage was achieved as above but with the cells being stored at -80°C.

2.4 Molecular biology methods

2.4.1 DNA electrophoresis

Gels electrophoresis of DNA was detected using 1% agarose gels made up in Tris-borate buffer (TBE) which comprised 90mM Tris, 90mM boric acid and 2mM EDTA pH 8.0. The agarose was melted into the buffer solution by means of a microwave and upon cooling down to pouring temperature 0.05µg/ml Ethidium Bromide was added.

Sample loading buffer contained 0.25% bromophenol blue and 40% (w/v) sucrose made up in water.

2.4.2 Plasmid restriction

Plasmid (5µg) was digested for 1 hour in a total volume of 50µl with 10-20 units of restriction enzyme. The supplied buffer (provided by Promega or Boehringer Mannheim) was used in accordance with manufacturers instructions.

5µl of the digest was run in a 1% agarose gel. A marker of 1kb standard ladder (Gibco-BRL) was used to identify fragments.

For PCR reactions it was sometimes necessary to use a smaller marker 500bp ladder. To check that the digest was successful the gel was viewed under a UV transilluminator.

The restriction digest mix was then cleaned up using a Wizard DNA Clean-Up Kit (Promega) with the final elution of DNA into 50µl sterile water.

2.4.3 PCR procedure; Site directed mutagenesis

2.4.3.1 Two stage PCR using Vent polymerase

PCR was used in a two stage procedure to generate site directed mutants of the enzyme (Higuchi *et al.*, 1988). Great fidelity is required for the generation of specific mutations and thus $Vent_R$ Polymerase was employed (New England Biolabs).

PCR external primers were designed upon the original multiple cloning site within the pTB361 plasmid. These primers incorporated the start codon (NdeI) at the 5' end and the stop codon (Bgl II) at the 3' end of the gene. Internal primers with the mis-match were designed with a T_m similar to that of the external primers so that any mismatching would not generate problems during thermal cycling.

2.4.3.2 PCR reaction mix

PCR reactions were carried out using DNA $Vent_R$ polymerase with appropriate buffers and salts. The reaction in a total volume of 100µl was set up comprising the following;

10µl of 10X reaction buffer, 10µl of 10X dNTP mix, 100pmol of each primer, 100ng of DNA and 10µl of 10X supplied magnesium buffer. DNA $Vent_R$ polymerase (1-2 units) was added for each reaction. Thin walled 200 µl PCR tubes were used within the PCR BioRad gene Cycler model number 10770. Thermal cycles of 3 min at 94°C (initial denaturation), with cycle repeats of 94°C, 55°C, 72°C, for 1 minute each followed by a final extension of 2 minutes at 72°C. Thirty of these cycles were carried out.

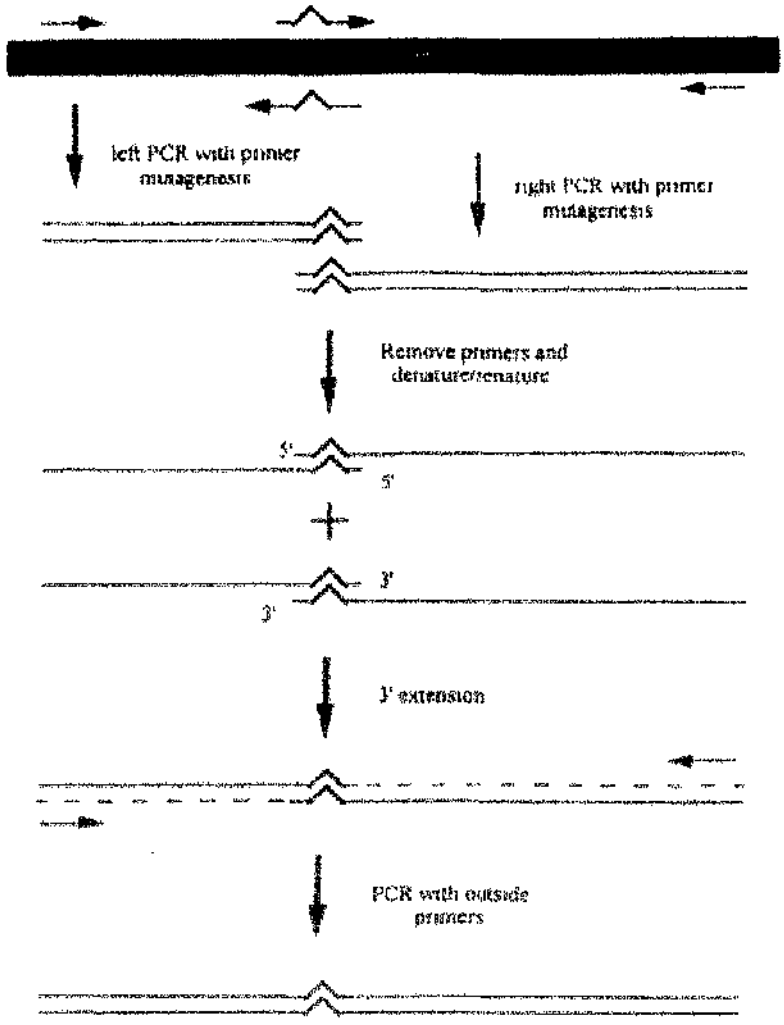


Figure 2.4.3.2: Site directed mutagenesis (Higuchi *et al.*, 1988).

Two separate complementary mutant oligos are generated in the first step PCR step followed by a second round where the complementary fragments are able to form the full length mutant insert ready for re-ligation into vector of choice.

2.4.3.4 Recovery of PCR products from reactions

10µl of the PCR product was run on a 1% (v/v) agarose gel to determine whether the PCR reaction had produced the desired fragment and whether other secondary PCR products

were generated. When only the desired fragment was observed, the PCR reaction mix was purified using the PCR Clean-Up Kit (Promega).

The final elution was made in 50µl sterile water and 10µl of this was run out on a gel to check that a good recovery was obtained. These fragments could then be used for the second round of PCR with the product of this second round, (the full length mutant insert) being used in the ligation mix.

2.4.4.1 Alkaline phosphatase treatment of restricted plasmid

Calf Intestinal alkaline phosphatase (CIAP) was added to the digested plasmid at a concentration of 1.0 unit per pmol 3' or blunt ends and 0.1unit per pmol of 5' ends. The molar amount of plasmid ends was calculated by;

$$\frac{\text{mass of plasmid (g)} \times \text{no. of bases}}{M_r \text{ of plasmid}} \qquad (M_r \text{ of base pair} = \sim 660)$$

Alkaline phosphatase treatment took place at 37°C for a period of 30 minutes in a total volume of 100µl. The DNA Clean-Up Kit was used to purify the plasmid.

2.4.4.2 Ligation of cut moieties

The concentration of DNA prior to ligation was checked either spectrophotometrically or estimated by the intensity of bands shown in gel electrophoresis. Generally several ratios of vector DNA and insert DNA were used in separate ligations. Ligations were performed in a total volume of 10µl with 1-3 units of T4 DNA ligase and incubated at 4°C overnight.

2.4.4.3 Plasmid isolation from colonies

Plasmid minipreps were performed using Wizard Mini™ preparations (Promega).

A single colony was used to innoculate 5 ml of fresh LB which was grown overnight with shaking, at 37°C. 3ml of the overnight growth sample was centrifuged and pelleted cells were used to isolate plasmid following the instructions in the above mini prep kit. For mass screening of colonies the lysozyme/boiling method of Holmes & Quigley (1981) was utilised.

2.5 Preparation of competent cells and transformation

A single colony of the appropriate strain of *E. coli* was picked from an agar plate or a loop was used to melt a small amount of culture from the top of a glycerol stock. This was used to inoculate a 20ml volume of LB which was then grown overnight at 37°C. From the overnight growth 2ml was used to inoculate 100ml fresh LB in a 250ml flask. The cells were grown at 37°C and with 200rpm shaking until they reached mid log phase (OD_{550}=0.35-0.5). The cells were then chilled on ice and centrifuged in a Beckman Model J-6B at 3,000g for 5 minutes at 4°C. The pellet of cells were then resuspended in 1/10 volume in sterile ice cold transformation buffer 1 (TFB1) (100mM RbCl, 50mM $MnCl_2$, 30mM KCl, 10mM $CaCl_2$ and 15% glycerol, added to water in sequence and then made to pH 5.8 with dilute acetic acid and filter sterilised). Incubation in the TFB1 took place for 90 minutes on ice after which the cells were centrifuged as before for another 5 minutes.

The pellet was then resuspended in TFB2 (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$ and 15% glycerol, adjusted to pH 7.0 using HCl and filter sterilised), 1/40 of the original starting volume of culture.

The resulting cells were then stored in 200µl aliquots ready for immediate use or snap frozen in a dry-ice/ethanol bath and stored at -80°C for an indefinite period without showing any significant reduction in transformation efficiency.

2.5.1 Transformation protocol

An aliquot (200µl) of frozen competent cells was slowly defrosted between thumb and finger prior to use then replaced back on ice.

The 10µl ligation mix was added to the cells and gently mixed putting the aliquot back on ice for a further 30 minutes. The cells were then heat shocked at 37°C for 90 seconds and replaced back on ice for 15 minutes. 800µl of LB was added to the cells (to bring the volume up to 1ml) and then incubated at 37°C for 60 minutes. Transformation controls were performed at the same time following the sample procedure minus the addition of DNA. Cells were centrifuged at full speed for 30 seconds prior to re-suspension in 50-100µl fresh LB and plated out on selective plates.

2.5.2 DNA Sequencing

The Molecular Biology Support Unit located in the Glasgow University campus was utilised for all sequencing runs. The Perkin Elmer ABI Prism™ Dye Terminator Cycle Sequencing Core Kit with Amplitaq®DNA Polymerase. Samples were prepared using QIAGEN mini preps for DNA isolation and the standard T7 primer together with internal primers specific for the known gene sequence were used.

The amount of DNA template was between 0.5-1.0µg and primer concentration was 3.2pmol. The procedure involved 25 cycle repeats of 96°C for 30secs, 50°C for 15secs and 60°C for 4mins.

2.6 Expression of proteins

2.6.1 Expression in pTB361

The expression plasmid pTB361 is a *cer* tet^R plasmid which contains a consensus T7 promoter immediately upstream from a multiple cloning site.

The inclusion of the gene for T7 lysozyme (which binds and inhibits T7 RNA polymerase) on plasmids pLysE and pLysS provides a means of controlling the basal levels of expressed protein prior to induction with IPTG. pLysS plasmid has the lysozyme gene inserted in the inverse direction to the *tet* promoter producing lower levels of enzyme and allowing greater mRNA production (Studier and Moffatt, 1986). These plasmids are compatible with pTB361 and confer chloramphenicol resistance. BL21(DE3) is *ompT* , *Lon* which may help to prevent proteolysis during purification procedure (Studier and Moffatt 1986).

The lysozyme produced from the pLysS plasmid is sufficient to enable lysis during either freeze-thaw or mild Triton treatment.

2.6.2 Protein expression

A 20ml overnight culture was prepared by selecting a colony from the expression strain of *E. coli* BL21 (DE3) pLysS pTB361 SK either the native or mutant variety.

On the following day the 20ml overnight culture was used to inoculate 500ml of fresh LB, containing the appropriate antibiotics, contained in a 2.5 litre flask. This was then grown overnight for preparation for the larger scale (5 litre) growth.

10 flasks each containing 500ml of fresh LB was inoculated with 35ml LB from the 500ml overnight growth. These were then incubated until the appropriate growth (OD_{600} ~ 0.500) was achieved. IPTG was then used to induce the protein expression and the flasks left to grow for a further 4 hours.

Harvesting the cells was achieved by centrifugation at 4,500g at 4°C for 15 minutes.

The cell paste was then transferred to an appropriate container and stored at -20°C.

2.6.3 SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (1970).

Acrylamide gels were made up using 70% (w/v) acylamide and 30% (w/v) bis-acrylamide; 3% stacking and 15% running gel. Polymerisation was achieved by the addition 0.03% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. The running buffer comprised Tris-HCl (3g/l), glycine (15g/l) and SDS (1g/l). Samples were denatured by boiling for 5 minutes after dilution in the loading buffer comprising 60mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol, 15% (v/v) β-mercaptoethanol and 0.0025% (w/v) bromophenol blue.

2.6.4 Protein staining

Protein was visualised on the polyacrylamide gels after staining with Coomassie blue. Coomassie reagents was prepared with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 10% (v/v) glacial acetic acid, 25% (v/v) methanol. Destaining was performed with several changes of a 10% (v/v) glacial acetic acid, 10% methanol solution.

2.6.5 Protein estimation

The methods of Lowry (1972) and Bradford (1976) were used to estimate protein concentration prior to preparing the protein solution for SDS-PAGE.

A premixed low range protein molecular weight marker (Boehringer Mannheim) was used to estimate the molecular mass of proteins on the gel, by comparison of the relative distance of migration with the markers of known molecular masses.

2.7 Protein purification of shikimate kinase

The purification protocol was based on a modification of that used for the purification of the *E. coli* shikimate kinase enzyme (Millar *et al.*, 1986). The method (Krell *et al.*,1997) required the presence of the dead end complex involving a substrate product mix of shikimate and ADP. However this method was adapted by reducing the salt concentration so as to prevent the protein from precipitating.

All steps after cell breakage were performed at 4°C. *E. coli* BL21 (DE3) pLysS pTB361 cells (10g) were resuspended in 20ml of buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$ and 0.4mM DTT, pH 7.5). The French Press was used to break the cells using two passes at a force of 950 psi. The material was then diluted to 100ml and centrifuged at 100,000g for 1 hour.

The supernatant from this was loaded directly onto the pre-equilibrated DEAE anion exchange column (30cm x 2.6cm diameter, flow rate 20ml/hr).

The column was then washed with buffer A until the absorbance at 280nm was below 0.3. Elution of shikimate kinase was achieved using a linear gradient of 800ml 0-300mM KCl with a flow rate of 48ml/hr and a fractions volume of 12ml.

Pooled fractions were made up in to 1.2M $(NH_4)_2\ SO_4$ by the addition of solid $(NH_4)_2\ SO_4$ these were applied to a phenyl Sepharose hydrophobic interaction column pre- equilibrated in buffer B (100mM Tris/HCl, 0.4mM DTT, pH 7.5). Elution was performed by using a gradient of 400ml 1.2M-0.0M $(NH_4)_2\ SO_4$ with a flow rate of 20ml/hr and a fraction volume of 6ml.

Pooled fractions were then dialysed overnight against buffer C (35mM Tris/HCl, 500mM KCl 2.5mM $MgCl_2$ and 0.4mM DTT, pH 7.5). This material was then concentrated by vacuum dialysis and glycerol added to 10% (v/v).

After concentration to a volume of 2 ml or less the sample was loaded onto the pre-equilibrated Sepharose 200 (superfine grade) column (120cm x 2.5cm) and eluted at a flow rate of 14ml/hour.

Fractions containing shikimate kinase activity were then pooled and dialysed against buffer C containing 50% glycerol stored at -20 °C.

2.8 Methods of analysis

2.8.1 Shikimate kinase assay

The assay for shikimate kinase was monitored 340nm. The assay depends on the coupling of the ADP formed to the pyruvate kinase and lactate dehydrogenase reactions, thereby leading to the oxidation of NADH which can be then measured at 340nm. Routine assays were carried out at 25°C in freshly made assay buffer (50mM triethanolamine pH 7.2, 50mM KCl, 5mM $MgCl_2$).

The assay consisted of the following of ATP 5mM*, NADH 0.2mM, shikimate 1.6mM* and phosphoenolpyruvate (PEP) 1mM *.

(* indicating neutralisation to pH 7.0 prior to storage at -20°C).

2.8.2 Circular dichroism

For a more detailed review of circular dichroism (CD) and its applications see Kelly and Price (1997) and references therein.

Folding of lysozyme was the first to be analysed using the CD method and in fact many thousands of proteins have had their structures analysed through the process of denaturation and refolding.

2.8.2.1 Preparation for CD analysis

Shikimate kinase was dialysed exhaustively into buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$, 0.4mM DTT, pH 7.6). Buffer A was used throughout the proceeding analytical protocols described and the enzyme concentration was varied for different experiments.

During near UV CD experiments 1mg/ml protein concentrations were required whereas for work in the far UV CD 0.2-0.5mg/ml was required.

CD measurements were made at 20°C using a JASCO J-600 spectropolarimeter.

For the denaturation profiles 8M guandinium chloride (GdnHCl) was made up in buffer A and appropriate volumes used to achieve the required molarity within the 1ml denaturation mix.

Refolding studies required much less protein for steady state analysis where the final concentration after dilution was only 0.09mg/ml for both CD and fluorescence work. However for the stopped flow work larger volumes were required because of the larger volumes required to use the equipment. The stock concentration in this case was 1mg/ml which would be diluted 10 fold for a series of runs.

A series of cells of pathlengths 0.5cm 0.05cm and 0.02cm were used in the experiments above depending on the concentration of protein.

2.8.3 Fluorescence studies

2.8.3.1 Preparation for fluorescence analysis

The protein was again dialysed extensively into buffer A prior to use. Fluorescence scans were carried out during denaturation using the same protein as was set up for circular dichroism.

The fluorimeter was set up using the excitation wavelength of 290nm, emission was recorded at 350nm and the sample loaded in a 1cm pathlength cell.

Fluorescence measurements were made at 20°C using a Perkin-Elmer LS50B spectrofluorimeter.

2.8.4 Stopped-flow CD and fluorescence

Stopped-flow measurements of the refolding process were made on an Applied Photophysics SX-17MU stopped-flow reaction analyser with a 10:1 mixing ratio.

In the CD mode the instrument used an umbilical cell which involves a dead time estimated as 8ms by using the well characterised refolding of lysozyme after denaturation.

Enzyme solution was made up at a concentration of 1mg/ml in 4.8M and 2.8M GdnHCl and this enzyme mix was used to fill syringe A. Syringe B was filled with 10ml of the appropriate refolding or control buffer.

2.8.5. 1) Differential scanning calorimetry (DSC)
2) Isothermal titration calorimetry (ITC)

Experiments were carried out in collaboration with Professor Alan Cooper, University of Glasgow, Biophysical Chemistry Group.

2.8.5.1 Differential scanning calorimetry (DSC)

The thermal denaturation of shikimate kinase was monitored using the method of DSC. The stabilisation provided by the binding of the nucleotides ATP, ADP and the dead end mix of ADP/shikimate to a number of shikimate kinase samples was determined by means of T_m and also thermodynamic constants. Protein ligand interactions have been monitored extensively in Cooper *et al.*, (1998, 1999).

Protein samples (1mg/ml ; 0.050mM) were exhaustively dialysed against buffer A and degassed briefly before loading into the DSC cell. All substrates were dissolved in dialysis buffer and the pH adjusted to pH 7.6. The rate of temperature increase was 50°C/hour over the range 20-90°C. Data collected were analysed using Microcal software.

2.8.5.2 Isothermal titration calorimetry (ITC)

The binding of nucleotide (ATP or ADP) to shikimate kinase samples at 25°C was determined using a Microcal OMEGA isothermal titration calorimeter following standard procedures (Wisemann, 1989, Cooper and Johnson, 1994). Protein samples (1mg/ml ; 0.050mM) were exhaustively dialysed against buffer A (as above section) and degassed briefly before loading into the ITC cell. Substrate (ATP or ADP) as dissolved in dialysis buffer and pH adjusted to pH 7.6.

A typical titration experiment involved a series of up to 25 x 10µl injections of ligand into the enzyme solution at 3 minute intervals with continuous stirring. Integrated heat effects on sample and controls were under identical conditions and analysed by standard techniques using Microcal ORIGIN software (1998).

Chapter 3 : Initial characterisation of mutants of shikimate kinase.

3.1 : Identification of the active site lysine in shikimate kinase II from *Erwinia chrysanthemi*

3.1.1 Abstract

The crystal structure of shikimate kinase (Krell *et al.*, 1998) suggested a number of different sites at which mutations could provide useful information.

Firstly the surface cysteine (C162) is involved in the dimerisation of the shikimate kinase; mutation of this residue would then permit the generation of different crystallisation conditions. The single lysine (K15) proved a good target to illustrate that this was indeed a very important residue in the active site and to investigate its role in co-ordination of the nucleotide substrate.

The ion pair thought to occur due to the interaction of the single lysine (K15) and the nearby cysteine (C13) was investigated through mutagenesis of the C13 to a serine. The activities of the mutant enzymes generated was in the order of wild-type>C162S>C13S where K15M and D34N had undetectable activity using the standard assay procedure.

The removal of the surface cysteine of C162S generated a much more soluble protein compared with the wild type. The activity of this mutant however was very similar to that of the wild type, however mutation of the internal cysteine (C13S) reduced the activity by 50% compared with wild type.

The lysine mutant had undetectable activity, however, it has recently been crystallised (John Maclean, University of Glasgow, Personal communication) which has allowed significant refinement to the previous crystal structure (Krell *et al.*, 1998).

3.1.2 Introduction

Site directed mutagenesis has become a very important and now relatively simple tool to explore the structural and functional significance of particular residues within proteins. This may involve the introduction of virtually any mutation at any site involving single base substitutions, short deletions or inserts. The use of this technique has grown enormously over the past ten years allowing us unlimited access to the manipulation of protein structure at the genetic code level.

A general review (Bordo and Argos, 1991) highlights the main aims of why such manipulation is required largely for;

1) identifying catalytic or structurally important residues: as will be illustrated in later chapters.
2) developing synthetic, novel genes for ease of expression in host organisms for much easier access to the particular protein of interest (Brocca *et al.*, 1998)
3) regulation of genes for industrial processes both with relation to controlling flux pathways for specific intermediates or end products of a pathway (Dell and Frost, 1993; Snell *et al.*, 1996).
4) engineering enzyme specificity to cope with novel substrates or simply to improve catalytic turnover (Sinisterra, 1996).

In this study the first point above was investigated, looking at the catalytic and structurally important residues and identifying the properties of single substitutions within shikimate kinase. For further review on other applications the other references highlighted above should be consulted.

In order to investigate and identify the catalytic residues it is important that no other unforeseen effect has been caused by mutation. Thus only one residue was substituted at a time. Extensive study into the 'safe substitutions' that are allowed within a protein were

investigated using the λ repressor and the codon most preferred for the mutant protein designed with reference to the highly and lowly biased gene table (Bulmer, 1988; Andersson and Kurland, 1990).

The choice of mutants to be made in shikimate kinase was selected from knowledge of the three dimensional structure of the enzyme. Multi-alignment programmes were used to align sequences and thus highlight conserved residues across a number of shikimate containing organisms which rely on the shikimate pathway.

In the alignment (Fig 3.1) it is possible to see well recognised nucleotide binding consenus sequences such as the Walker type A motif (Walker *et al.,* 1982) between residues 10 and 20 which contained the single 'invariant' lysine.

Firstly, however the removal of the ability of the shikimate kinase to form a disulphide bond was investigated by looking at the external cysteine (C162). This had been found to form a disulphide bond during previous crystallisation trials and was thought to be the main contributing factor accounting for the lack of density around the lid domain (Fig 3.2). Thus removal of the possibility of disulphide bond formation may bring about more favourable crystallisation conditions. A 'safe' mutation for this was selected to be serine and thus the mutant C162S was generated.

Secondly, the single lysine within shikimate kinase structure (K15) was thought to be essential for catalysis so a mutation analogous to that made of adenylate kinase (Tian *et al.,* 1990), where the two lysines were mutated to methionine (K21M and K27M), was carried out resulting in the mutant K15M of shikimate kinase.

In order to learn more about the catalytic mechanism of shikimate kinase other residues targeted were C13 and D34. Firstly the C13 was thought to contribute to the ion pair formation with the neighbouring lysine (K15) and thus a conversion of the cysteine to serine generating C13S was selected as a third mutant. The C13S mutant was hoped to

have little effect on the overall activity of the enzyme since serine residues exist in the catalytic triad mechanism, investigated greatly, in the mechanism of the serine and thiol proteases.

D34N was generated in order to identify the effect that would occur if the active site magnesium was to be dislodged. The change from an aspartic acid to an asparagine is a rather drastic mutation which would perhaps knock out the ability of the aspartic acid to form the ligand with the free magnesium.

Investigation of this series of mutants C162S, K15M, C13S expression and purification provide the basis of this chapter.

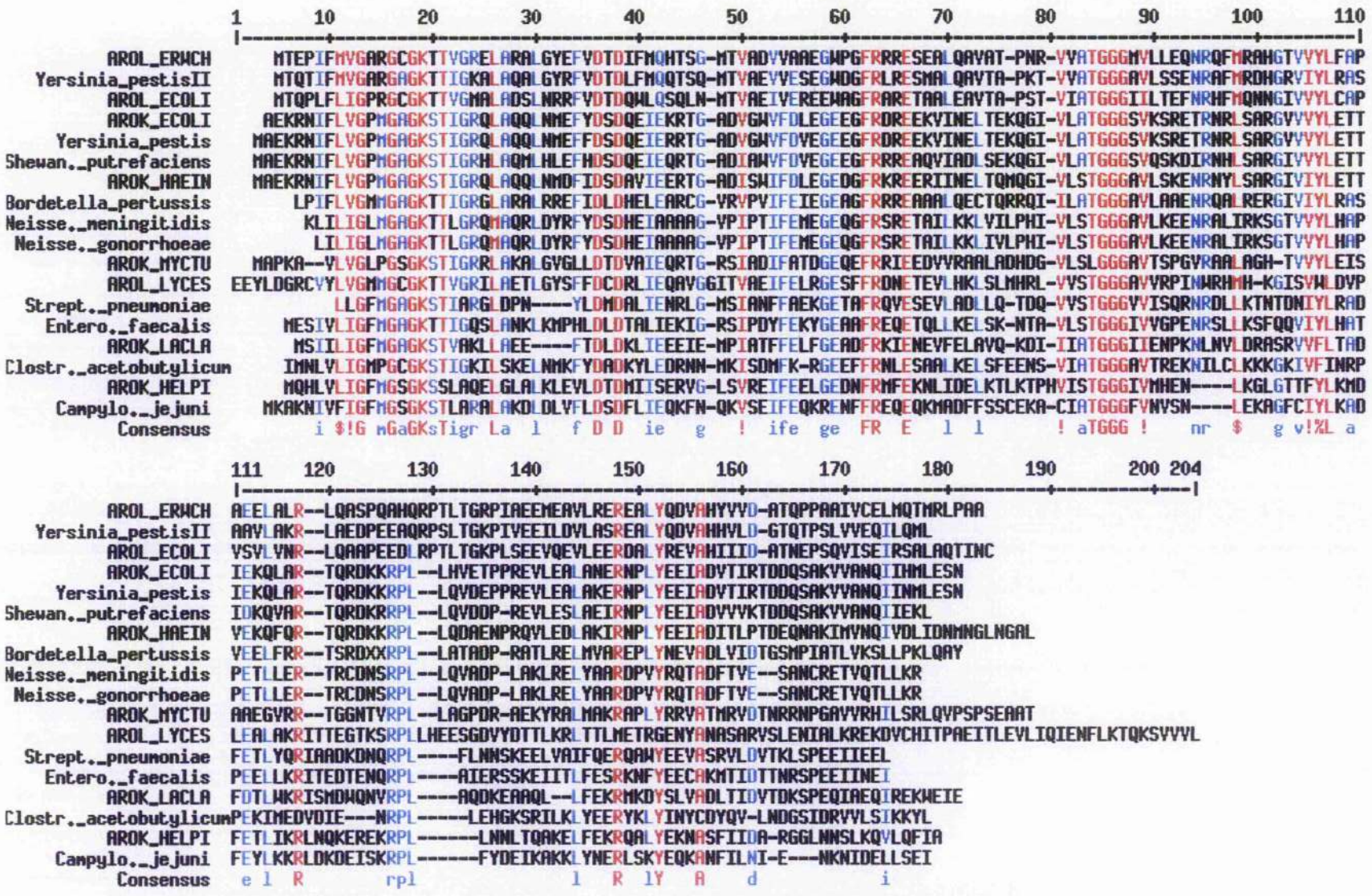


Figure 3.1 : Multi alignment of a number of shikimate kinases (I and II) highlighting conserved regions.

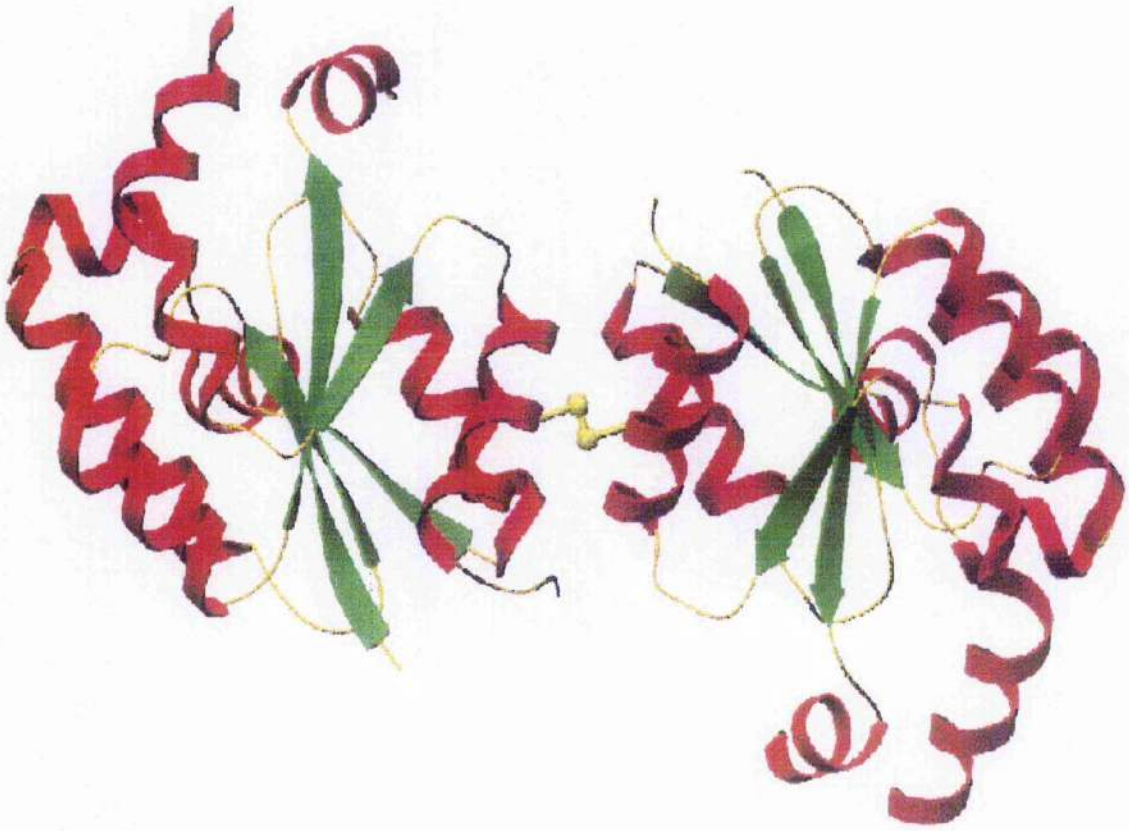


Figure 3.2: Dimer of shikimate kinase formed by the disulphide bond between the external cysteines (C162) highlighted in yellow.

3.1.3 Methods

Full information regarding the methods involved in generating the site directed mutants has been given in material and methods section 2.4.

The final mutant PCR products were cloned into pTB361 and amplified in *E. coli* DH5α. The resulting plasmid was purified and sequenced. The confirmed mutated plasmid was then used to transform *E. coli* BL21 (DE3) pLysS. Protein expression from this inducible strain was monitored.

Colonies that showed good expression were used to generate approximately 10g of cells in a large 5 litre growth. Purification then followed using the method in section 2.7. There were no major changes to the protocol for the purification of wild type and that of the mutant proteins except that during purification of the mutants K15M and D34N the fractions containing the enzyme were detected using SDS-PAGE since the enzyme activity was undetectable in the standard assay. Fractions containing the over-expressed protein were pooled and the purification protocol continued.

3.1.4 Results

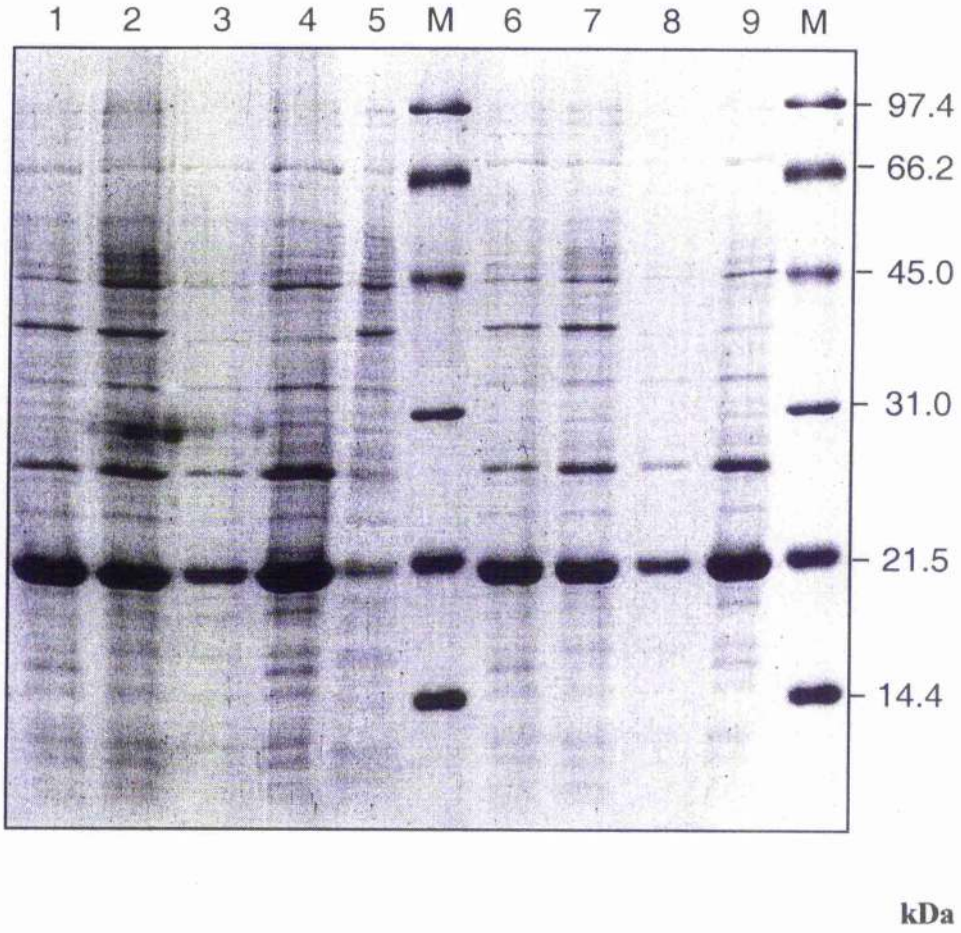


Figure 3.3 : SDS-PAGE over-expression gel showing the ability of the mutant plasmids to express shikimate kinase.

All samples were taken 4 hours after induction with IPTG and prepared as described in method and materials section 2.6.3.

Lane M : Molecular weight markers

Lane 1 and 6 : BL21 (DE3)pTB361pWT SK (15µl, 10µl)

Lane 5 : BL21 (DE3) pTB361pWT SK (5µl)

Lane 2 and 7 : BL21 (DE3)pTB361pC13S SK (15µl, 10µl)

Lane 3 and 8 : BL21 (DE3)pTB361pK15M SK (15µl, 10µl)

Lane 4 and 9 : BL21 (DE3)pTB361pC162S SK (15µl, 10µl)

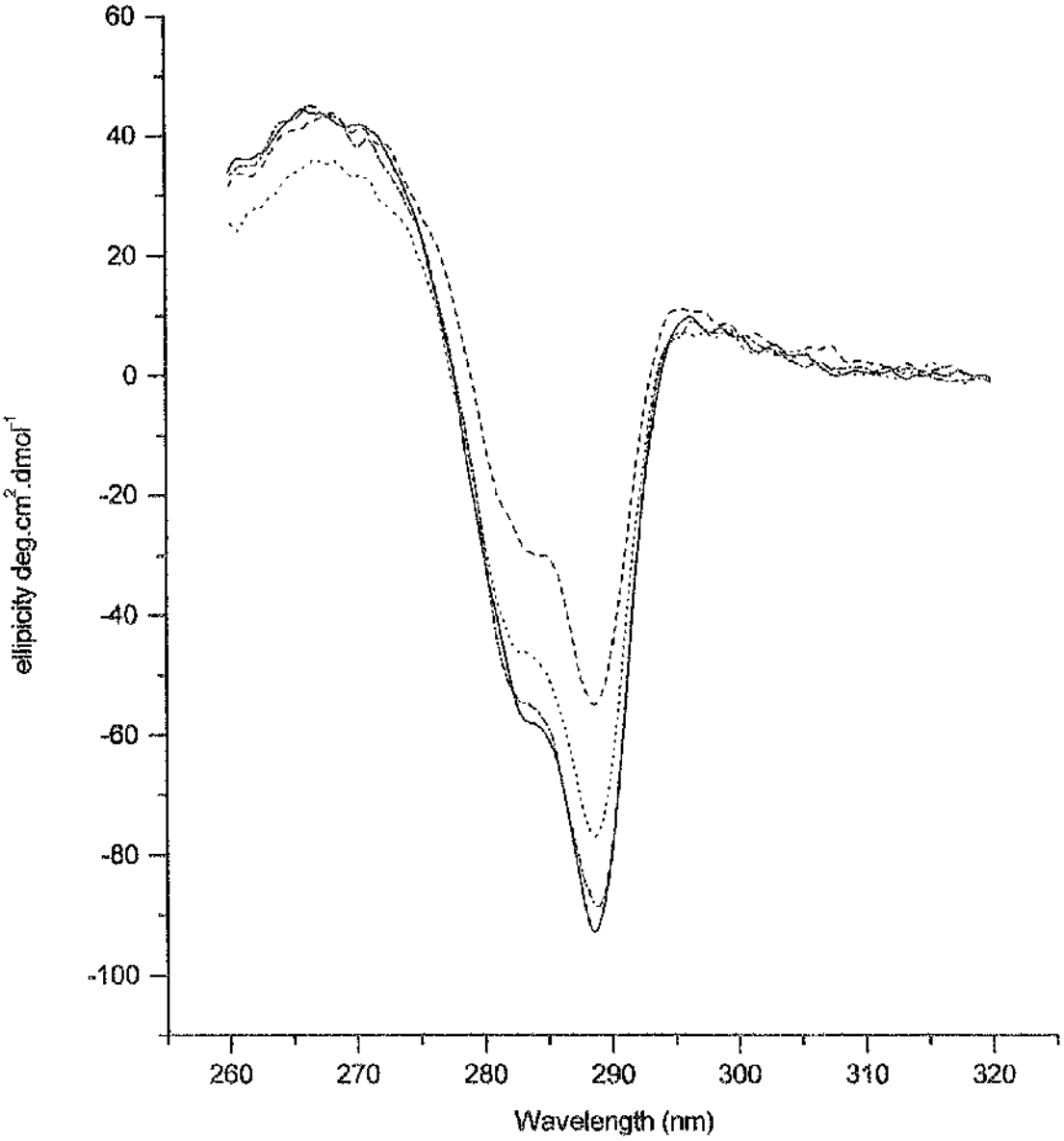


Figure 3.4 : The near UV CD spectra of shikimate kinase.
Dash ; K15M, dot; C13S, dot/dash; C162S and the solid line is the native shikimate kinase.

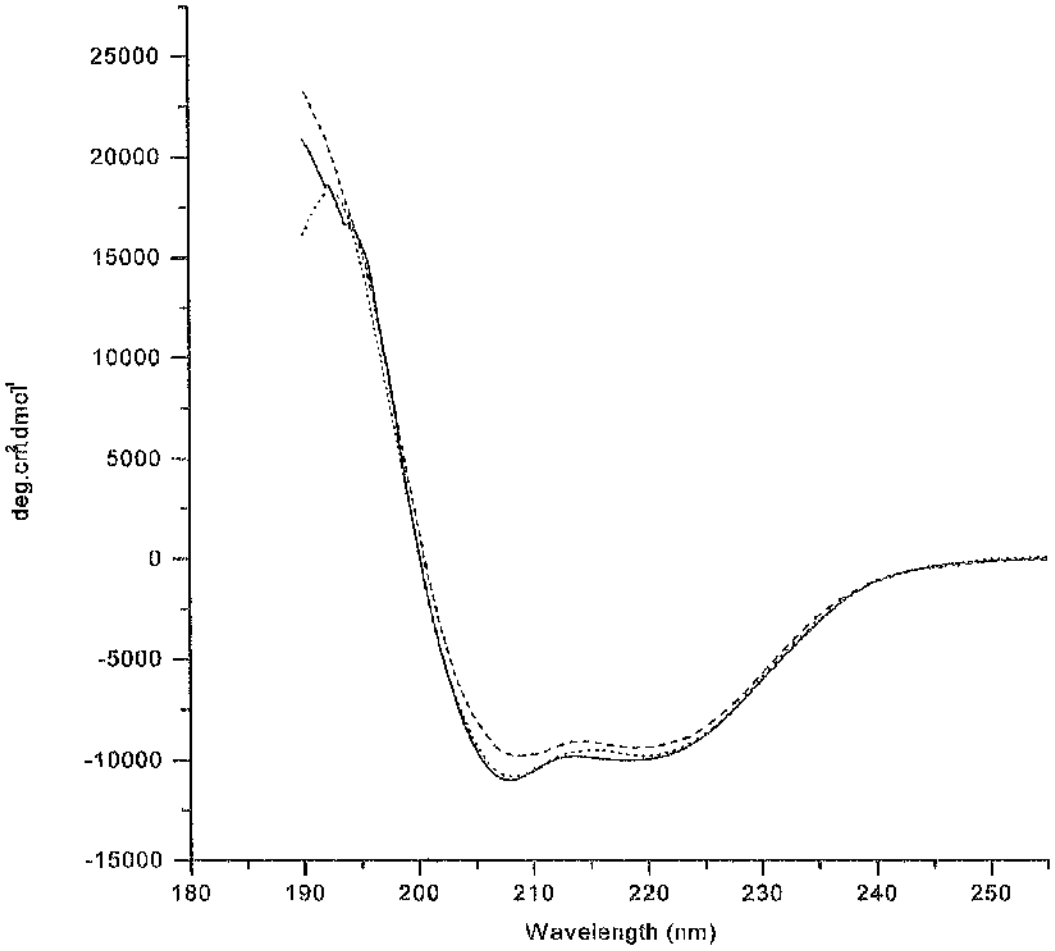


Figure 3.5 : The far UV CD spectra of the; wild-type: solid line, C13S:dot, K15M:dash shikimate kinase.

Enzyme	α-helix (%)	β-sheet (%)	remainder (%)
Wild type	29 ± 0.69	26 ± 1.2	46 ± 1.5
C162S	22 ± 2.3	40 ± 3.5	38 ± 5.3
C13S	24 ± 2.8	42 ± 4.2	35 ± 6.4
K15M	21 ± 1.3	33 ± 2.2	46 ± 2.7

Table 3.1: CONTIN analysis of secondary structure content of wild type and mutant shikimate kinases from the far UV CD.

3.1.5 Crystallisations

Prior to crystallisation the enzyme was exhaustively dialysed into 10mM Tris/HCl, pH 7.5, 0.5mM DTT and then concentrated to a concentration of 15mg/ml using Centricon-10 concentrators (Amicon, Stonehouse, Gloutershire, UK). Crystals were grown by Dr. John Maclean using varying conditions. The dead end complex containing the substrate/product mix was set up using 2.5mM of shikimate and ADP with a final concentration of 10mM $MgCl_2$. Sitting drop vapour diffusion was used to obtain the crystals with the protein 8-10mg/ml and pH readjusted to pH 7.5. 2µl of protein with equal volume of reservoir solution. Crystals of C162S mutant were obtained in 20% PEG 600, 100mM Tris pH 8.6, 2.5mM ADP, 2.5mM Shikimate, 0.5mM DTT, for K15M the resulting solution which generated crystals was 10% PEG 8000, 100mM Tris pH 8.0, 2.5mM ADP, 2.5mM Shikimate, 0.5mM DTT.

The structure of the K15M mutant was solved by molecular replacement using native shikimate kinase (Krell *et al.*, 1997). Two K15M molecules were found in the asymmetric unit which were essentially identical and these when compared to the original shikimate kinase wild type showed an almost perfect alignment (Fig. 3.6).

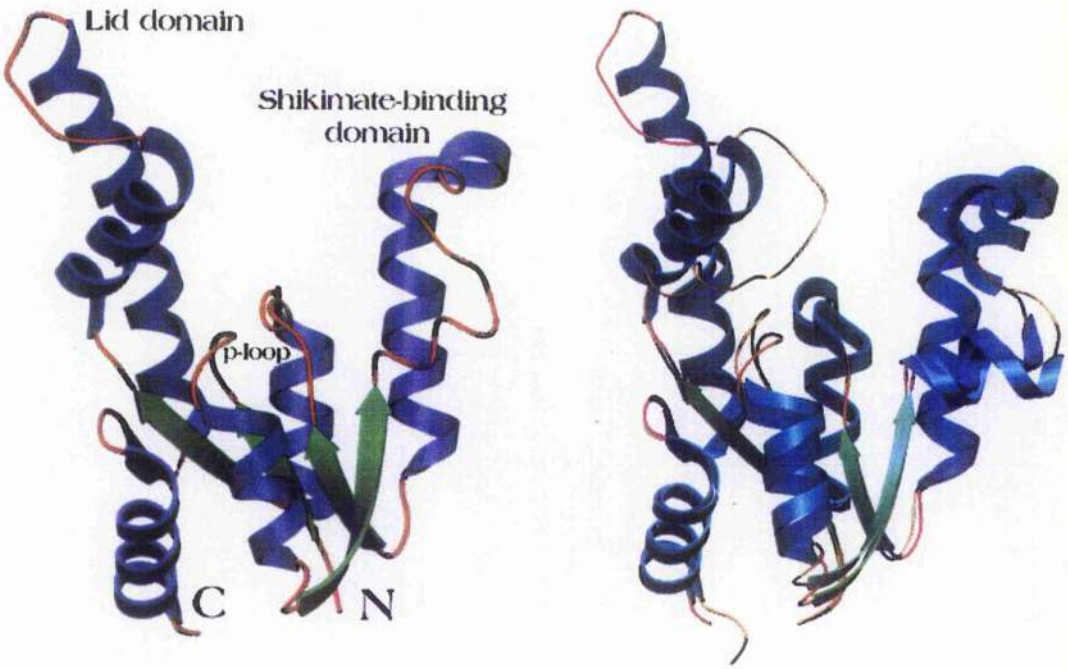


Figure 3.6 : Overlay of the K15M structure (purple) with the native (blue) shikimate kinase.

Enzyme	K_m or K_d* (Shikimate) μM	K_m or K_d* (ATP) μM	k_{cat} (sec^{-1})	k_{cat}/K_m (shikimate)
E. coli				
SK I	20,000[a]	-	-	-
SK II	200[a]	160[a]	27.1[b]	1.36×10^5
E. chrysanthemi				
wild type	307 / 700*	620 / 2 560*	34.7	1.29×10^5
C162S	282	670	40.1	1.62×10^5
C13S	75	235 / 1 530*	22.6	3.42×10^5
K15M	670*	620*	n.d.	n.d.
D34N	n.d.	n.d.	n.d.	n.d.

Table 3.2: **The kinetic parameters of wild type and mutant shikimate kinase type II from *E. chrysanthemi* compared with *E. coli* isoenzymes I and II.**

K_m values were obtained using Lineweaver Burk plots. The assay mix to measure K_m (shikimate) contained 5mM ATP and 3mM shikimate for K_m (ATP).

a) Values taken from De Feyter and Pittard (1986), b) Averaged values values taken from Millar *et al.* (1986).

*K_d values gained from fluorescence quenching experiments they represent the disscociation constants of one ligand in the absence of other ligands (see Chapter 4). n.d. not determined.

3.1.6 Discussion

The host strain *E. coli* (BL21 (DE3) pLysS) containing the mutant plasmids were able to grow and express shikimate kinase at much the same level as the wild type as seen on SDS-PAGE (Fig 3.3). The mutant enzymes could then purified as the wild type (see material and methods section 2.7). It was, however, rather difficult to isolate and purify the K15M and D34N mutants because of their very low catalytic activity. Thus purification was monitored by means of SDS-PAGE with fractions containing a band of the correct molecular mass pooled for subsequent purification. The D34N mutant was shown to be unstable except in the presence of 20% glycerol. The properties of this mutant were not investigated in detail.

Results of the tertiary and secondary structure as shown in Fig 3.4, 3.5 and Table 3.1 illustrate the overall similarity of the structures of the wild type, C13S, C162S and K15M enzymes. The CONTIN analysis, (discussed in Kelly, 1997) (Table 3.1) of the far UV structure of the mutant enzymes, varies very little in the percentage of alpha-helical structure present. This is perhaps the most important analysis from the far UV CD as it illustrates that the core protein structure is unaffected by the mutant changes. The overall values gained for the beta sheet and remainder percentages may suggest a slight perturbation of the structure but the crystallography data clearly indicates that no major change has taken place (Fig. 3.6).

The mutagenesis of C162 was based upon the premise that it was the cause of the dimerisation seen within the crystallographic unit cell. Removal of this ability therefore would hopefully result in more favourable crystallisation conditions. Although the C162S mutant was not crystallised in this work it was possible to see that the enzyme was five fold

more soluble than the wild type. Kinetic parameters of the C162S mutant were very similar to the wild type.

The C13S mutation had only a slightly reduced k_{cat} value but was shown by analysis of the steady state kinetics and fluorescence measurements to bind both substrates with a higher affinity (See Table 3.2).

In the crystal structure the aspartic acid (D34) was seen to bind the shikimate and stabilise a water molecule for co-ordination of the essential magnesium ion. Thus, a rather drastic mutation to asparagine involving a change in charge type would be expected to lead to the loss of metal ion binding and thus result in reduced capacity to bind the nucleotide.

Although crystallisation trails have been carried out with these mutants unfortunately these have not generated any crystals for diffraction.

The K15M mutant however, was crystallised in the method described in section 3.1.5. Unfortunately, in the crystallised material, it appeared that another mutation had occurred although this had not been apparent from in the earlier mass spectrometric analysis of the purified material. A proline to leucine mutation at position 116 was detected.

However, the crystal structure of the K15M mutant is essentially super-imposable on that of the wild type showing only slight perturbation around the P-Loop where the methionine has caused the arginine 110 in the lid domain to move 2.7 Angstroms towards it. Another ion pair formed between Asp32 and Arg11 can be seen in the K15M mutant may underlie the increased stability that is observed in this mutant. This is discussed in greater detail in chapter 5.

Chapter 4 Fluorescence studies on shikimate kinase

4.1 Use of fluorescence quenching to study the environment and mobility of the tryptophan in shikimate kinase II from *Erwinia chrysanthemi.*

4.1.1 Abstract

The quenching agents NaI and succinimide were used to quench the intrinsic fluorescence of the single tryptophan that exists within shikimate kinase (W54). When comparing the two sets of quenching data it became apparent that an increase in the Stern-Volmer constant (K_{SV}) and thus the ability of the NaI to quench the tryptophan fluorescence, compared with the neutral compound succinimide, was due to the presence of three positively charged arginines within a distance of 0.5-1.1 nm of the tryptophan.

4.1.2 Introduction

The degree of fluorescence quenching of tryptophan residues by various reagents has been exploited to yield valuable information about the conformation of proteins (Freifelder, 1976).

The general mechanism for the quenching of an excited state luminescent state A* by quencher Q is ;

$A^* + Q \rightarrow A^*\text{---}Q \rightarrow A + Q + \Delta$

The quenching of the tryptophan (fluorophore) is dependent on its exposure to the quencher and the environment in which it resides. Conformational changes that occur due to the binding of ligands may increase or decrease the exposure of the tryptophan and thus result in a change in the fluorescence (Eftink and Ghiron, 1976). The accessibility of the

fluorophore to the solvent is indicated by the magnitude of the Stern-Volmer constant (K_{SV}) value (shown overleaf).

$$\frac{Fl}{Fl_{exp}(V\,[Q])} = 1 + K_{SV}\,[Q]$$

Where Fl is the original fluorescence value, Fl_{exp} is the recorded fluorescence after each aliquot of quencher, V is volume of quencher, [Q] is the concentration of quencher and K_{SV} is the Stern Volmer constant for the dynamic quenching process.

Sodium iodide (NaI), acrylamide and succinimide have all been used as quenching agents (Eftink and Ghiron, 1976). NaI itself is a polar compound, acrylamide and succinimide are neutral compounds, succinimide being about 20% larger than the acrylamide as calculated from Van der Waals atomic increments. By calculating the K_{SV} ratios measured from quenching studies using succinimide and acrylamide, the exposure of the tryptophan can be assessed (Eftink and Ghiron, 1984).

4.1.3 Methods

Shikimate kinase was dialysed for 2 hours in Buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$, 0.4mM DTT pH 7.5) to a concentration of about 1.5-2 mg/ml.

The final concentration of enzyme used in each quenching experiment was 0.2mg/ml.

Stock concentrations of quencher; NaI, succinimide and acrylamide were made up at 2M in the appropriate buffer.

Fluorescence measurements were carried out as described in the material and methods (Section 2.8.3) using a Perkin Elmer LS50B spectrofluorimeter. Excitation wavelength of 290 nm and emission wavelength of 350nm were used. The values were obtained from the READ mode setting and collected every 5 seconds after the addition of 10µl of the 2M stock concentration of quencher. The values were corrected for the effect of dilution and then used to construct the Stern-Volmer plot (F_o / F_{corr} vs. [Q]). The slopes of this plot gives the Stern-Volmer constant (K_{sv}).

4.1.4 Results/Discussion

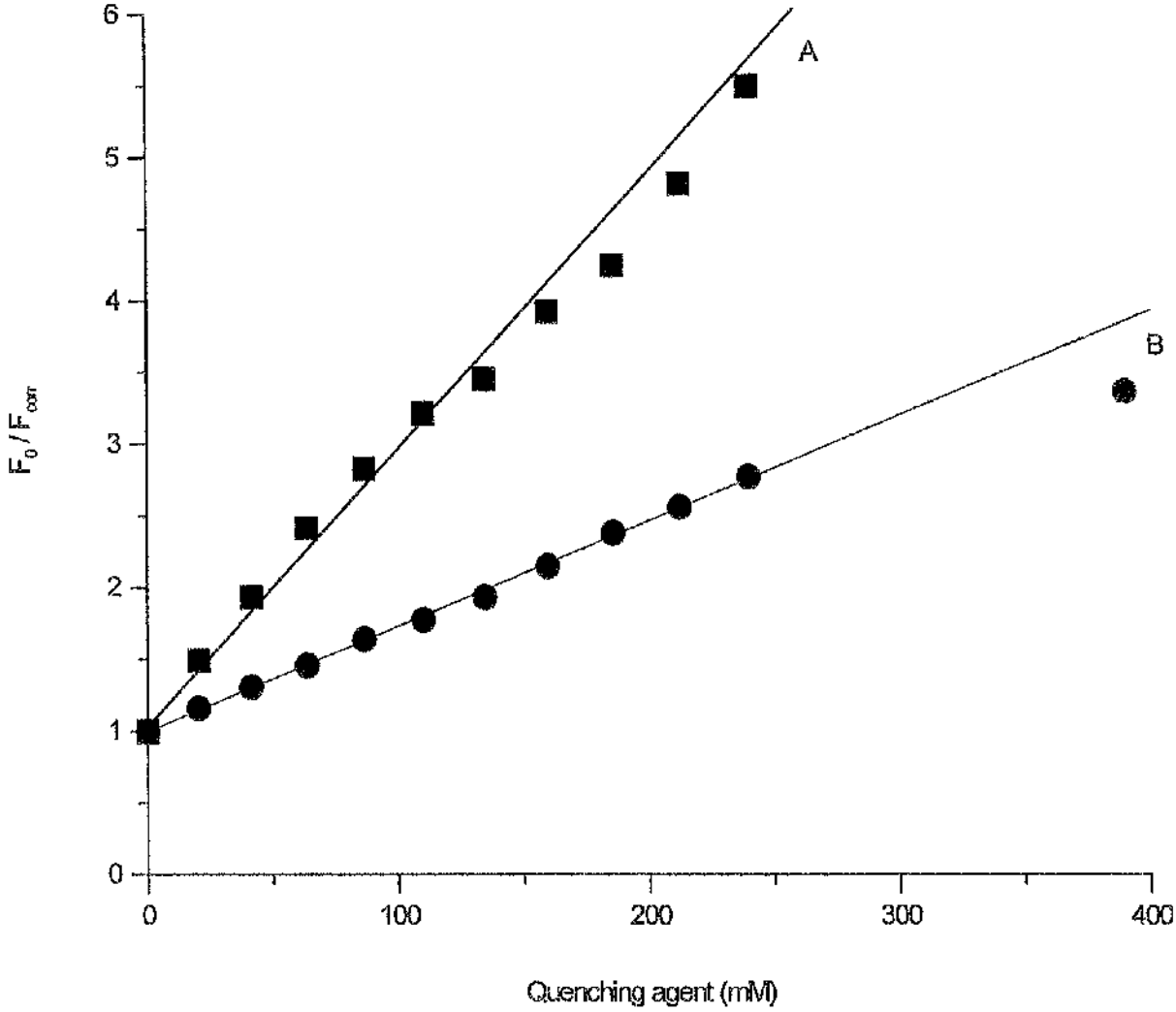


Figure 4.1: **Stern-Volmer plot showing the quenching of shikimate kinase by NaI (A) and succinimide (B).**

Stern-Volmer constants (K_{SV}) of 18.68 M^{-1} and 6.56 M^{-1} respectively.

Enzyme	NaI (2M)* (K_{SV} M^{-1})	Succinimide(2M)* (K_{SV} M^{-1})	Acrylamide(2M)* (K_{SV} M^{-1})
E. chrysanthemi SKII			
wild type (wt)	18.68	6.56	9.51
wt + ADP (2mM)	15.42	5.63	8.70
wt + shikimate (2mM)	5.49	3.87	5.82
wt +ADP + shikimate (2mM)	3.88	3.09	3.56

Table 4.1: The calculated mean Stern Volmer constants (M^{-1}) for the fluorescence quenching of shikimate kinase fluorescence in the absence and presence of ligands.

* Quenching agent used

The data shown above in Table 4.1 illustrate a common trend in the K_{SV} values obtained with each quenching agent in the presence of added ligands. This trend represents a decrease in K_{SV} indicating that the tryptophan is becoming less accessible to the quenching agent. The magnitude of K_{SV} for succinimide in the absence of ligands indicates that the tryptophan is quite highly exposed to the solvent (Idziak *et al.*, 1997). This conclusion is consistent with the value of the fluorescence emission maximum (350nm) which is close to that of tryptophan model compounds (356nm). There is however a relatively small difference between the wild type alone and wild type in the presence of 2mM ADP, thus indicating that the nucleotide brings about little if any structural change in relation to the position of the tryptophan.

However in the presence of 2mM shikimate the K_{SV} is reduced considerably thus indicating that the binding of shikimate causes a significant change in the environment of the tryptophan indicating that it has become significantly less exposed to the solvent.

There is also a change in the fluorescence emission maximum of the enzyme from 350nm to 345nm in the presence of 2mM shikimate as seen in standard fluorescence scans, in addition the circular dichroism spectra of the enzyme in the presence of shikimate also indicate some secondary structure changes (data not shown). In the dead end complex (ADP-shikimate) an enhanced effect is seen and the K_{SV} values is at its lowest value ($3.09M^{-1}$ for succinimide) indicating that the tryptophan is at its least accessible. It is interesting to note the fact that the NaI in the above case has the greatest Stern-Volmer constant and this in fact is higher than that seen with the model compound N-acetyltryptophan amide (NATA). The reason for this was apparent from inspection of the crystal structure (Krell *et al.*,1998). As can be seen in Figure 4.2 there are three positively charged arginine residues within 10nm of the tryptophan residue which clearly attract the negatively charged iodide ion leading to enhanced quenching.

The three arginine residues (Arg 58 (5nm), Arg 11 (9nm), Arg 139(10nm); distances from the tryptophan residue are mentioned in brackets) surrounding the tryptophan (W54) generate the electrostatic density depicted in the upper right red region in Fig 4.2.

The removal of the positively charged environment provided by the arginines however can be brought about by the perturbation of structure and one way to do this is by introducing a denaturing agent. Addition of 2M guanidinium chloride (GdnHCl) a chaotropic agent was used to disrupt the protein structure. When the quenching experiment was carried out in the presence of 2M GdnHCl the resulting Stern-Volmer constant was reduced to 5.80 M^{-1} in the case for NaI, a value similar to that observed for the titration with succinimide.

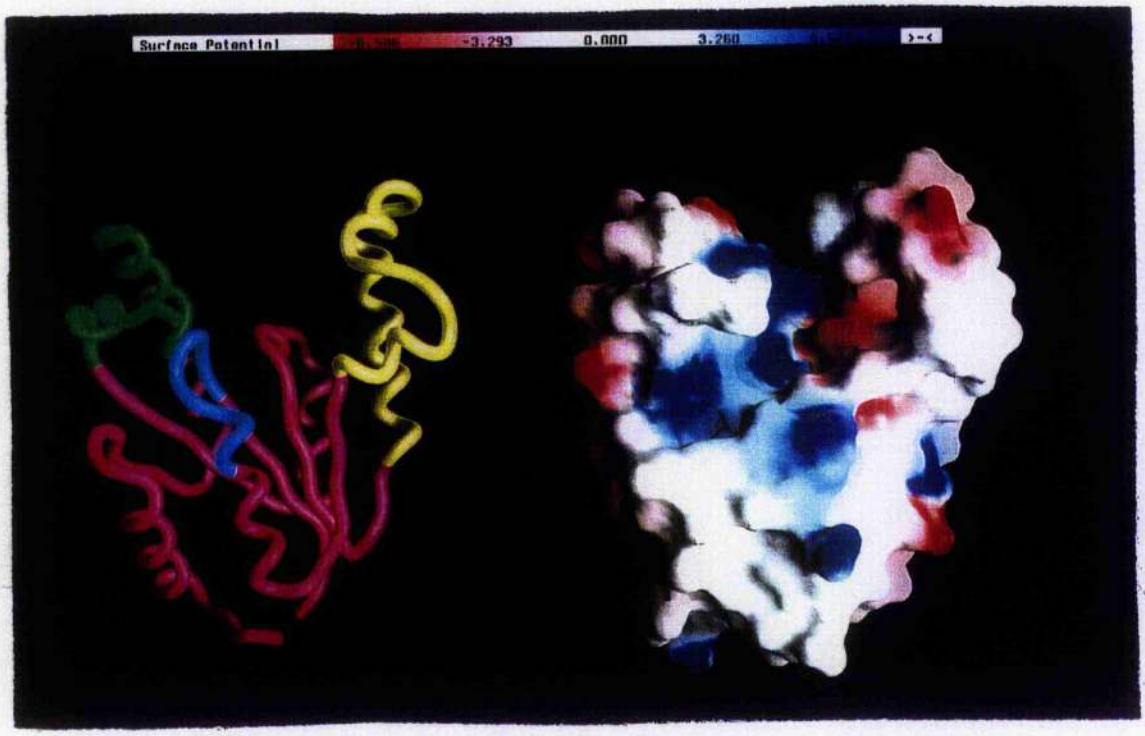


Figure 4.2 : Electrostatic surface representation of shikimate kinase.

Blue and red regions represent positive and negative potential. Surface charge was calculated using GRASP (Gourley *et al.*, 1994)

The positively charged environment around W54, is illustrated as the upper red region on the right lobe (A more precise location of the W54 was given in the introduction section Figure 1.7). The positive potential in this region clearly accounts for the enhanced quenching seen with NaI. This agent had been originally thought to be only able to quench completely exposed tryptophan residues due to the fact that NaI is hydrated and charged (Eftink and Ghiron, 1976) however these results have proved otherwise and in fact the NaI is able to quench, more effectively, the tryptophan which lies in a positively charged environment. More detailed analysis on model compound with tryptophan located in a positively charged region may prove interesting regarding this finding.

4.2 Binding of substrates to shikimate kinase

4.2.1 Abstract

Shikimate kinase undergoes conformational changes upon substrate binding; this is an example of 'induced fit' which was first recognized by Koshland (1959).

The conformational changes can be exploited to monitor the binding of substrate. For instance, binding of shikimate leads to substantial quenching of the fluorescence of shikimate kinase. From the titration of the enzyme with shikimate, the value of K_d can be derived as 750μM. This is somewhat higher than the apparent K_m (300μM) for this substrate derived from an analysis of steady state kinetics. In the presence of nucleotide (ADP) the fluorescence titration shows that the K_d for shikimate is reduced to 320μM, a value close to the value obtained from kinetics. The reduction in K_d value is consistent with the proposal of substrate synergism.

Quenching of the fluorescence by shikimate alone gave rise to a binding curve which fitted well to a hyperbolic binding model. However the binding of nucleotide monitored by fluorescence quenching was rather different and could not be fitted to a hyperbolic model. The data however fitted moderately well to the Hill equation with a Hill co-efficient of 2.66 for ATP binding and 1.58 for ADP binding.

Since the binding of nucleotides, monitored by Isothermal titration calorimetry (ITC) gave binding affinities resulting in K_d values of the order of 25-40μM then a possible explanation for this behaviour and the previous result gained for the Hill equation could be the presence of a second nucleotide binding site.

4.2.2 Introduction

Substrates upon binding may cause structure alterations in the protein of study for instance the induced fit noted by a number of studies dating back to 1950s (Koshland, 1959; Yankeelov and Koshland, 1965).

The presence of the single tryptophan in shikimate kinase provides a convenient probe for monitoring the structural changes which occur on binding substrates. This information can be compared with that available on the homologous enzyme adenylate kinase. Examples of engineering tryptophans within adenylate kinase from *E. coli* has been shown in Bilderback *et al.*, (1996).

The elucidation of crystal structure of adenylate kinase has made it is possible to see the induced fit movement on an atomic level and this has been well documented in a number of studies (Schulz *et al.*, 1990; Gerstain; 1993; Muller *et al.*, 1996 and Sinev *et al.*, 1996ab).

A movie generated from various liganded forms of adenylate kinase can be found on the internet;

http://bioinfo.mbb.yale.edu/MolMovDB/db/movie

4.2.3 Methods

Substrates, shikimate, ADP and ATP (10mM and 50mM stock solutions) were made up in buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$, 0.4mM DTT pH 7.5) and the pH adjusted if necessary to pH 7.5. Shikimate kinase was dialyzed against buffer A for two hours and then used at a concentration of 0.2mg/ml. Titrations were carried out in a similar way as described in section 4.1 above using the READ mode setting on the spectrofluorimeter. Substrate solutions rather than quenching agent stocks were used; generally 10 x 10µl aliquots of 10mM stock solution followed by 5x 10µl aliquot additions of the 50mM substrate stock solution.

It was necessary to use the model compound NATA to correct the titration data for any possible inner filter effects and for dilution caused by addition of ligand.

Data collected were then analysed by the program 'Leonora' (Cornish-Bowden 1996) which fitted data to a hyperbolic saturation curve giving the K_d and the limiting degree of fluorescence quenching. Alternatively the data could be analyzed by means of a double reciprocal plot (Lineweaver Burk plot) as shown in Fig 4.5.

4.2.4 Results/Discussion

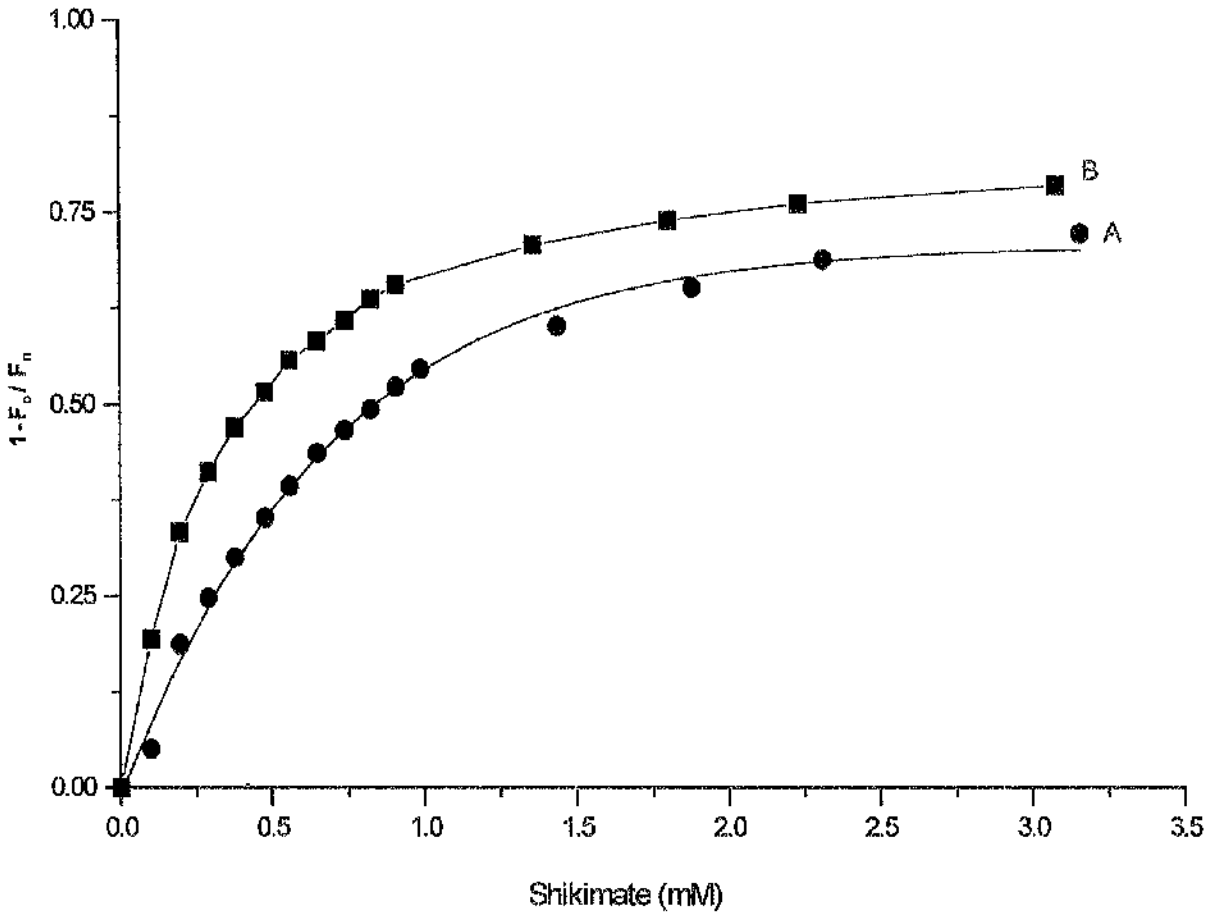


Figure 4.4 : **The binding of shikimate to shikimate kinase as monitored by fluorescence quenching.**

A typical Michaelis-Menton curve is obtained after titration of shikimate upon shikimate kinase.

A and B represent data obtained in the absence and presence of ADP (1.5mM) respectively.

$1-F_o/F_n$ is the resulting degree of quenching when corrected for dilution and filter effects using the model compound NATA.

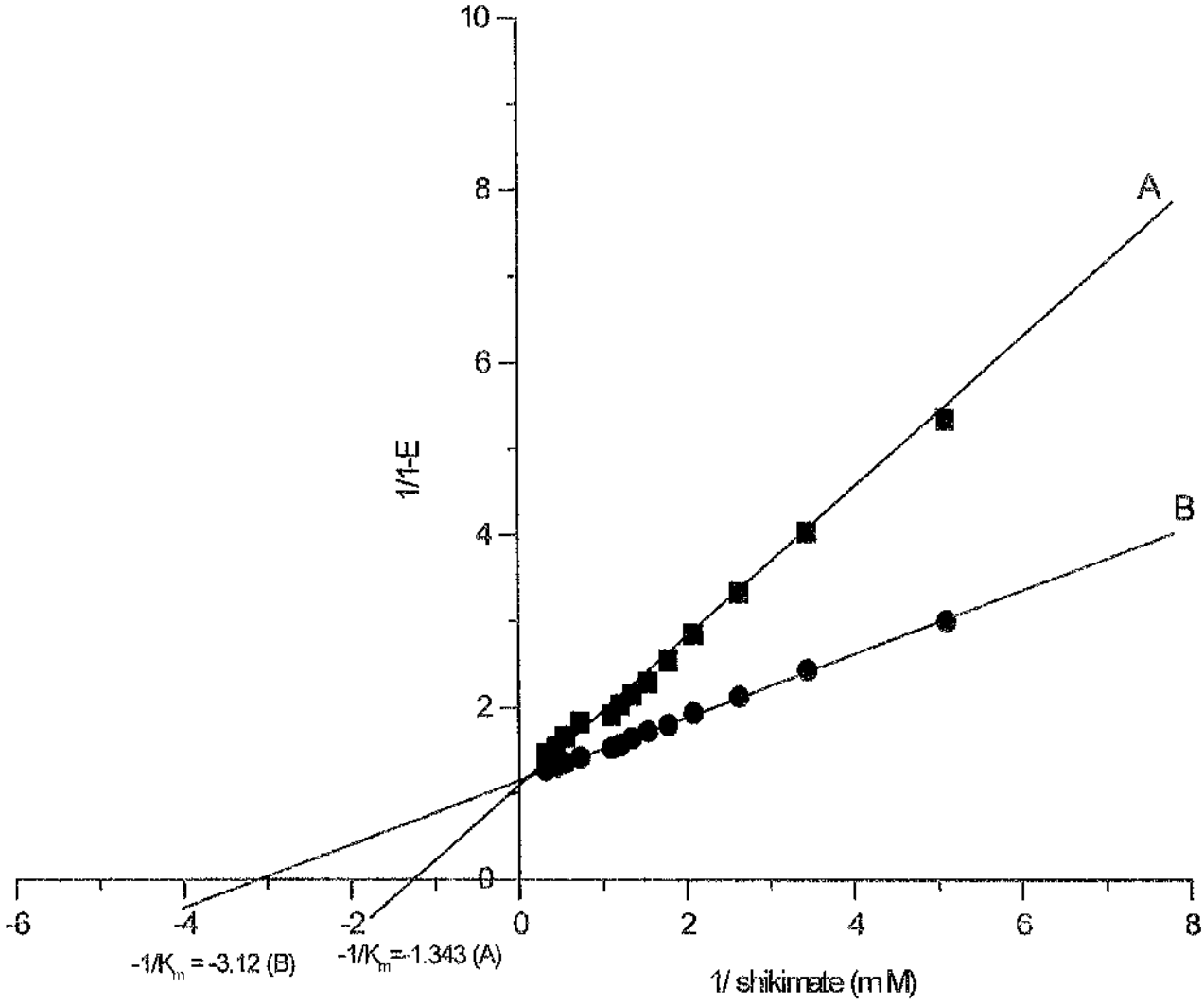


Figure 4.5: Double reciprocal plot showing the differences in K_d values for shikimate obtained in the absence (A) and presence (B) of ADP (1.5mM).

K_d values were 745μM and 320 μM respectively.

The K_d values obtained for the binding of shikimate to shikimate kinase were similar to those obtained in steady state kinetics. The dissociation constant in the absence of nucleotide was 700 μM; this is reduced in the presence of 1.5mM ADP to 320μM which is comparable to the K_m value obtained from an analysis of steady state kinetic data. The two fold increase in affinity of the enzyme for shikimate in the presence of nucleotide is consistent with the proposal of substrate synergism. Substrate induced structural changes which underlie such synergism have been well documented in a number of kinases.

Phosphoglycerate kinase has been observed to undergo a hinge motion with a positional displacement of 2.7nm for the ends of the domains relative to the unliganded structure (Bernstein *et al.*, 1997), complete hinge closure for this enzyme has been brought about by the synergistic combination of both substrates. Adenylate kinase has been studied in much greater detail and detailed structures of differently liganded forms the enzyme have been combined together to form a movie of ligand binding (Schulz *et al.*, 1990; Gerstain, 1993; Muller *et al.* 1996; Sinev *et al.* 1996ab). Movement of the hinge domain within adenylate kinase takes place with a displacement of up to 3.0nm.

This movement, correlated with the results shown above, illustrates that where the tryptophan is located, between helix three and four of shikimate kinase, there must be a degree of flexibility. This 'degree' of flexibility however could only be quantitatively resolved by much more detailed work using actual fluorescence energy transfer experiments (Bilderback *et al.*,1996) or the ability to crystallize a number of differently bound states of shikimate kinase as has been done with adenylate kinase.

Analysis of the binding of the nucleotide, ADP, to shikimate kinase proved to be more complex.

It was found that the fluorescence quenching data would not fit firstly to a simple hyperbolic curve, and since consequently the double reciprocal (Lineweaver Burk) plot was not linear (Fig 4.6) the binding data could be fitted to a Hill plot (Fig 4.7a,b) from which it was possible to estimate a Hill coefficient.

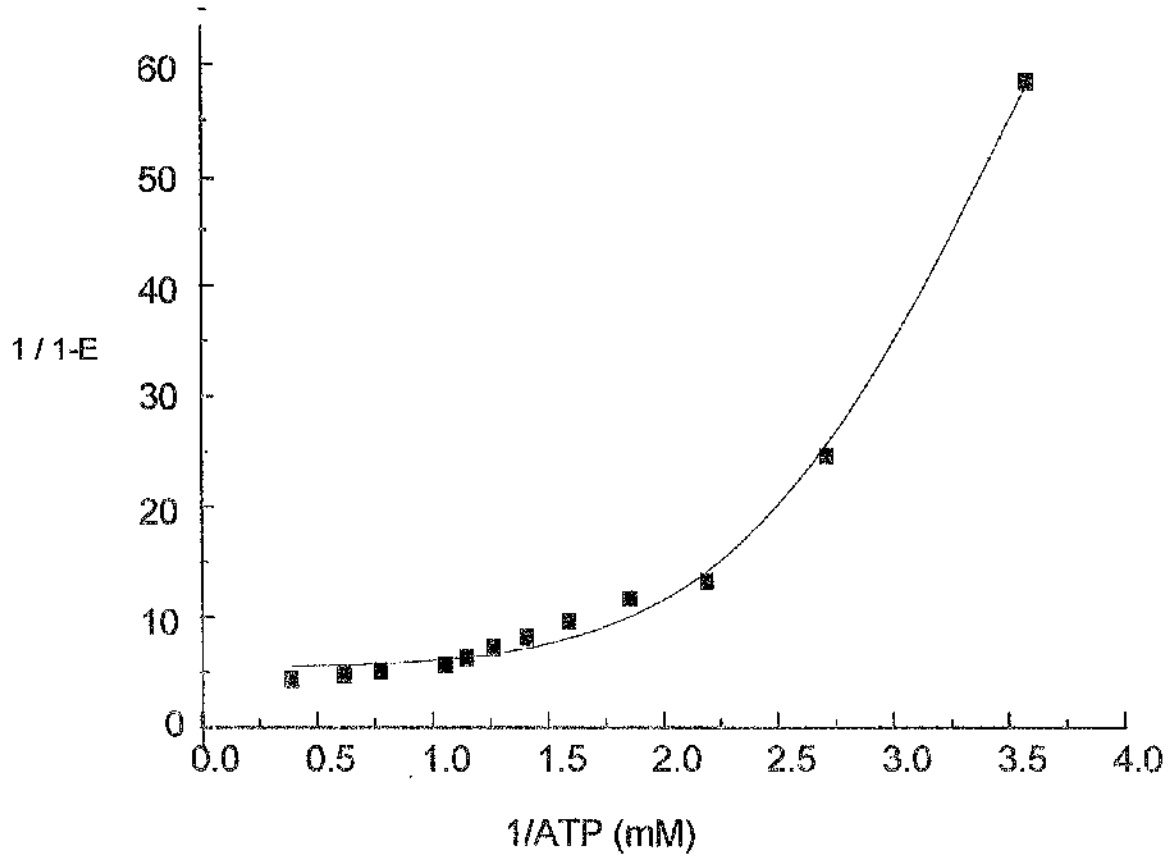


Figure 4.6: Double reciprocal plot of the fluorescence quenching of shikimate kinase by ATP.

Illustration that the binding is of a non-hyperbolic type.

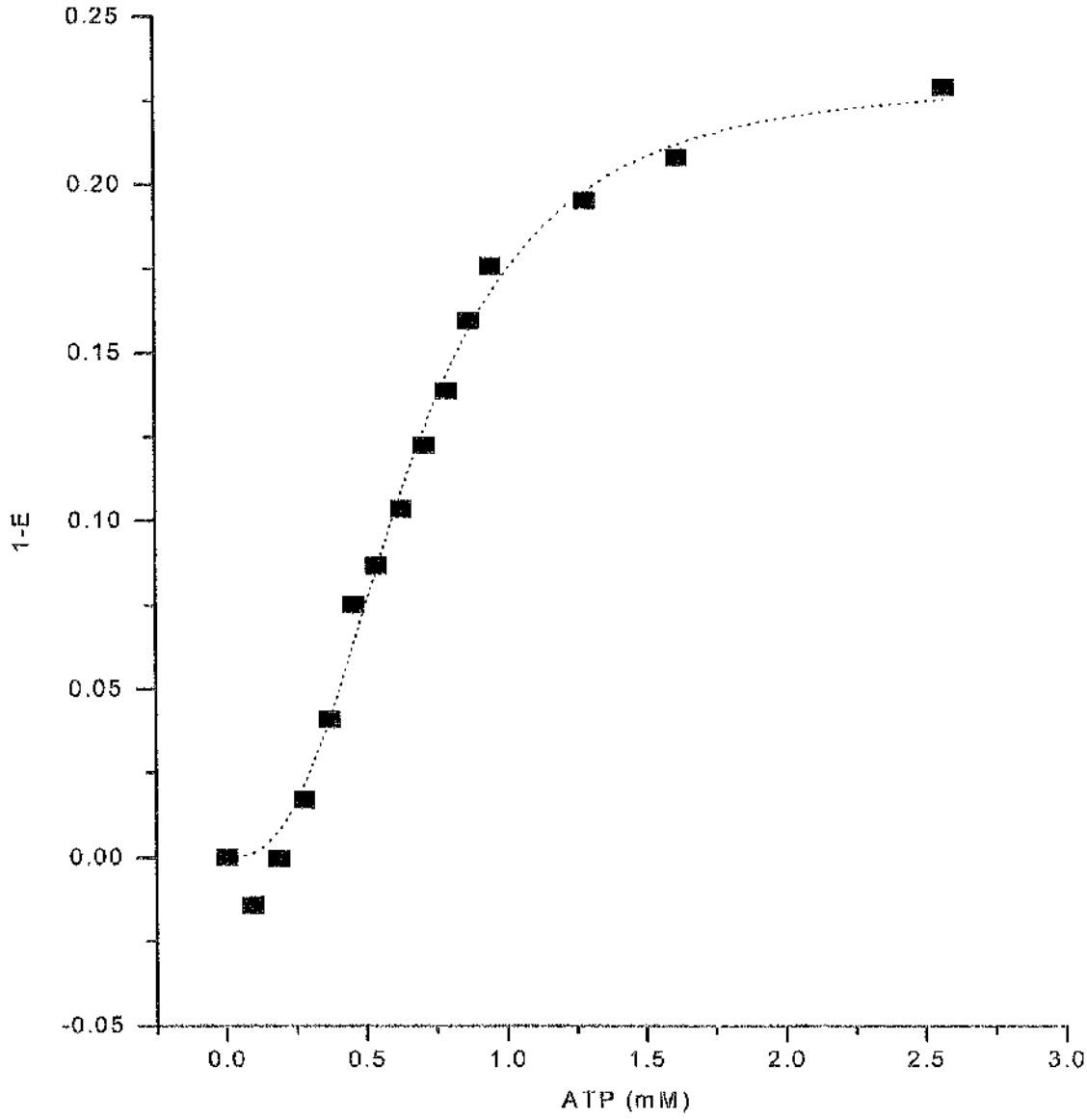


Figure 4.7a : Data for the ATP quenching of shikimate kinase fluorescence fitted to the Hill equation.

The parameters obtained from the fitting process were ;

Limiting value of (1-E)	=	23.1± 0.007%
Concentration at half saturation	=	658± 0.023μM
Hill co-efficient, h	=	2.66 ± 0.21

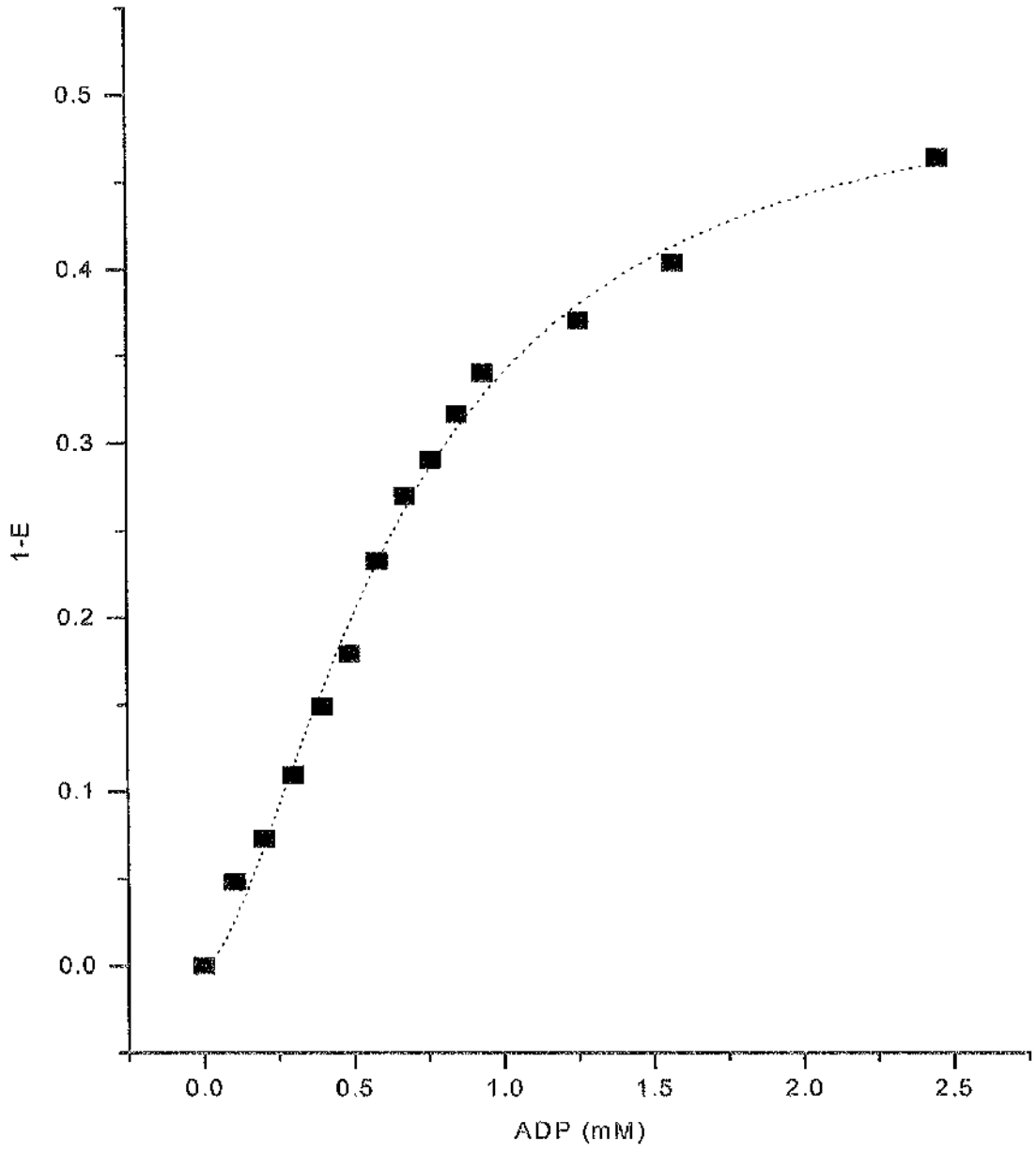


Figure 4.7b : Data for the ADP quenching of shikimate kinase fluorescence fitted to the Hill equation.

The parameters obtained from the fitting process were ;

Limiting value of (1-E)	=	52.0± 0.0235%
Concentration at half saturation	=	669± 0.047μM
Hill co-efficient, h	=	1.579 ± 0.115

The kinetic data for the apparent nucleotide binding gives a K_m of 700 μM. This is then comparable to the data that are obtained from the Hill plot in both 4a and 4b where the concentrations at half saturation are 658μM and 660μM for ATP and ADP respectively.

The nucleotide titrations clearly do not fit a simple hyperbolic curve and consequently the data fitted to the Hill plot suggest some degree of co-operativity in nucleotide binding . The Hill co-efficient gained in each of the above mentioned cases is 2.66 for ATP and 1.58 for ADP.

This demonstration of co-operativity indicates that there may be one or more additional binding sites for nucleotide in shikimate kinase.

Further experiments in this chapter used isothermal titration calorimetry (ITC) as another means of measuring substrate binding.

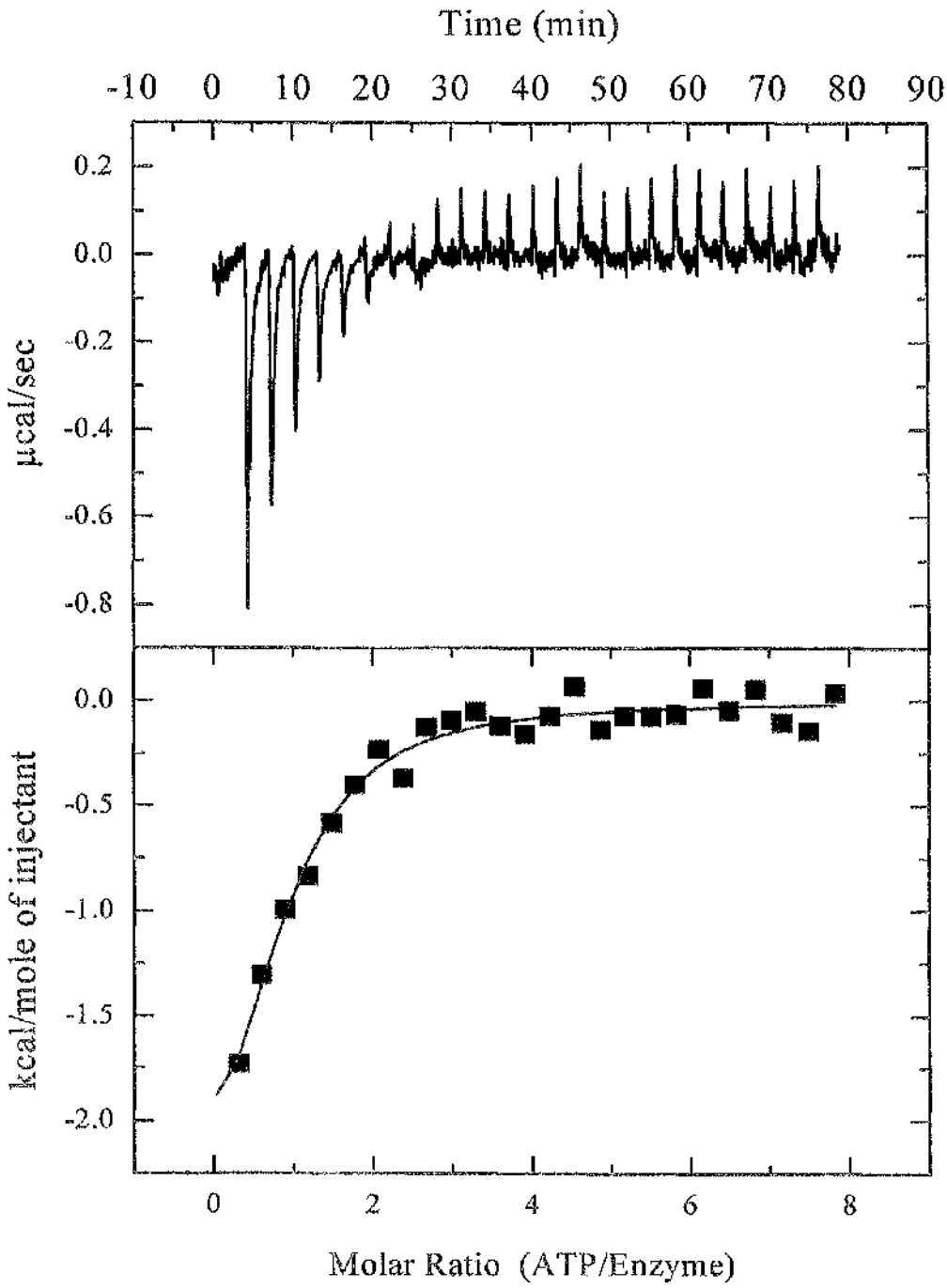


Figure 4.8 : The binding of ATP to shikimate kinase (wild type) as monitored by isothermal titration calorimetry.

Enzyme	Number of sites	Dissociation constant (μM)	Enthalpy (cal/mol)
E. chrysanthemi (SK II)			
wild type + ATP	0.83	24.3	2923
	0.66	22.5	3422
wild type + ADP	1.12	34.8	3124
C13S mutant + ATP	0.60	43.3	2394

Table 4.2 : Nucleotide binding to shikimate kinase type II from *E. chrysanthemi* as monitored by isothermal titration calorimetry.

The ITC data shown above indicate that ATP binding is much tighter (23μM) than is suggested by the apparent K_m (700 μM) derived from an analysis of kinetics. The ITC traces appear to fit in a satisfactory manner to a one site model, which would conflict with the conclusion reached from the fluorescence quenching data (Fig 4.7).

Problems were encountered with shikimate titrations in ITC; when using the same initial concentration of shikimate (2mM stock solution). Titrations with the shikimate from this stock gave no reasonable binding curve. High shikimate concentrations (up to 50mM) gave rise to a high signal to noise ratio. Thus the values derived from the fluorescence quenching are the most useful to comment on the shikimate binding (Fig 4.4).

The two methods used give very different results for the binding of nucleotides to the enzyme. In principle the ITC method should give the more reliable data since it is based on the enthalpy changes which accompany complex formation. The values for K_d obtained by

ITC are significantly lower than either the apparent K_m from analysis of steady state kinetics or the concentration range over which fluorescence is observed. Since addition of nucleotides leads to trivial quenching of protein fluorescence by an inner filter effect (Price, 1972), it is necessary to correct for this by performing parallel titrations of the model compound, NATA, with the nucleotide. When these corrections have been carried out the actual degree of quenching of shikimate kinase fluorescence is quite small, up to about 23%, Figure 4.7a. Although this type of method has been used successfully to analyse the binding of nucleotides to various kinases (Price, 1972) it is possible that the method may not be appropriate in the case of shikimate kinase. To investigate this possibility titrations should be performed with proteins such as lysozyme or bovine serum albumin which do not bind nucleotides. Shikimate kinase may indeed possess two binding sites for nucleotides, a high affinity site which does not lead to fluorescence quenching but which can be detected by ITC and a low affinity site, binding which is accompanied by a small enthalpy change but by significant fluorescence quenching. Final resolution of the situation will only be possible when the binding is analyzed by a direct binding method such as equilibrium dialysis.

Chapter 5 : Chemical and thermal denaturation of shikimate kinase type II from *Erwinia chrysanthemi.*

5.1 Abstract

The unfolding of shikimate kinase was studied both using both chemical (increasing concentrations of guanidinium chloride (GdnHCl) and thermal denaturation. Chemical denaturation was measured by the loss of secondary structure, as monitored by far UV Circular Dichroism (CD) and measuring the loss of tertiary structure as monitored by fluorescence changes.

Shikimate kinase was denatured both in the absence and presence of the substrate shikimate and ATP. Thermal denaturation was observed by the use of Differential Scanning Calorimetry (DSC) and the effects of ligands on the stability of the enzyme were judged by changes in T_m and the thermodynamic parameters (ΔH_{cal} and $\Delta H_{v'H}$). Thermal denaturation was also monitored by changes in the secondary structure, using far UV CD.

The concentration of GdnHCl which gave 50% denaturation was shown to be 1.25M and the total loss of structure was seen at 4M GdnHCl.

Thermal stability studies showed that the general temperature of 'unfolding' and collapse of structure was seen at 39°C. This unfolding temperature was increased in the presence of saturating concentrations of the nucleotide substrates ATP and ADP (2mM) to 47°C. No dramatic increase is seen in the presence of shikimate at 2mM.

It became apparent when monitoring the chemical denaturation in the presence of substrate (shikimate) by fluorescence that a two stage unfolding occured. This was illustrated by the loss of shikimate binding capability between 0 and 1M GdnHCl prior to the total unfolding of the enzyme. These observations can be interpreted as indicating that low concentrations of GdnHCl gave rise to small perturbations within the structure which lead to the disruption of the shikimate binding site.

5.2 Introduction

A wide variety of biophysical techniques are used for the study of protein denaturation including circular dichroism, fluorescence and differential scanning microcalorimetry. These have provided powerful ways to follow the characteristics of intermediates that occur within the protein folding pathway.

Studies on the denaturation of adenylate kinase by GdnHCl have been undertaken (Zhang, *et al.* 1993, 1998). Denaturation studies of proteins using DSC have also been well documented (Cooper *et al.* 1999). DSC is a very accurate way of determining the thermal transition and can be used to determine the absolute thermodynamic data from thermally induced transitions. The technique has been used to show how thermal stability can be affected by experimental conditions; for example glucose will protect glucokinase against both thermal and proteolytic degradation (White *et al.,* 1990). The effect of buffer composition used during DSC has been investigated in particular looking at the stabilisation that Zn^{2+} offers carboxypeptidase B (Conejero-Lara *et al.,* 1991), and the scan rate utilised has been shown to affect the denaturation of yeast phosphoglycerate kinase (Galisteo *et al.,* 1991). The formation of enzyme ligand complex via a Schiff base which is part of the catalytic mechanism of dehydroquinase type I has been shown to have great effects on the stability of the enzyme (Kleanthous *et al.,* 1991).

The unfolding of the adenylate kinase and the shikimate kinase can be compared since both are monomeric kinase enzymes of similar molecular mass and with similar overall structure.

5.3 Methods

Shikimate kinase was prepared as described in Section 2.7. The enzyme was dialysed against two changes of Buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$, 0.4mM DTT pH 7.6). Incubation in GdnHCl (made up in buffer A) was carried out for 1-2 hours. All measurements were made at 20°C; for fluorescence, measurements were made using a Perkin Elmer L550 spectrofluorimeter in 1ml cuvettes. CD spectra were recorded on a JASCO J-600 spectropolarimeter using a 0.05cm pathlength cell. Fluorescence scans were carried out on each sample using the excitation wavelength of 290nm and emission at 350nm using slit widths of 2.5nm bandpass adjusted to cater for different protein concentrations. For each sample the wavelength of maximum emission (λmax) and the intensity of fluorescence at 347nm were recorded. From the changes in these parameters the % change in structure at each concentration of GdnHCl could be calculated. For the DSC measurements the enzyme was also extensively dialysed against buffer A. Enzyme concentration was adjusted to 1mg/ml by the addition of dialysis buffer. Solutions of ligands were also prepared in the dialysis buffer in order to keep the ionic strength constant. Data were obtained using a Microcal-MCS system (Cooper *et.al.*, 1999) and the Microcal Origin package (Microcal Software Inc.) was used to analyse the data in order to determine the T_m and thermodynamic parameters.

5.4 Results/Discussion

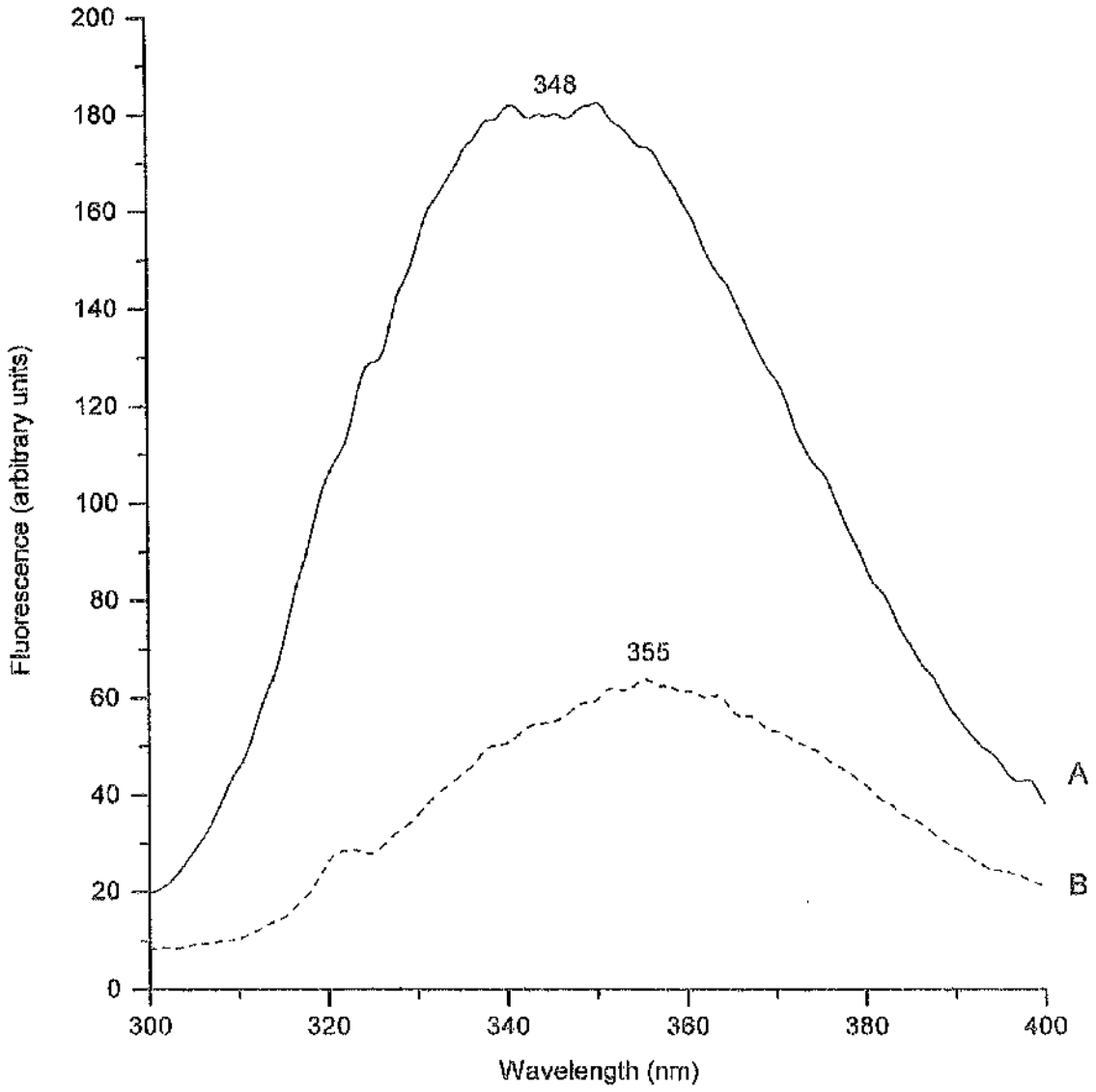


Figure 5.1 :Denaturation of wild type SKII in presence of GdnHCl illustrating the change in intensity and the shift in the emission maximum from the native to denatured states.

A; native state (0M GdnHCl), B; denatured state (5M GdnHCl).

Spectra were recorded at 20°C in a 1cm pathlength cuvette over the wavelength range 300-400nm. SKII was set up at a protein concentration of 0.2mg/ml with increasing concentrations of GdnHCl but for clarity only the scans for native and fully unfolded enzyme are shown.

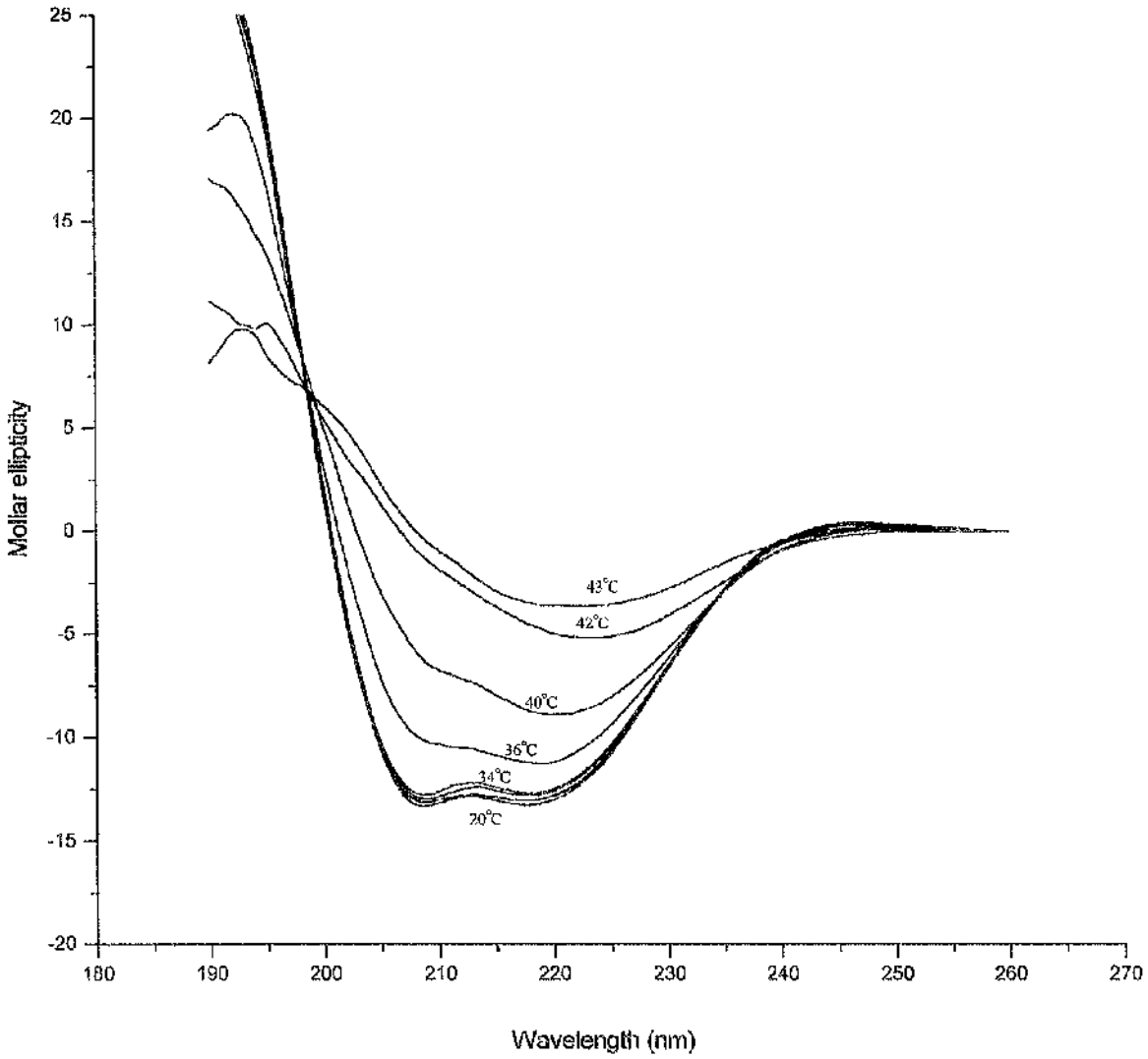


Figure 5.2 : Secondary structure changes during thermal denaturation as monitored using Circular Dichroism.

Protein concentration of 0.5mg/ml were used and CD spectra recorded at the temperatures indicated. Increasing concentrations of (GdnHCl) as with the increasing temperature above also show loss of secondary structure. Such degree of structure loss is monitored using the wavelengths of 208nm and 225nm.

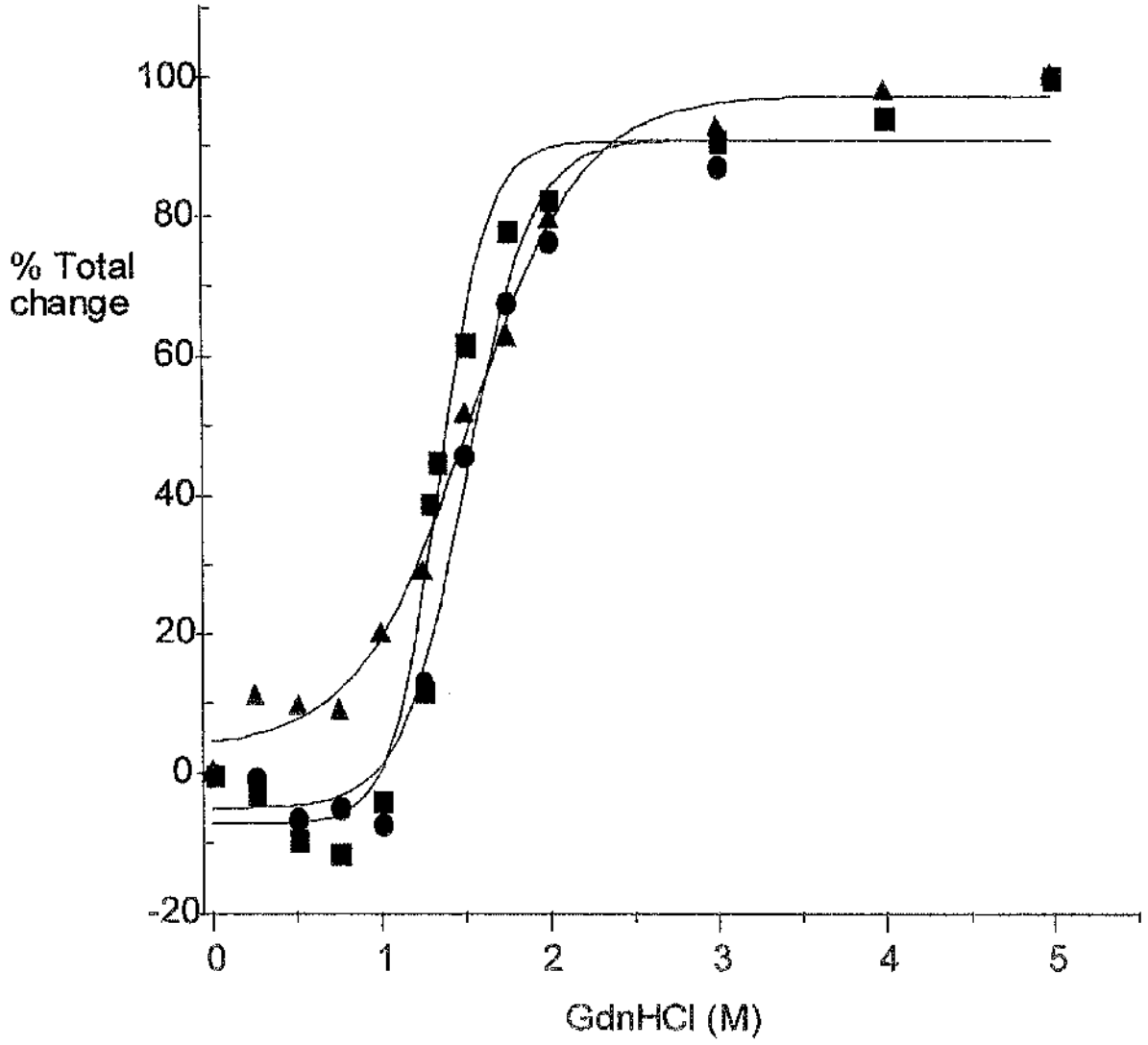


Figure 5.3 : Secondary structure denaturation profile of shikimate kinase.

Unfolding was monitored by changes in ellipticity at 222nm of shikimate kinase (0.2mg/ml) in the presence of increasing [GdnHCl].

Squares :wild type; circles :C13S; triangles:K15M.

The changes in the fluorescence and far UV CD of shikimate kinase on incubation with GdnHCl for 2 hours were monitored. In each case incubation with both 2.8M and 4.8M GdnHCl lead to essentially complete loss of structure as indicated by:

i) the shift of the fluorescence emission maximum from 348nm to 355nm (Fig. 5.1). The latter value is essentially identical to that of the model compound NATA which has a λ (356nm).

ii) the more than 10 fold decrease in ellipticity at 225nm. Data in Fig 5.3.

The changes illustrated in Figure 5.3 illustrate the percentage of total change in secondary structure of shikimate kinase between 0 and 6M GdnHCl. There is a small increase in the intensity of the ellipticity at 222nm between 0 and 1M GdnHCl, giving rise to negative values in Fig. 5.3 in this range. This increase is not shown in the K15M mutant and therefore it might be concluded that the secondary structure of the K15M is somewhat more stable than that of the wild type enzyme. Comments regarding this stability are discussed in greater detail later.

The changes in fluorescence are almost superimposable on those seen in the CD, indicating an essentially identical pattern of denaturation.

The sharp nature of the changes in CD and fluorescence between 1 and 1.3M GdnHCl suggest that the overall secondary and tertiary structures of the SK are lost in a process in which the entire monomer unfolds in a highly co-operative fashion.

In the presence of substrate, 2mM shikimate, the fluorescence data during denaturation show an unusual biphasic behaviour (Figure 5.4).

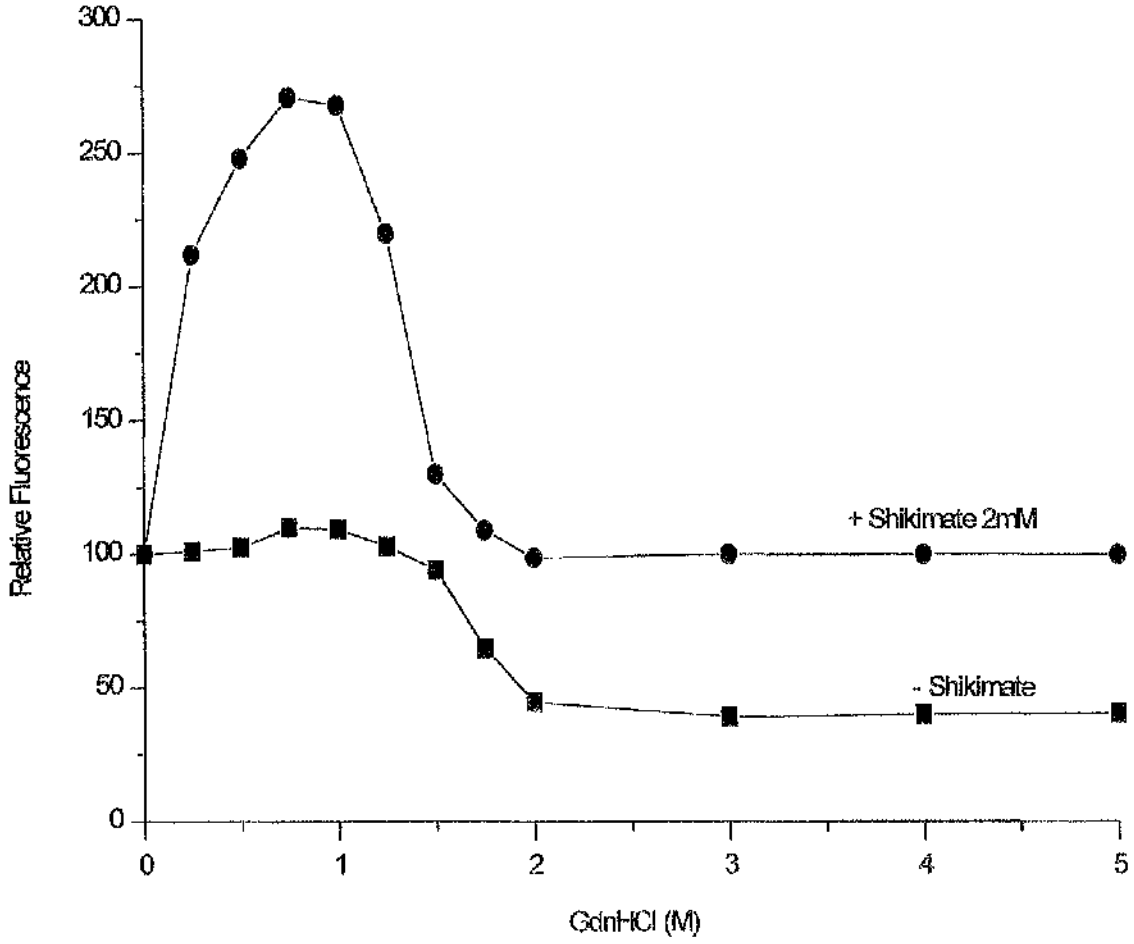


Figure 5.4 Denaturation of shikimate kinase in the presence of substrates.

Fluorescence changes seen in GdnHCl denaturation of shikimate kinase in the presence (circles) and absence (squares) of 2mM shikimate.

For ease of comparison, the fluorescence values at 0M GdnHCl are normalised to 100. The actual value in the presence of shikimate is 35, reflecting the quenching accompanying the binding of ligand (Fig 4.4).

In the presence of substrate shikimate it can be seen that the shikimate binding domain is lost prior to the complete unfolding of the enzyme. This is illustrated by the 2.5 fold increase in fluorescence seen in Figure 5.4 corresponding to the quenching by shikimate. The concentration of GdnHCl which leads to the dissociation of the substrate shikimate is not sufficient to lead to significant loss of the overall secondary and tertiary structure, since there are no changes in the far UV or fluorescence in the presence of 1M GdnHCl. Small increases in ellipticity observed at low concentrations (0.5M) of GdnHCl have been noted for a number of enzymes and have been interpreted as indicating that the low levels of the denaturant can stabilise proteins by altering the solute/solvent forces.

It is not possible to study the unfolding of protein by fluorescence in the presence of nucleotide since binding of nucleotide brings about a much smaller quenching of fluorescence. In addition the presence of nucleotide leads to a large 'inner filter' effect and so any possible dissociation of ligand would be difficult to detect.

Thermal denaturation provides another means to observe any possible ligand induced stability. Shikimate kinase was monitored by means of differential scanning calorimetry (DSC) which provides a number of thermodynamic parameters related to the protein stability. In the presence of ligands, both shikimate and ATP and the dead end complex shikimate plus ADP it was possible to measure the degree of stabilisation provided by the ligands.

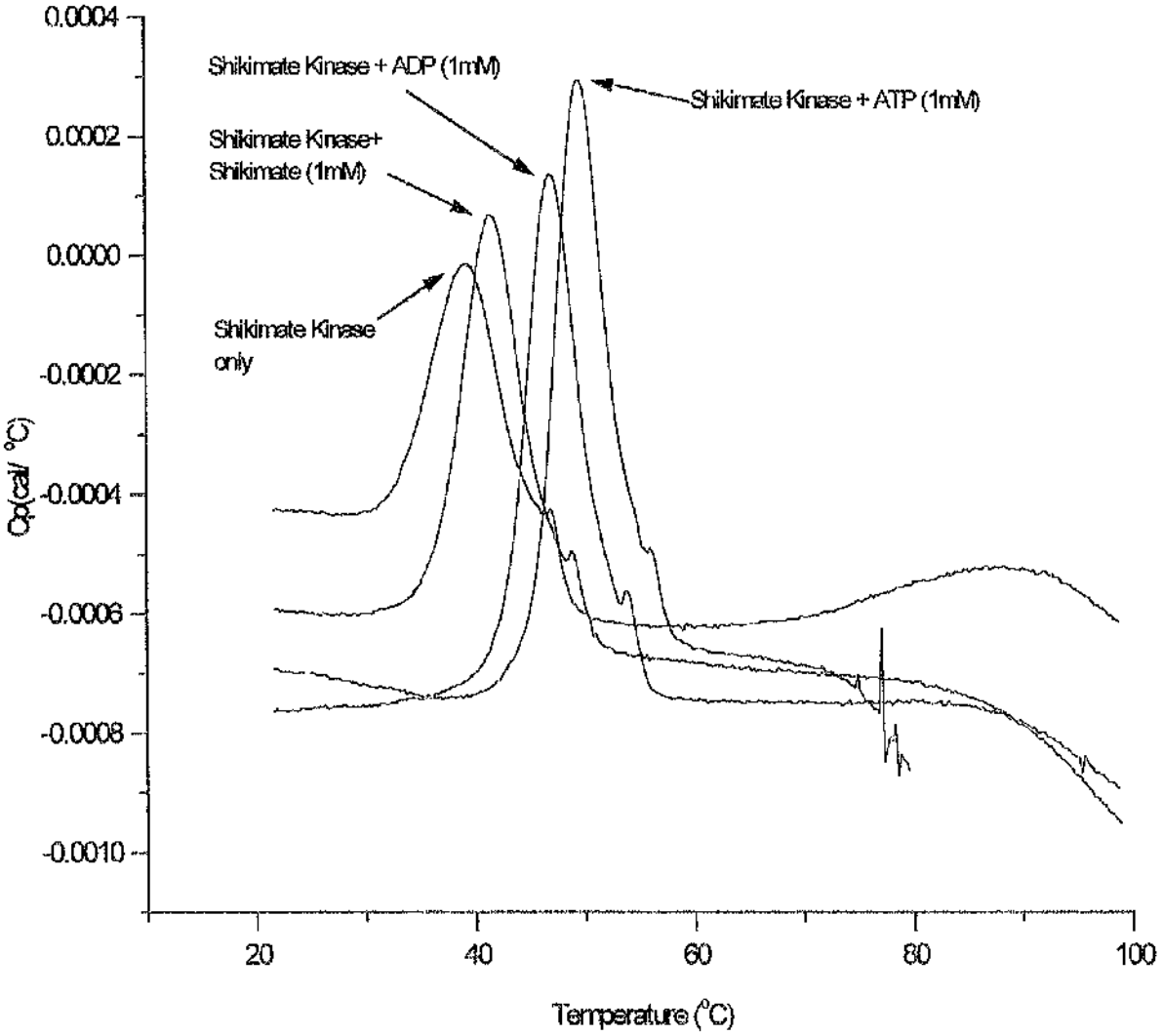


Figure 5.5 : Differential scanning calorimetry scans showing shift in the T_m of shikimate kinase in the presence of the various ligands indicated.

Enzyme	Conditions	T_m (°C)	ΔH_{cal} (J/mol)	ΔH_{vH} (J/mol)
wild type	no ligand	**39.70**	7.37×10^4	8.95×10^4
50°C/ hour	0.1mM ATP	**42.11**	7.62×10^4	9.35×10^4
	1mM ATP	**48.12**	9.98×10^4	1.18×10^5
10°C / hour*	no ligand	**36.13**	5.37×10^4	1.04×10^5
	1mM ATP	**45.01**	7.88×10^4	1.4×10^5
C13S	no ligand	**39**	7.62×10^4	9.35×10^4
	1mM ATP	**46.13**	9.85×10^4	1.21×10^5
K15M	no ligand	**43.87**	4.66×10^4	1.49×10^5
	1mM ATP	**45.0**	5.05×10^4	2.06×10^5
wild type	no ligand	**39.65**	6.72×10^4	8.7×10^4
	2mM shikimate	**41.75**	8.04×10^4	1.04×10^5
	2mM ADP	**47.26**	9.03×10^4	1.17×10^5
	2mM ATP	**49.88**	9.75×10^4	1.25×10^5
	2mMADP/Shi	**50.21**	6.85×10^4	1.81×10^5

Table 5.1 : DSC results showing the T_m and thermal stability of shikimate kinase type II from *E. chrysanthemi* in the absence and presence of ligands.

All measurements unless otherwise indicated* were taken with a scan rate of 50°C/hour. The protein concentration was 1mg/ml.

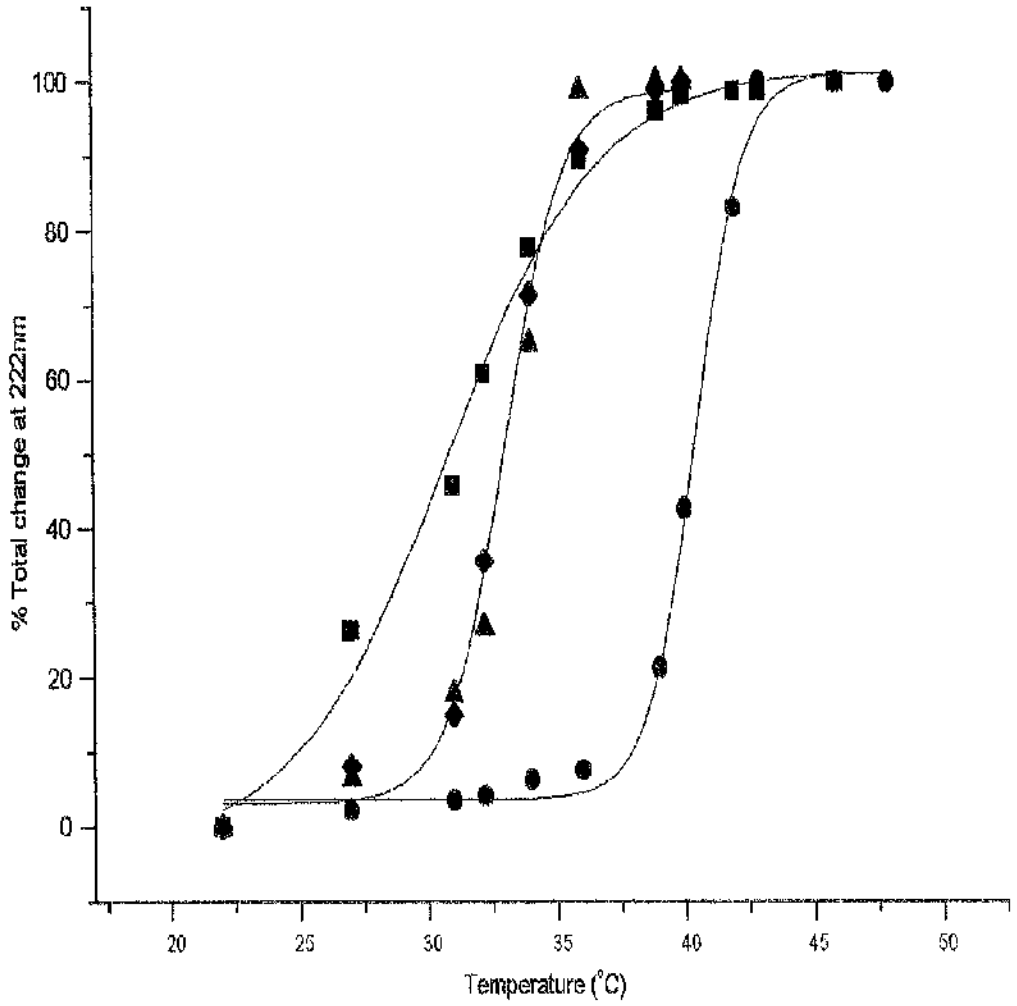


Figure 5.6: Thermal denaturation profile monitored by changes in ellipticity at 222nm.

The stability of the secondary structure was monitored in the absence (squares) and presence (diamonds) of 0.1mM ATP and in the presence of 1mM shikimic acid (triangles).

The maximum stability is seen in the presence of 1mM ATP (circles).

In these experiments the temperature was increased by 10°C/hour.

5.5 Further Discussion

The thermal denaturation studies illustrate the effect of ligands on the values of T_m (Table 5.1). In the absence of any ligand, the C13S and wild type enzymes show a very similar T_m (39°C), in the presence of 1mM ATP this is increased to a T_m of 46°C and 48°C for C13S and wild type respectively. The addition of 2mM ATP, which presumably leads to enhanced saturation of the enzyme, increases the T_m to a value of 50°C. Shikimate however, does not offer the same protection since the change in T_m of wild type shikimate kinase increases by only 2°C in the presence of 1mM shikimate. The dead end complex of ADP (2mM) plus shikimate (2mM) gives a T_m of 50.21°C; thus very little extra effect additive effect is seen when shikimate is added to the nucleotide-enzyme complex.

These data therefore show that the nucleotide offers greater stabilisation than shikimate. This can be understood since there are a greater number of residues involved in co-ordinating the nucleotide compared with those required for shikimate co-ordination.

The general stability of the C13S mutant does not seem to be affected since values gained with the C13S are comparable to those seen in the wild-type. In the presence of 1mM ATP the T_m for C13S is 2°C lower than the wild type; this may indicate that the ATP is not as tightly bound or, as well co-ordinated to the structure of the C13S mutant as to the wild type.

The most interesting results however are those seen for the K15M which show a significantly higher T_m (44°C) in the absence of ligand; this is increased only to 45°C in the presence of 1mM ATP. The data indicate that the K15M mutant has intrinsically greater stability than the wild type enzyme. Addition of nucleotide causes only a minor increase in T_m which indicates that binding or co-ordination of ligand to the mutant is weaker than to the wild type.

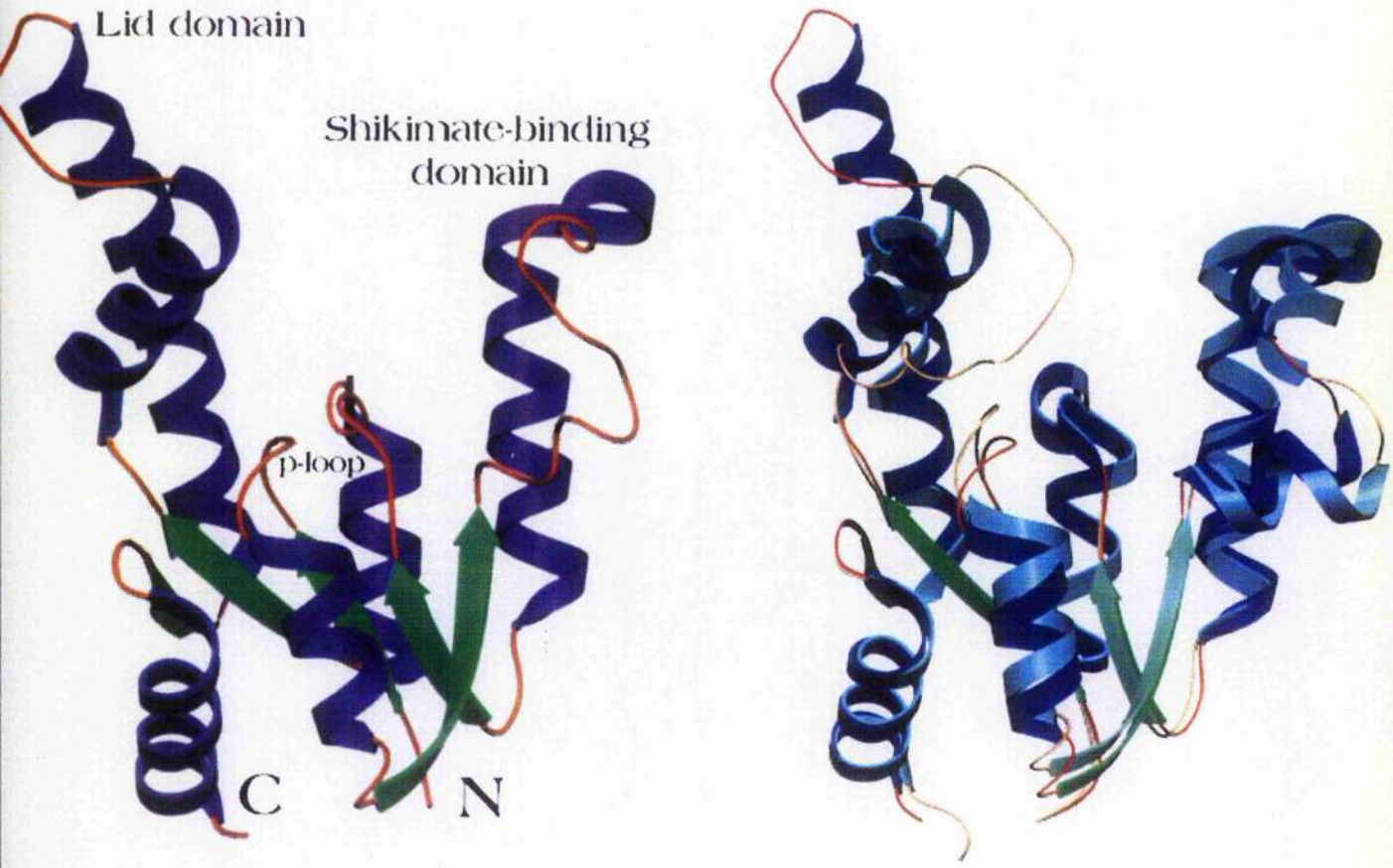


Figure 5.7 : Topology of shikimate kinase wild type and mutant K15M.

Comparison of the K15M mutant shikimate kinase (left) in the open conformation showing the regions which compose the active site. The overlaid wild type (pale colours) and K15M (dark colours) are shown on the right.

These figures were kindly provided by Dr. John Maclean (University of Glasgow, crystallography department, personal communication).

Figures 1.6 and 1.7 in the introduction may aid in locating the named residues.

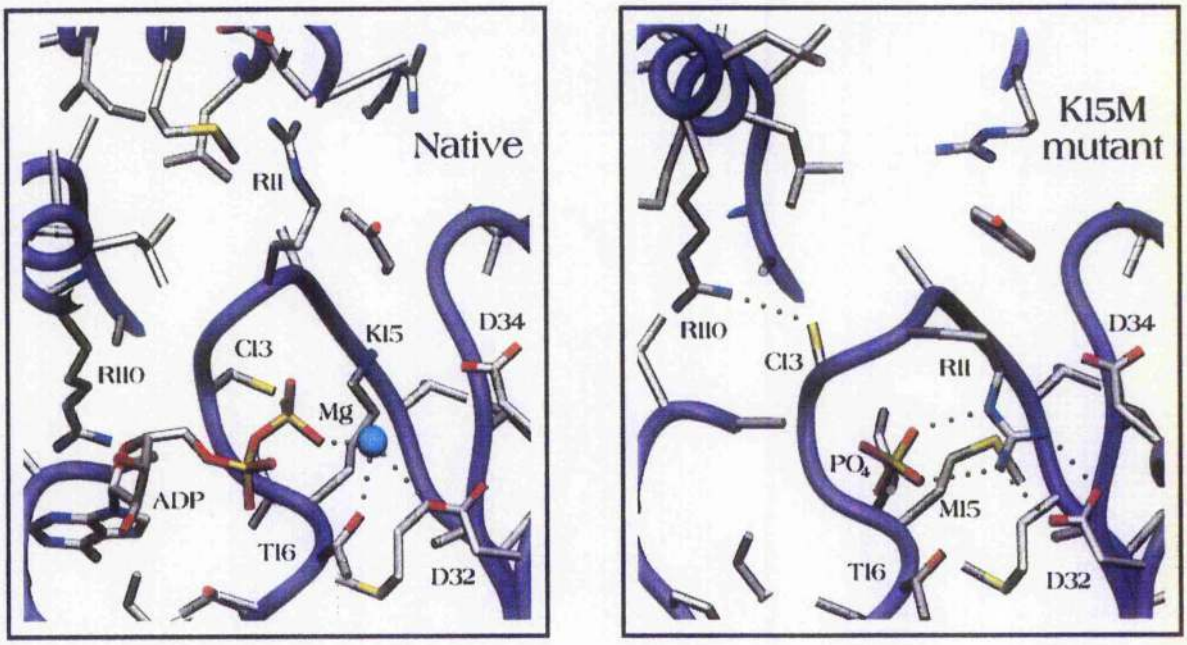


Figure 5.8a : Alterations in the P-loop region of shikimate kinase between K15M and wild type enzymes.

Note the formation of the new ion pair between C13 and R110 which is formed in K15M but which is not seen in the wild-type. The side chain of R11 has been shifted into the Mg^{2+} binding site where it forms ion pairs with the side chains responsible for the Mg^{2+} binding in the wild type, that is D32 and T16. The R11 is also involved in coordinating the phosphate which is located in the active site within the K15M mutant

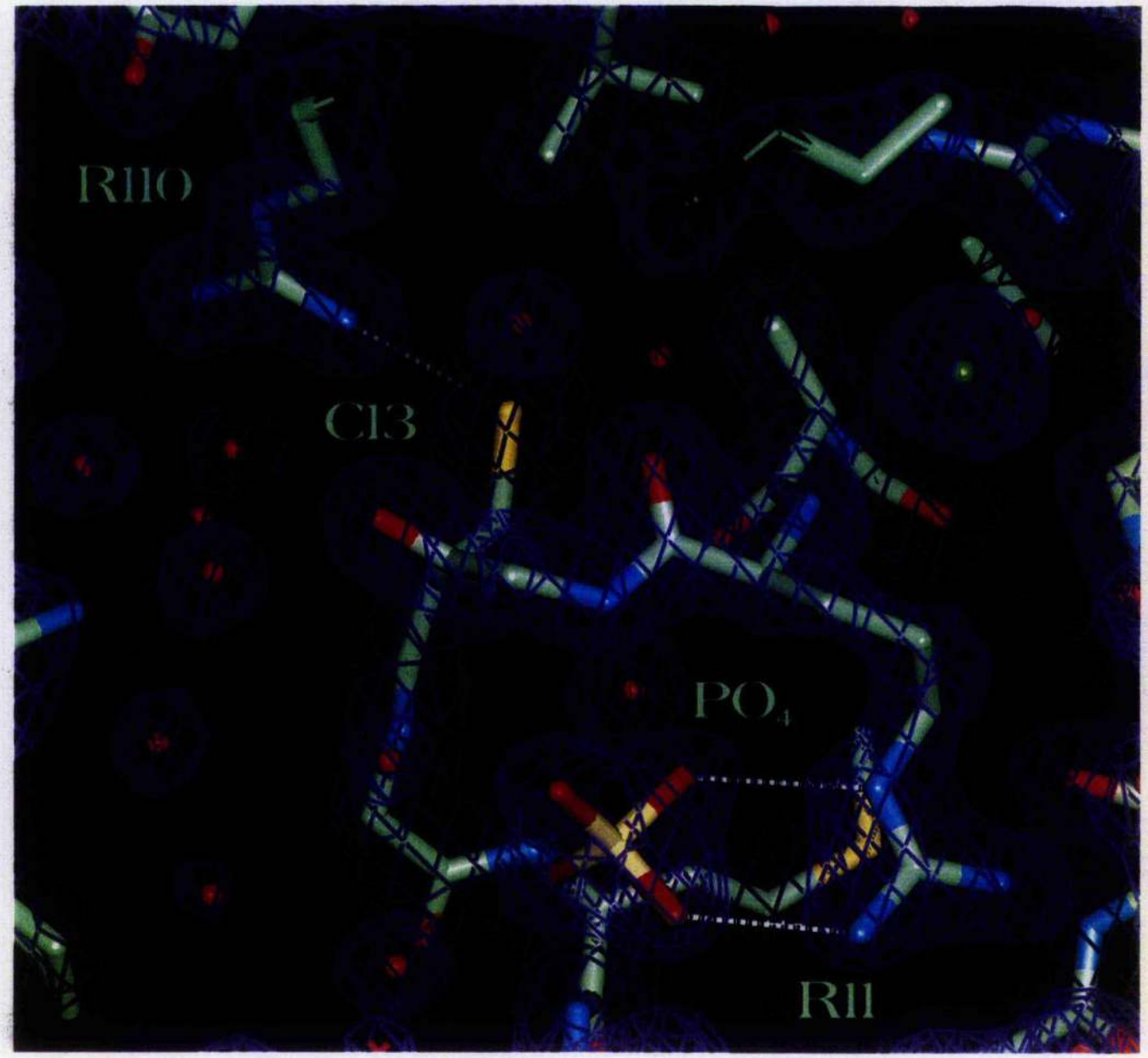


Figure 5.8b : Electron density around the P-loop in the K15M mutant. The map shows the new ion pairs seen in the K15M mutant, (those between C13 and R110, and between the side chains of R11 and D32 and T16), which are not present in the wild type enzyme.

The new ion pairs formed in the K15M mutant may be the reason for the elevated T_m of this enzyme compared with that of wild type, since these ion pairs are known to have greater stability than is observed on co-ordination of Mg^{2+} alone (Barlow and Thornton 1983).

The presence of the ion pairs explain why no enhancement of ellipticity is observed at low concentrations of GdnHCl in the K15M mutant compared with the wild type (Fig 5.3).

Chapter 6 : Refolding Of Shikimate Kinase type II from *Erwinia chrysanthemi*

6.1 Abstract

The refolding of shikimate kinase was initiated by dilution of enzyme solutions in the presence of the denaturant guanidinium hydrochloride (GdnHCl). The refolding upon the dilution of GdnHCl from both 4.8M to 0.44M and from 2.8M to 0.25M GdnHCl was monitored using circular dichroism (CD) and tryptophan fluorescence. Both manual mixing and stopped-flow methods were carried out to record the time courses of changes of secondary and tertiary structure respectively. Rapid regain of secondary structure took place with almost 100% of the total change happening within the dead time of the manual mixing procedure (15 sec). Using stopped-flow mixing it was possible to detect some of the final stages of the regain of secondary structure but even so 60% of the secondary structure was formed in the dead time (8msec).

Manual mixing using fluorescence showed 60% of the total fluorescence regained during the dead time (15sec) however stopped-flow techniques allowed the initial stages of fluorescence changes to be monitored. A bi-phasic curve was obtained indicating very rapid initial folding accounting for 10% of the total change followed by a slower process with rate constants of 0.006 and 0.24 sec^{-1} respectively.

In the presence of ANS however there was interference with the regain of native structure and during refolding on the manual mixing time scale, aggregation was seen illustrating incorrect folding for at least a fraction of the enzyme present.

There was a dramatic increase in ANS fluorescence during refolding of shikimate kinase compared with that observed in the initial (2.8M) or final (0.25M) concentrations of GdnHCl. During refolding ANS apparently binds strongly to hydrophobic residues exposed in an intermediate. Once complexed with ANS this intermediate is not able to refold correctly and release the ANS, but would appear to be prone to aggregate formation.

6.2 Introduction

The study of protein folding generally involves trying to understand the process by which a fully unfolded protein (U) matures into the folded native protein (N) and identifying any intermediates that may lie between the two states (Bycroft *et al.*,1990; Creighton, 1990, 1996; Radford *et al.*, 1992). Protein folding has been proposed to occur via transient partially ordered intermediates. In many proteins these intermediates have been classified as being of the 'molten globule' type. This is a state where the protein is reasonably compact and has native or near to native secondary structure but non-native fluctuating tertiary structure. The molten globule is postulated as an important intermediate in the protein folding pathway (Ohgushi and Wada, 1983) and is thought to appear early in the folding process of many proteins as a results of a rapid hydrophobic collapse of the polypeptide chain (Jones *et al.*, 1995).

The refolding pathway when studied on a very small time scale such as stopped-flow is able to elucidate the initial stages of protein structure formation which still remain a major unsolved area of modern structural biology (Kim and Baldwin, 1990).

Steady state refolding studies in proteins invariably show very rapidly formed intermediates. Secondary structure and indeed some enzyme activity are often formed within the dead time of manual mixing and so it is necessary to use stopped flow CD, (Kuwajimi *et al.*, 1987 and 1996; Price *et al.*, 1999) and fluorescence (Engelhard and Evans 1996) to observe the rapidly formed intermediates.

Such studies have been undertaken to map out the folding pathway of shikimate kinase and to identify some of the rapidly intermediates involved.

The reagent ANS (8-anilino-1-naphthalene sulphonate) is a commonly used probe for the molten globule state of proteins (Ptitsyn, 1990; Semisotov *et al.*,1991). ANS binds extensively to the hydrophobic sites of the molten globule; as the tertiary structure becomes

more native-like these sites disappear and ANS is released. Accordingly the increase and decay of ANS fluorescence has been used as a probe for the transient molten globule state during protein folding (Ptitsyn, 1995).

Apart from its intrinsic scientific interest and commercial importance, such as the development of methods for recovery of expressed proteins from inclusion bodies, understanding protein folding will shed light on many defective diseases which are known to occur due to incorrect protein folding and processing. Human genetic diseases can be due to mutations in proteins which influence their trafficking and lead to retaining of protein in the endoplasmic reticulum or incorrect processing (Bychova, 1995). One of the most common genetic diseases in humans is cystic fibrosis (CF). The membrane associated, N-linked glycoprotein (CFTR) is incorrectly folded due to lack of full glycosylation (Cheng *et al.,* 1990; Denning *et al.,* 1992) this leads to failure of an epithelial cell chloride channel to respond to cAMP thus leading to cystic fibrosis.

A review of defective protein folding as a basis of human disease is found in Thomas *et al.,* (1995). Misfolding and indeed the ability to form very stable structure due to the misfolding has been shown to cause the pathogenicity of scapie, with the infectivity due to structural changes in the prion protein (Gasset *et al.*, 1993,). A similar situation occurs in the bovine spongiform encephalopathy epidemic (Purdey, 1996a,b; Lasmezas *et al.,* 1997; Hope *et al.*, 1999).

Formation of amyloid plaques in Alzheimer's disease may be a consequence of a conformational transition from the largely α-helix native state to a β-sheet structure in the amino terminal region of the amyloid β-peptide (Soto *et al.*, 1995; Crawford *et. al.*, 1998).

6.3 Methods

GdnHCl was made up as an 8M stock solution in buffer A (35mM Tris/HCl, 5mM KCl, 2.5mM $MgCl_2$ and 0.4mM DTT, pH 7.5). ANS was also made as a 1mM stock solution in buffer A and stored in the dark. Both solutions were made up fresh.

Protein was dialysed as standard for at least two hours into buffer A and stock concentrations made up as required mostly from 1mg/ml in the denaturing solution prior to refolding.

Refolding was initiated by an 11 fold dilution reducing the concentration of GdnHCl from 2.8M to 0.25M. This was monitored in both a steady state fluorimeter and the stopped-flow apparatus using the appropriate wavelengths for protein fluorescence at (excitation 290nm; emission 350nm) and ANS fluorescence (excitation 380nm; emission 480nm).

Both steady state and stopped-flow results over varying time courses are shown.

The values regarding rates of refolding during tertiary and secondary structure regain with reference to denatured and refolded enzymes are also given.

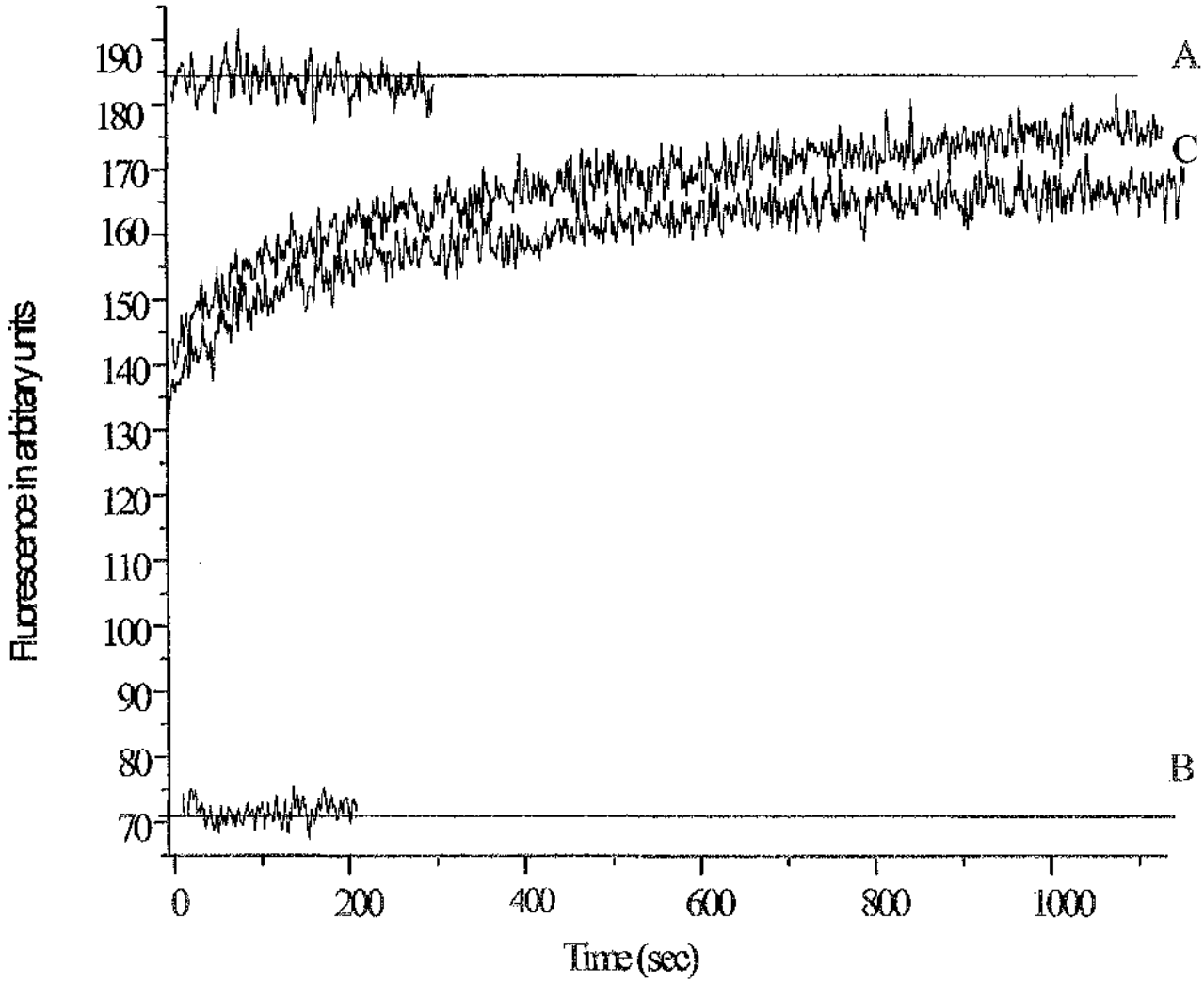


Figure 6.1: The refolding of shikimate kinase as monitored by protein fluorescence

Shikimate kinase 0.09mg/ml (C) was monitored at 350nm after dilution from 4.8M-0.44M GdnHCl. A dead time of 10 seconds occurred prior to recording data points.

(A) Native state shikimate kinase in 0.44M GdnHCl

(B) Denatured state shikimate kinase in 4.8M GdnHCl

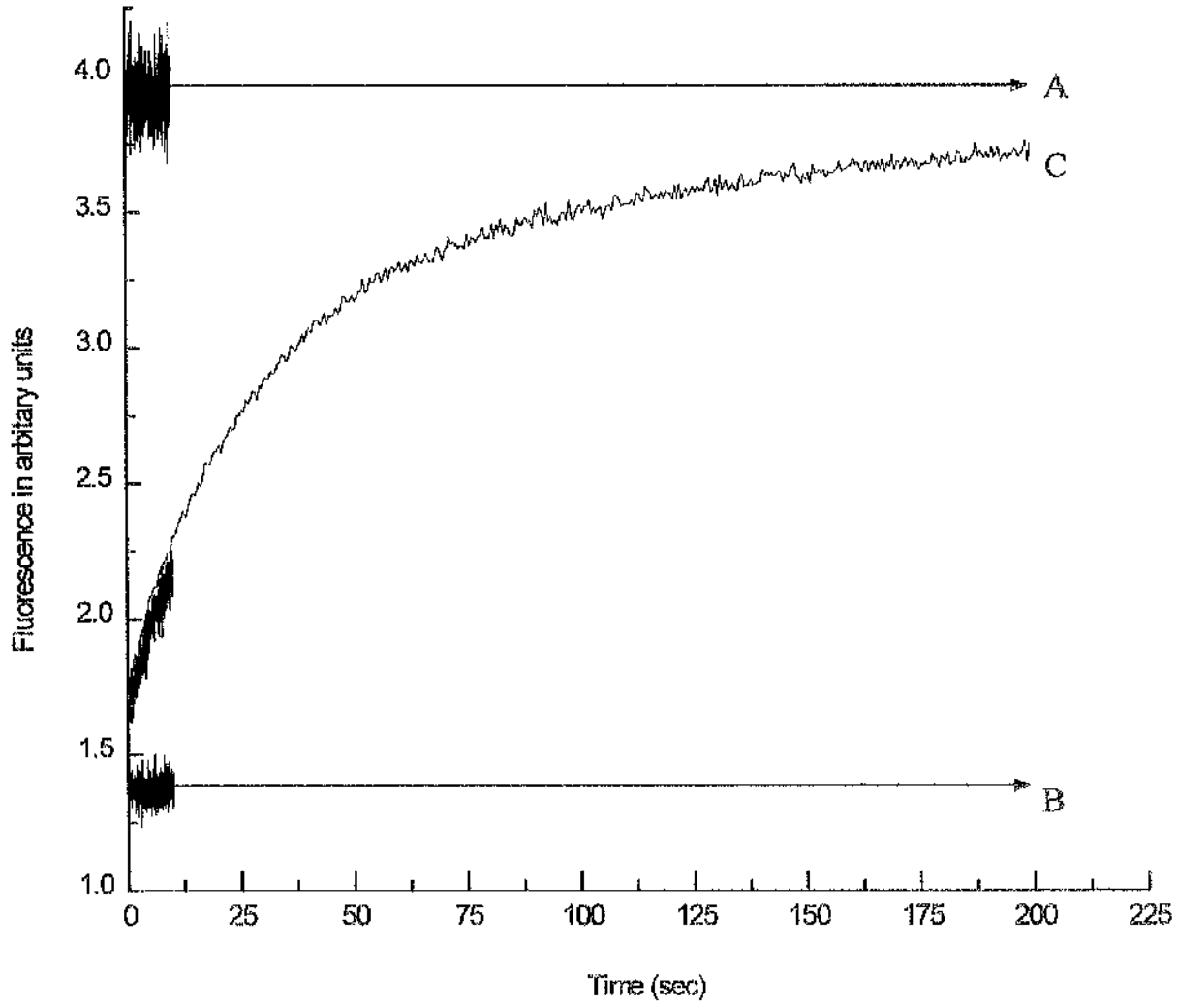


Figure 6.2: **The refolding of shikimate kinase as monitored by stopped-flow protein fluorescence.**

Shikimate kinase 0.09mg/ml (C) was monitored at 350nm after dilution from 2.8M-0.25M GdnHCl. There is only 1-3msecond of dead time when using the stopped flow apparatus.

(A) Native state shikimate kinase in 0.25M GdnHCl

(B) Denatured state shikimate kinase in 2.8M GdnHCl.

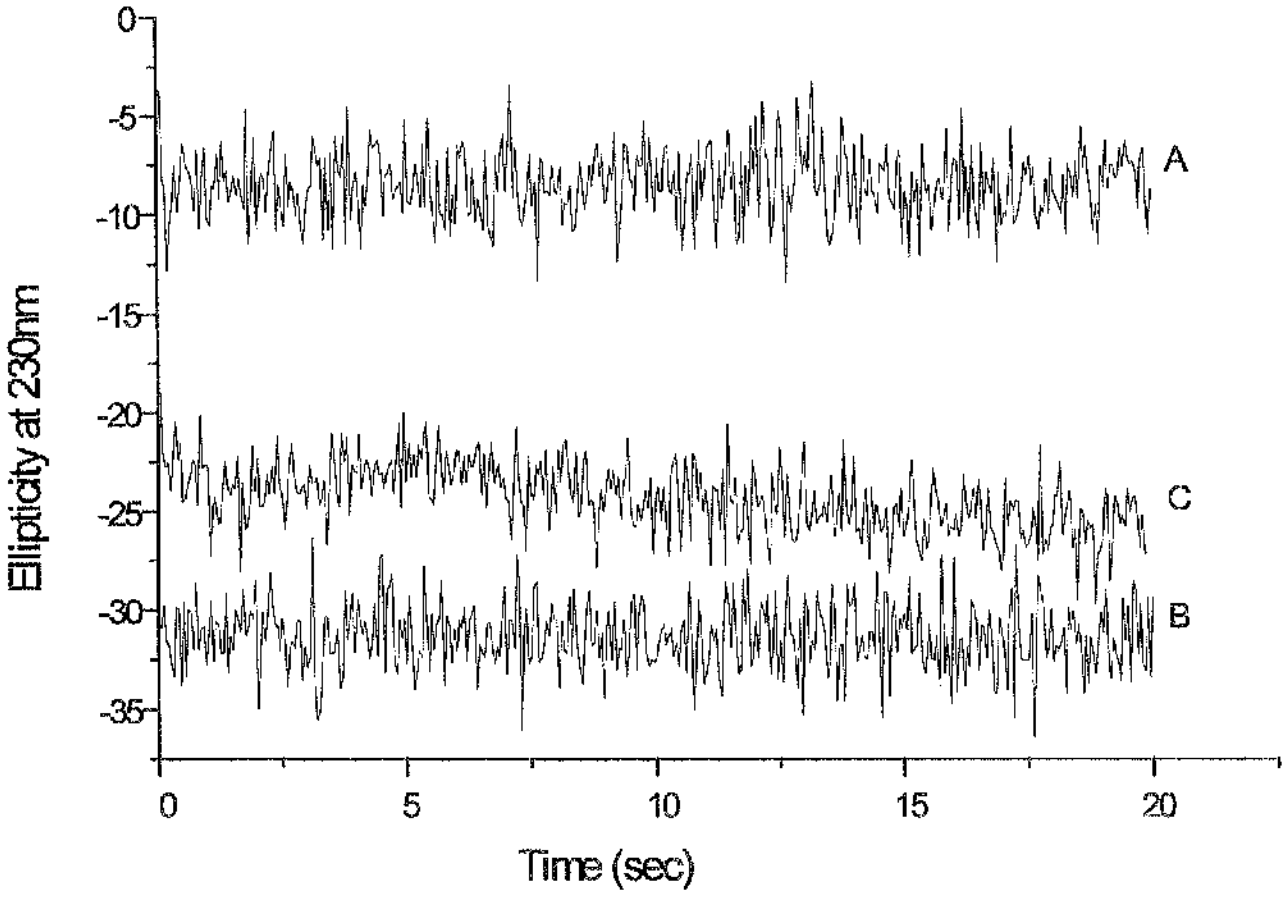


Figure 6.3: The refolding of shikimate kinase as monitored by stopped-flow CD.

Shikimate kinase 0.09mg/ml (C) was monitored at 230nm after dilution from 2.8M-0.25M GdnHCl.
(A) Denatured state shikimate kinase in 2.8M GdnHCl
(B) Native state shikimate kinase in 0.25M GdnHCl
(C) Refolding enzyme diluted from 2.8-0.25M GdnHCl.

Fitting the data shown in C to a first order process gave a first order rate constant of 0.125 sec^{-1}. The change occurring in the dead time accounts for 60% of the total structural structure again.

Enzyme *E. chrysanthemi* (SKII)	Regain % of total		
	CD	Fluorescence	Activity
wild type (no ligands)	97.2 ± 3.8	95 ± 2.3	83 ± 4.2
w.t+ATP (1mM)	87.3 ± 6.0	81 ± 5.4	75 ± 6.3
w.t.+shikimate (1mM)	97.9 ± 4.1	85 ± 3.9	80 ± 3.8

Table 6.1: **Regain of CD (secondary structure), fluorescence (tertiary structure) and activity of shikimate kinase 30 minutes after refolding had been initiated by mixing.**

Enzyme Conc.	k_1 (sec^{-1})	Amplitude of first phase (%)	k_2 (sec^{-1})	Amplitude of slow phase (%)
0.045mg/ml*	0.062	49.4	0.0074	50.6
	0.067	52.4	0.0085	47.6
0.09mg/ml*	0.042	61.8	0.0077	38.2
	0.038	60.5	0.0067	39.5
0.09mg/ml **	0.235	62.3	0.0063	37.7

Table 6.2: **Values of the actual rates and amplitudes of the fluorescence changes on refolding of shikimate kinase following dilution of GdnHCl from 4.8M to 0.44M* and 2.8M to 0.25M** dilution.**

** Concentration of GdnHCl (2.8M-0.25M). The regain of tertiary structure is four times faster for the initial stage of refolding indicated by the rate constant being 0.235 sec^{-1}.

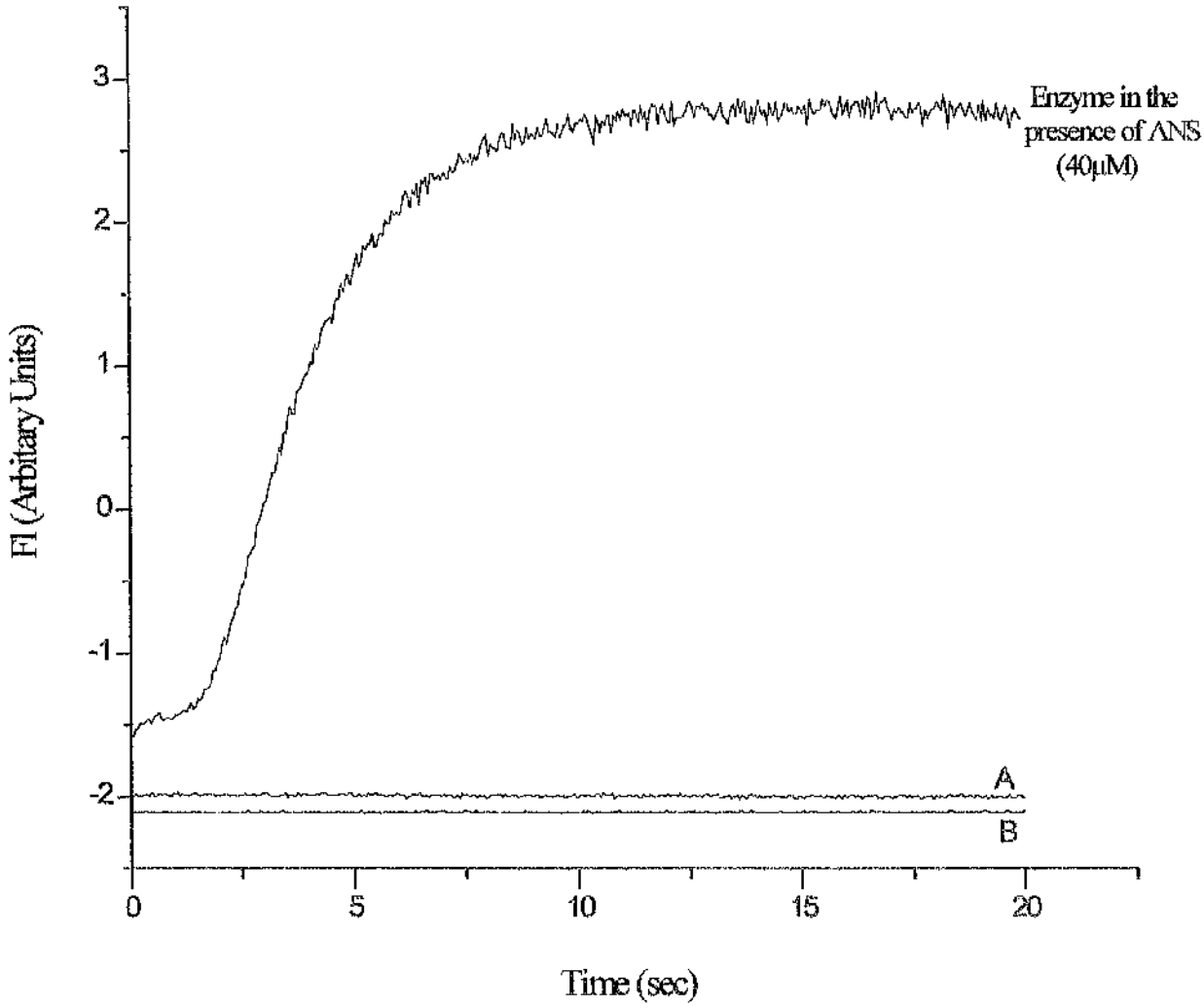


Figure 6.4: Changes in ANS fluorescence during refolding of shikimate kinase in stopped flow apparatus.

Shikimate kinase (0.09mg/ml) was refolded in the presence of ANS (40μM). ANS fluorescence monitored at 480nm (excitation 380nm).

(A) Native enzyme 0.25M GdnHCl +ANS (40μM)

(B) Denatured enzyme 2.8M GdnHCl +ANS (40μM)

k (rate constant obtained from data after 2.5 seconds) = 0.264sec^{-1}

6.5 Further Discussion

6.5.1 Denaturation

Incubation at 20°C at concentrations of 2.8 M GdnHCl or greater is required to bring about complete unfolding of shikimate kinase as indicated by changes in fluorescence (See chapter 4). In practice, a 2 hour incubation in GdnHCl (2.8M and 4.8M) was used out to bring about total denaturation prior to the initiation of refolding. Refolding was initiated by dilution of the denatured enzyme with 10 volumes of buffer both in manual and stopped flow mixing methods thus reducing the final concentration of GdnHCl to 0.44M and 0.25M respectively. It should be noted that the greatest degree of dilution obtainable in the stopped-flow apparatus was 11 fold.

6.5.2 Changes in fluorescence during regain of structure

The refolding of shikimate kinase via the regain of tertiary structure as monitored by fluorescence reveals more of the early stages of tertiary structure acquisition.

A biphasic curve results which is in common with other studies where a fast and then slower process for fluorescence regain has been observed (Semisotov *et al.,* 1991; Elove *et al.,* 1992). Refolding of shikimate kinase however gave a biphasic process with rate constants of k_1 0.24 and k_2 0.007 sec^{-1} for the faster and slower phases respectively.

The activity was also monitored during refolding and 80% activity was recovered after 5 minutes. Due to the nature of the assay it was not possible to study activity recovery on a shorter time scale.

It would have been interesting to have monitored the fluorescence changes over a shorter time period but when the increment of time at which readings are taken is reduced to less that 10msec then there is a greater signal to noise ratio which makes interpretation more difficult.

The speed at which regain of secondary structure has occurred during refolding has been well documented in the burst phase stages of the refolding of lactoglobulin (Kuwajimi, 1996).

6.5.3 Changes in CD during refolding

It was found using manual mixing techniques and monitoring the secondary structure regain by following the ellipticity at 225nm that refolding of shikimate kinase at a concentration of 50μg/ml occurred largely in the dead time of mixing (about 10 -15seconds), with no further changes observed (data not shown). The CD spectrum of enzyme after 15 minutes refolding was very similar in shape to the spectrum obtained from a control enzyme sample which had not been subject to the cycle of denaturation and refolding.

By using stopped-flow mixing it was possible to monitor the formation of secondary structure and show that it was a fast process which when monitored at 230nm, showed that 62% of the secondary structure was formed in the dead time (8msec). This result is comparable to a number of systems studied in this fashion where a large proportion of the secondary structure is regained within the dead time of the stopped-flow apparatus. In cytochrome c 44% of the structure is regained (Elove *et al.*, 1992) and the refolding of β-lactoglobulin also shows a very rapid regain of secondary structure (Kuwajima *et al.*,1987, 1996; Arai *et al.*,1998). From such studies it can be said that the very rapid regain of the secondary structure is a common feature in the folding of globular proteins. However, there is a some what slower process of regain which complements the data gained in the biphasic curve of fluorescence regain, this remainder of the secondary structure was regained in a process that can be fitted to a rate order constant of 0.125 sec^{-1}. Such a biphasic process has also been seen in the multimeric protein dehydroquinase (Price *et al.*, 1999) where the slow phase of the CD changes has a rate constant of 0.07sec^{-1}. The final refolded structure appears to have 91% total regain of structure.

The results above illustrate the limitations of conventional rapid mixing techniques to study the refolding of such a small monomeric protein as shikimate kinase. Thus in trying to identify any intermediates that may exist hydrophobic fluorescence molecules such as ANS can provide valuable information.

6.5.4 Changes in ANS fluorescence during refolding

The refolding of shikimate kinase shows a somewhat different pattern when the experiment is conducted in the presence of ANS. There was a 20 fold increase of ANS fluorescence during refolding; however the ANS fluorescence does not show any subsequent decline as has been found during the refolding of a number of other proteins.

The initial binding of ANS does not seem to take place very quickly upon refolding as there is a 'lag' phase which lasts for about 2 seconds from the start of refolding. Following this there is a much enhanced binding of the ANS which gives rise to the 20 fold increase in fluorescence. The increase seems to reach a plateau after about 10 seconds. Comparing the extent of regain of activity of wild type enzyme in the absence and presence of ANS we can see that there is a significant discrepancy between the values obtained. 80% of the activity is regained in the refolding of shikimate kinase in the absence of ANS, with only 60% regained in the presence of ANS. The 20% of mis-folded protein could account for the enhanced binding of ANS seen in Fig 6.4. It would be necessary to study the extent of refolding in the presence and absence of ANS over a range of protein concentrations in order to substantiate this proposal.

Chapter 7: General Discussion

7.1 Structural studies - Ligand binding

The single tryptophan residue (W54) that exists within shikimate kinase type II of *Erwinia chrysanthemi* was a useful indicator of structural changes which make up the most part of this study. These changes in the enzyme structure were induced upon ligand binding and through chemical and thermal denaturation and monitored through CD spectroscopy and fluorescence measurements.

Sequential changes in substrate binding were seen during titrations with shikimate in the absence and presence of nucleotide (chapter 4). The reduction in the dissociation constant from 700 μM to 320μM in the absence and presence of nucleotide respectively illustrates a two fold increase in affinity of the enzyme for shikimate which is consistent with the proposal of substrate synergism. The changes observed during ligand binding have been shown during the study of the enzyme phosphoglycerate kinase using three dimensional data obtained from crystallographic studies. Ligand binding caused the enzyme to undergo a degree of hinge motion with a positional displacement of 2.7nm for the ends of the domains relative to the unliganded structure (Bernstein *et al.*, 1997). Complete hinge closure for this enzyme has been brought about by the synergistic combination of both substrates and further evidence for the importance of a proline residue involved in the hinge movement has been illustrated in McHarg *et al.*, (1999).

Perhaps though, the most investigated kinase of all has been adenylate kinase. A number of different structures in various liganded states have been observed through crystallographic analysis (Schulz *et al.*, 1990; Gerstain *et al.*, 1993; Muller *et al.*, 1996; Sinev *et al.*, 1996) giving detailed information as to the key residues required for substrate binding and indeed catalysis.

This study of shikimate kinase illustrated a tightening of the enzyme structure as observed by monitoring the tryptophan using fluorescence quenching experiments. Full crystallographic evidence for such liganded states was beyond the scope of this study but certainly in the future it may be interesting to compare these with those that already exist with adenylate kinase.

Future work to observe the actual 'degree' of flexibility however could only be quantitatively resolved by much more detailed work using actual fluorescence energy transfer experiments (Bilderback *et al.*,1996) or as has been mentioned the ability to crystallize a number of differently bound states of shikimate kinase.

7.2 Stability studies

The strength of ligand binding and the effect of that the 'tightened' structure had on stability during denaturation was investigated in depth using a number of techniques. Both chemical and thermal stability gave interesting results from wild type and mutant SK II enzymes.

7.2.1 Chemical denaturation

A very co-operative unfolding of the secondary and tertiary structure was found during chemical denaturation (Fig. 5.3) with GdnHCl. This profile was not unlike that of the unfolding of adenylate kinase (Zhang *et al.*, 1998) yet the mid-point of denaturation was somewhat elevated from the 0.7M GdnHCl found with adenylate kinase to that of 1.3M with the shikimate kinase and thus indicates that the SKII is more stable than the adenylate kinase mentioned.

In the presence of shikimate during denaturation the increase in fluorescence between 0.25-1.0M GdnHCl (Fig. 5.4) is indicative of the loss of the shikimate binding site prior to the co-operative loss of structure.

There seems to be no obvious difference in the wt, C13S and C162S mutant during chemical denaturation but the K15M showed some indication of stabilization in the lower (0 to 1M) GdnHCl concentrations. This stability was indeed true when thermal denaturation was investigated using differential scanning microcalorimetry.

Although there seemed to be no obvious difference in the secondary and tertiary structure loss in shikimate kinase other studies do illustrate a difference upon GdnHCl denaturation (Tsou *et al* 1983, 1986, Uversky and Ptitsyn, 1994; Zhang *et al.*, 1993, 1998). This is generally found when larger protein structures containing dimers and trimers, as single and mulitiple active structures combined, exist.

7.2.2 Thermal denaturation

The greatest stabilization during thermal denaturation is seen in the wild type shikimate kinase in the presence of both substrate and product mix (shikimate and ADP). An 11 degree change from a T_m of 39°C (no ligand) to that of 50.21°C in the presence of shikimate (2mM) and ADP (2mM) (Table 5.1).

Such stabilization through ligand binding has been investigated in the enzyme dehydroquinase type I. Binding of ligand to this enzyme formed a Schliff base which increased the T_m to a great extent from 57°C wild type unbound to 96°C with the covalently bound ligand (Kleanthous *et al.*, 1991). Although this is much more elevated than that seen in the shikimate kinase it give further evidence for significant ligand induced stabilization.

The use of DSC has been well documented in many other examples which can be found in Cooper (1999).

Studies on the mutants in particular the K15M mutant were very interesting both from the aspect of stabilization and with the progress made on the crystallization front (Dr. John Maclean personal communication). Evidence for the existence of a second ion pair is the reason that we have given for the elevated T_m seen with the K15M which is only marginally increased in the presence of saturating concentrations of nucleotide.

Structural stability as has been suggested by Fry *et. al* (1986) states that lysine residues are suggested to have the function of enabling conformational changes to take place; thus removal of the lysine may have reduced all important flexibility of the structure associated with ligand binding and catalysis.

7.3 Refolding

The pattern of refolding as seen in chapter 6 has been studied in some depth.

Concentrations of 2.8 M GdnHCl or greater is required to bring about complete unfolding of shikimate kinase as indicated by changes in fluorescence from which refolding was initiated.

A biphasic curve results which is in common with other studies where a fast and then slower process for fluorescence regain has been observed (Semisotov *et al.*, 1991; Elove *et al.*, 1992). Refolding of shikimate kinase however gave a biphasic process with rate constants of k_1 0.24 and k_2 0.007 sec^{-1} for the faster and slower phases respectively.

Stopped-flow mixing was the only way in which to monitor the rapid regain of secondary structure and even so when monitored at 230nm, 62% of the secondary structure was formed in the dead time (8 msec). This result however, is comparable to a number of systems studied in this fashion where a large proportion of the secondary structure is

regained within the dead time of the stopped-flow apparatus. This very rapid regain of the secondary structure is a common feature in the folding of globular proteins.

The refolding of shikimate kinase shows a somewhat different pattern when the experiment is conducted in the presence of ANS. A 20 fold increase of ANS fluorescence during refolding was seen which did not show any subsequent decline as has been found during the refolding of a number of other proteins. Thus it was assumed, and indeed seen, that some aggregation was occurring that led to the partial mis-folding in the presence of ANS.

In order to investigate these findings further experiments with varying ANS and protein concentrations would need to be followed monitoring using the stopped-flow apparatus.

Appendix 1 : Investigating the importance of glycosylation of the enzyme *Candida rugosa* by site directed mutagenesis.

(This appendix consists of the work that was carried out within Universita' degli studi di Milano, dipartimento di fisiologia e biochimica, Italy over a four month period March-July 1998 under the supervision of Dr. M. Lotti).

A.1 Abstract

This project seeks to illustrate the role that glycosylation plays within Lipase 1 enzyme of *Candida rugosa*. It covers the strategy that was employed in order to generate site directed mutants, the amplification of the gene within *Escherichia coli* and describes some preliminary results on the activity of the mutants when expressed in *Pichia pastoris*.

The growing interest in the organic synthesis field regarding the reaction catalysed by lipases, namely the hydrolysis of esters of glycerol, has arisen due to the great number of enantiomerically pure and optically active compounds that can be produced. This has stimulated detailed investigation of the mechanism of lipase catalysed reactions and factors determining their stereoselectivity (Boland *et al.*, 1991; Dordick, 1992).

Glycosylation may play a role in the substrate selectivity or in the stabilisation of the protein. This study seeks to investigate the role that glycosylation plays in the mechanism of lipases.

Prior to site directed mutagenesis the lipase enzyme from *Candida rugosa* had to undergo a total synthesis due to the presence of non-universal codon usage for serine (Brocca *et al.* 1997). The synthetic gene was then manipulated at the three sites of glycosylation; asparagine 291, 314 and 351.

Expression of the various mutant enzymes was achieved after subcloning the plasmids in *E. coli*, the order of activity was found to be N291Q>N314Q>N351Q.

A.2 Introduction

Lipases catalyse the hydrolysis of esters of glycerol. These enzymes exist across the phylogenetic tree from bacterial to mammalian species and the basic reaction is shown below.

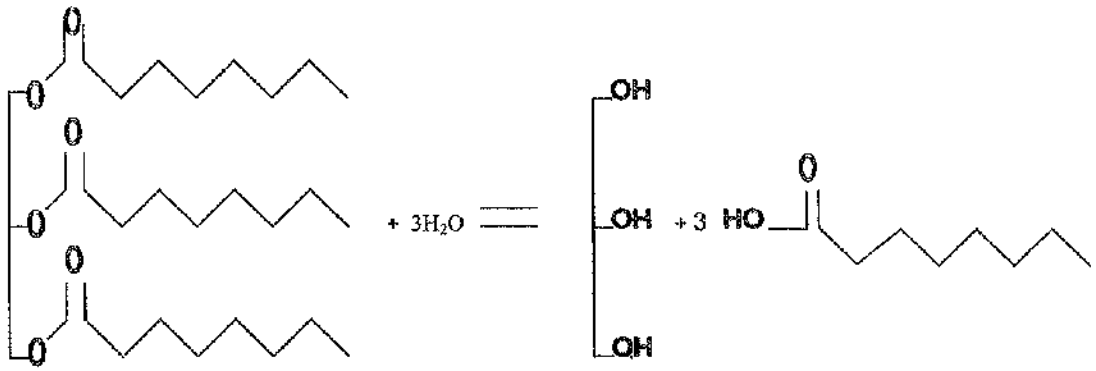


Figure A.2.1: Reaction of the Lipase enzyme. Adapted from Schrag *et al.*, 1991.

The activity of the lipases can be seen to utilise a catalytic triad similar to that of the well known protease family (Schrag *et al.*, 1991). In *C. rugosa* the active site serine (Ser 209) exists within the sequence Gly-X-Ser-X-Gly and is located in a unique supersecondary structure at a tight bend between a β-strand and α-helix buried within the structure (Cygler *et al.*, 1992). Comparison of the secondary structure elements of lipases have revealed a particular hydroylase fold (Ollis *et al.*, 1992) as well as a lid domain consisting of one or two loops which regulate access of substrate to the active site.

Regulation of substrate access is the molecular basis by which stereoselectivity of substrates is achieved. This is termed 'interfacial activation' a mechanism in which the presence of a lipid interface allows the lid domain to open and activate the enzyme. The stabilisation of the open form of the lid domain has been observed in the three dimensional

crystal structure of the enzyme (Grochulski *et al.* 1994).

The kinetics of lipases can not be analysed using the standard Michaelis-Menten equations. This is due to the fact that it is necessary to take into account the kinetics of adsorption of the enzyme onto the lipid interface which leads to activation of the enzyme; this has been described by Jaeger *et al.* (1994). A new model for explaining the observed kinetics of the enzyme is shown in Fig 7.2.

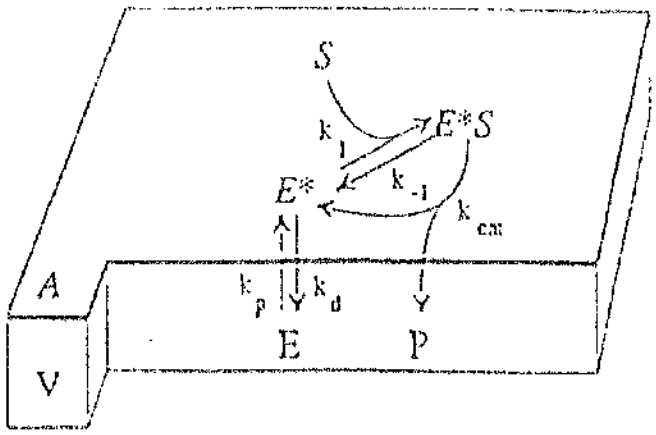


Figure A.2.2: New Model for studying lipase kinetics (Jaeger *et al.* 1994).

A and V indicate the external and internal environment respectively, of the lipid interface.

There are two steps to describe the kinetics of catalysis by lipolytic enzymes;

1) the physical adsorption of the enzyme at the lipid interface could include activation of the enzyme (Brzozowski *et al.*, 1991; Van Tilbergh *et al.*, 1993),
2) the formation of the enzyme/substrate complex which can be hydrolysed to give the product and regenerate the adsorbed enzyme.

These are represented above with the rate constants for such adsorption (k_d) and dissociation (k_p).

Lipases have been used traditionally in the flavour development of some foods such as Italian hard cheese (Steads, 1986). Now, with the advance of recombinant DNA

technology, it is possible to investigate the use of lipases in many other applications including the manufacture of detergents containing an extracellular lipase, and hydrolysis of lower quality oils and fats to generate more highly refined lipids. Lipases can also be used to generate the building blocks for the production, for example of pesticides and the synthesis of various chiral drugs such as α-blockers. In fact, 25% of current methods employed for preparing chiral chemical compounds use lipases as biocatalysts (Sinisterra, 1996). Another area of interest concerns the targeting of these enzymes in pathogenic bacteria and fungi which are known to produce extracellular lipases as virulence factors (Jaeger *et al.,* 1994).

A.2.1 *Candida rugosa* and the total synthesis of the synthetic gene Lip1.

Candida rugosa is a hemiascomycetous fungus which is a good producer of lipases which exhibit high activity in hydrolysis and synthesis.

The secreted lipase enzymes from *C. rugosa* exist as a mixture of five isoenzymes encoded on different genes. Of these, Lip1 protein, is the most prominent (Lotti *et al.,* 1993). These genes however could not be expressed in a standard expression system such as *S. cerevisiae* due to the fact that *C. rugosa* utilises an unusual codon first described by Kawaguchi *et al.*, (1989). The codon CUG which, universally encodes a leucine, in *C. rugosa* encodes a serine, in fact, up to 40% of the serines are encoded in this way (Lotti *et al.*, 1993) including the active site serine and thus protein generated in standard expression systems is inactive and not secreted (Fusetti *et al.*, 1996).

Preliminary mutations attempting to replace 50% of the CUG triplets restoring the serines also resulted in the lipase being inactive (Brocca, *et al.,* 1998) and thus a full scale synthesis of the gene was carried out generating fully functional lipase with all the CUG triplets encoding a serine.

The synthetic gene was optimised for expression in the *Pichia* host since this host is becoming an increasingly attractive host for production of proteins on an industrial scale (Tschopp *et al.*, 1987). The genetic manipulations were carried out on this synthetic gene.

A.2.2 Glycosylation/Mutagenesis strategy

Candida rugosa Lip1 is a glycosylated protein with the sugar content accounting for up to 5% of the total mass (60kDa) of the protein.

The post-translational modification of glycosylation is seen in many proteins formed by a O- or N-glycosidic bond to the R-group of serine/threonine or an asparagine side chain respectively. The role of the sugar moiety could be crucial in processes such as protein folding, oligomer assembly, secretion or catalysis. However, although the mechanisms of glycosylation are known, the functional consequences of the sugar moiety are poorly understood. The glycosylation sites within the Lip1 enzyme have been identified in the sequence (Longhi *et al.*,1992) and in the recent three dimensional crystal structure (Grochulski *et al.*, 1994) to occur on the asparagines (Asn) 291, 314 and 351 which all lie within the consensus sequence of Asn-X-Ser/Thr.

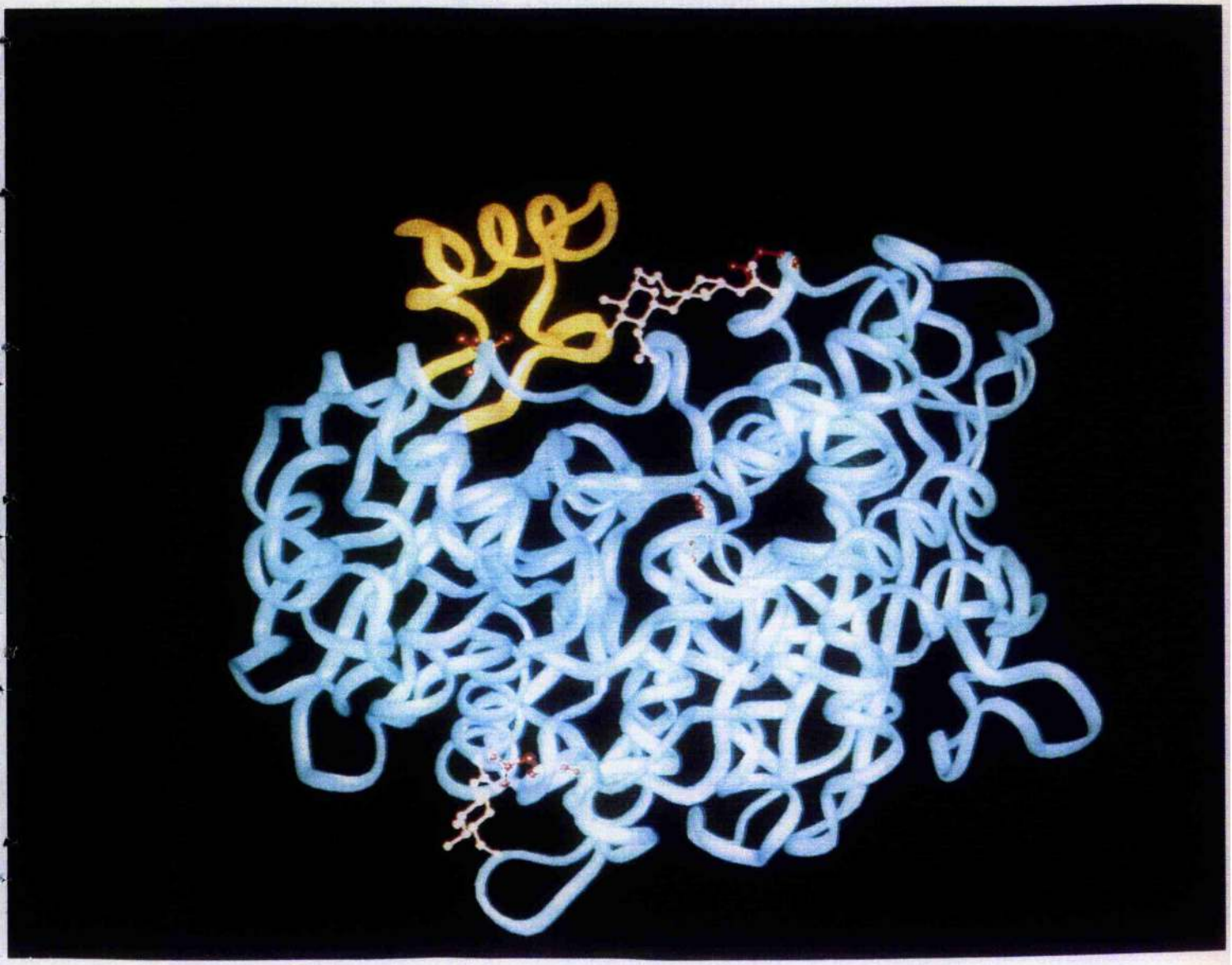


Figure A.2.3: The Crystal Structure of Lip 1 from *C. rugosa*

Asn residues (red) are shown with attached sugars (white).

The lid domain is shown in yellow. In particular the Asn 351 N-acetylglucosamine (NAG) is thought to have some interaction with the stabilisation of the lid domain when the lipase is in the active (open) confirmation and it is the effect of removal of the sugars that is investigated in this project.

A.3 Procedure.

A 3.1 Site directed mutagenesis

Site directed mutagenesis is a useful tool for probing the importance of particular residues within a protein structure. In this case, however, a mutagenesis strategy was employed to generate null mutants of glycosylation; accordingly the most 'silent' of mutations was to be made. It was decided that the substitution of the asparagine with glutamine (Asn->Gln) was the most silent as all hydrogen bonds were retained. The function of the human insulin receptor in the β-subunit have been investigated in a similar way by mutation of each of the four N-glycosylated sites with the substitution of Asn by Gln (Leconte *et al.*, 1994).

A two stage PCR reaction requiring two complementary mutant oligonucleotides for each mutation. The oligonucleotides were ordered from M-Medical (Florence).

Primer	Sequence		Length
E.Forward	5'^{411}	GTGGATCTTTGGTGGTGG 3'	18-mer
E.Reverse	5'^{1470}	GAAAGCGTTGTTGTAGATCAAG 3'	22-mer
Q291F.	5'^{911}	CTTGGAAGACGCGACTCAGAACACCCCTGG 3'	31-mer
Q291R.	5'^{941}	CCAGGGGTGTTCTGAGTCGCGTCTTCCAAAG 3'	31-mer
Q314F.	5'^{984}	CCAGACGGTGTTCAAATCACCGACGAC 3'	27-mer
Q314R.	5'^{1111}	GTCGTCGGTGATTTGAACACCGTCTGG 3'	27-mer
Q351F.	5'^{1097}	CTTCTTCTTTGCAAGTTACCACTGATGCCC 3'	30-mer
Q351R.	5'^{1126}	GGGCATCAGTGGTAACTTGCAAAGAAGAAG 3'	30-mer

Figure A.4: Oligonucleotide primers designed for site-directed mutagenesis of *lip1* gene from *C. rugosa*.

Site directed substitutions occurred within the underlined codon.

The template for the mutagenesis was the shuttle vector pPICZαB plasmid available from Invitrogen which has the antibiotic resistance gene for Zeocin allowing for selection of positive transformants.

The gene *lip1* was inserted into the vector so as to be under the strong control of the *AOX1* promoter allowing for the induced expression of lipase in the presence of methanol.

The mutagenesis was carried out using *pfu* DNA polymerase in order to give greater fidelity. The standard reaction conditions were: 50pmol of each primer and 0.3μg-0.5μg DNA template in 100μl total volume. The reaction followed a two stage PCR reaction firstly in the production of two fragments containing the mutation in a selected region of DNA and secondly the PCR reaction to generate the full length insert.

The PCR cycle involved an initial denaturation of 94°C for three minutes and followed by twenty five cycles of 94°C, 55°C, and 72°C each for one minute with a final extension of 72°C.

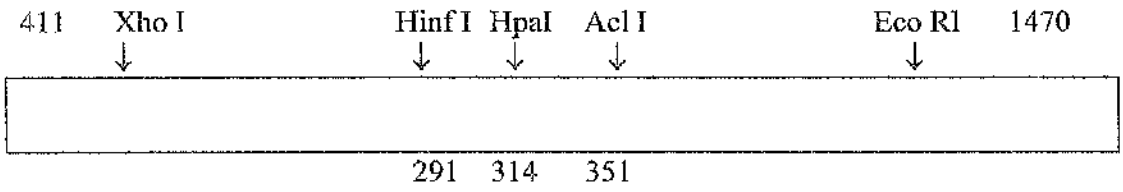


Figure A.5 : Full length PCR product.

This shows the positions of the Asn residues and restriction sites utilised in a) ligation into vector (Xho I, EcoRI) and b) in the diagnostic digests (Hinf I, Hpa I and Acl I).

Re-ligation of the insert into the plasmid was achieved using the unique Xho1 and EcoR1 restriction sites. Standard ligation conditions were used and the product formed was used to transform *E. coli* strain JM101. These transformed cells were grown on low salt LB

containing the antibiotic zeocin (25μg/ml). Positive colonies were selected and mutations confirmed through diagnostic digests and analysis of products. The sequence was verified by DNA sequencing carried out by M-Medical.

Transformation of *Pichia pastoris* strain X-33 was achieved with the bicine method and electroporation (Becker and Guarente 1991). The freshly transformed cells were firstly plated out onto solid medium (YPD). Secondly, positive colonies were transferred onto minimal tributyrin-methanol plates and expression of the lipase was induced by methanol. Plates were incubated at 30°C for 48 hours.

Positive colonies secreted lipase which could be identified by the presence of a clear halo around the colony compared with the opaque tributyrin medium (Figure 7.7).

A four day induced expression of lipase was carried out in order to correlate the activity of the lipase with the actual amount of enzyme present within both supernatant and cell extract samples.

The selected colony was grown initially in a 5ml overnight (YPDS) culture. This culture was then used to inoculate a 30ml BMGY medium which would support growth for a four day expression. Induction of growth was carried out using methanol when the culture had reached an OD_{600} of 12. Methanol was added daily (250 μl) and the medium was supplemented daily with a concentrated (5x) nutrient YP medium (4ml).

Samples for analysis on SDS-PAGE were prepared from both the supernatant and the cell extract. The cells from 1ml aliquot were centrifuged at 3,000 rpm for 5 minutes. The supernatant was taken from the cell pellet and protein concentration estimated. The remaining cells were then solubilised in the presence of sterile silica beads in ice-cold extraction buffer (Tavare *et al.*, 1988). Re-centrifugation occurred to sediment the silica beads and the remaining cell extract sample used.

Aliquots of the two samples, supernatant and cell extract, from each mutant were run on an SDS-PAGE (not shown) and transferred electrophoretically onto Amersham Hybond-ECL nitrocellulose membrane. Immuno-blotting (Fig A.6a and A.6 b) using a raised primary anti-body against the lip1 and secondary anti-IgG was carried out as in Brocca *et al.* (1998). It can been seen (Fig. A.6 a and b) that there is production of lipase enzyme both secreted and within the cell extracts although the results show variations between the samples.

A.4 Results/Discussion

A

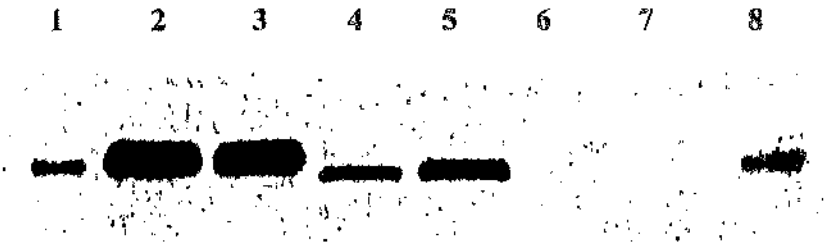


Figure A.6a: Secreted lipase 1 enzyme from *P. pastoris* as detected by Western blotting.

The supernatants, from the transformed *P. pastoris* containing one of each of the lipase enzymes, were prepared after a four day induced expression;

Lane 1: Wt (com.), 2: Wt (syn.), 3: Q291, 4: Q314, 5: Q351,

6: Double mutant Q314+Q351, 7: Triple Mutant Q291+Q314+Q351

8: Wt (com).

The supernatants (A) from both wild type (commercial and synthetic) and mutant Q291 are all seen to have migrated to the same extent indicating that there are identical in size. The results are consistent in that the wild type and Q291 are present to a similar extent when

compared against each other, yet these are both present to a greater extent than that seen in the mutants Q314 and Q351. The gel shows that the Q314 and Q351 proteins are migrating further than those of the wild type and Q291. This is expected as the reduction in mass seen with the loss of sugar moieties due to the inability to become glycosylated has caused them to migrate further.

There seems to be no secretion of the double or triple mutant (Lane 6 and 7); this could suggest that the lipase enzyme has not been secreted and thus that the sugar groups might contribute to translocation of the protein.

B

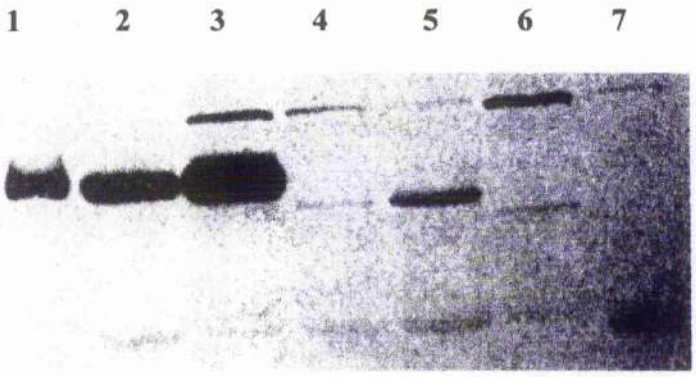


Figure A.6b: Cell extract samples containing the lipase 1 enzyme from *P. pastoris* as detected by Western blotting.

The cell extracts from the transformed *P. pastoris* containing one of each of the lipase enzymes, were prepared after a four day induced expression;

Lane 1: Wt (com.), 2: Wt (syn), 3: Q291, 4: Q314, 5: Q351,

6: Double mutant Q314+Q351, 7: Triple Mutant Q291+Q314+Q351

In the cell extracts (B) a similar picture can be seen as far as the single mutation products are concerned, although there is also evidence of a product in the case of the double mutant

Q314 + Q351. Some partial products of possibly degradation (lower bands) or the presence of attached accessory proteins encountered during the secretion process could also be contributing to the higher bands seen in this gel.

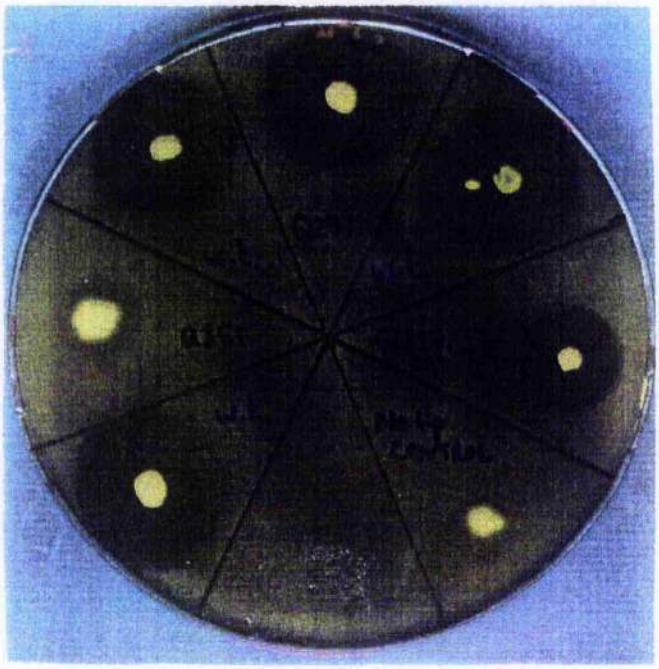


Figure A.7: Colony growth on a tributyrin plate of *P. pastoris.*

A colony of each of the transformed cultures of *P. pastoris* containing the wild type and mutant lipase was plated out onto a fresh tributyrin plate and the extent of the opaque area around each colony growth was related to the activity of the lipase secreted.

The activity of the various lipase enzyme is shown on the above tributyrin plate (Fig. 7.7). Colonies of similar size were grown from the original transformed *Pichia* and allowed to grow for 48 hours. The activity shown above indicates the following hierarchy of expression as noted by the size of halo around each colony: wild-type>Q291>Q314>Q351. Such differences in the size of the halo generated around each colony could be related primarily to the extent of expression as these have been noted to be different (FigA.6 a,b) or it might be that the actual activity of the enzymes are different.

Concluding remarks

These initial results are encouraging and have given some idea as to the effect of the mutations on protein size (Fig A.6 a,b) and possible expression and activity levels (Fig. A.7). It is anticipated that future work will look in greater detail at the size of the secreted product, the secretion mechanism, and ultimately the investigation of the activity and kinetics of the purified protein.

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