# Studies of Isocitrate Dehydrogenase from *Bacillus caldotenax*

by

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## Abbreviations

The abbreviations used in this thesis are those recommended by the Biochemical Society, London, except for those listed below:

| | |
|---|---|
| Bicene | N,N-bis (2-hydroxyethyl) glycine |
| BSA | Bovine serum albumin |
| CAPS | Cyclohexylaminopropane sulfonic acid |
| CS | Citrate synthase |
| DNase | Deoxyribonuclease |
| DTT | Dithiothreitol |
| fplc | Fast protein liquid chromatography |
| hplc | High pressure liquid chromatography |
| ICDH | Isocitrate dehydrogenase |
| ICL | Isocitrate lyase |
| IMDH | 3-Isopropylmalate dehydrogenase |
| IPTG | Isopropyl-β-thiogalactoside |
| MS | Malate synthase |
| Mops | Morpholino propane sulphonic acid |
| NADP | Nicotinamide-adenine dinucleotide phosphate |
| OAA | Oxaloacetate |
| PEP | Phosphoenol pyruvate |
| Pi | Orthophosphate |
| PTH | Phenylthiohydantoin |
| RNase | Ribonuclease A |
| SDS | Sodium dodecyl sulphate |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| Tris | Tris (hydroxymethyl) aminoethane |
| X-gal | 5-Bromo-4-chloro-3-indoyl-β-galactoside |

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## Summary

Most micro-organisms contain an NADP-dependent isocitrate dehydrogenase (ICDH) activity. During growth of micro-organisms on acetate as the sole carbon source the two enzymes of the glyoxylate bypass, isocitrate lyase (ICL) and malate synthase (MS), are induced. These two enzymes are required to generate the intermediates of the TCA cycle that are used for biosynthesis. Under these conditions there is competition between the TCA cycle enzyme ICDH and the glyoxylate bypass enzyme ICL for available isocitrate. In *E. coli* this competition is overcome by the partial phosphorylation and inactivation of ICDH. This mechanism of control has been observed in other enteric bacteria.

In recent years there has been increasing interest in the study of thermophilic bacteria due to their possible applications in industrial processes. Despite the interest in these organisms very little is known about their central metabolism. This study has concentrated on the central metabolic enzyme ICDH from the thermophile *Bacillus caldotenax*. During the course of this study the enzyme has been purified, its regulatory properties studied and the gene for ICDH has been cloned and sequenced.

A purification procedure for *B. caldotenax* ICDH was developed using ammonium sulphate fractionation followed by ion-exchange and dye-ligand chromatography. *B. caldotenax* ICDH is a dimer of subunit molecular weight 46,000 daltons and is similar in size and subunit composition to *E. coli* ICDH. The similarity between these two enzymes was further established by the fact that *B. caldotenax* ICDH cross-reacted with an antibody raised to *E. coli* ICDH.

*In vitro* phosphorylation studies demonstrated that it was possible to inactivate *B.caldotenax* ICDH, using purified *E. coli* ICDH kinase, in the presence of ATP. The observed decrease in ICDH activity was associated with the phosphorylation of ICDH. The stoichiometry of phosphorylation of *B. caldotenax* ICDH was determined by comparison to be the same as that of *E. coli* ICDH, i.e .1mol of phosphate/mol of subunit.

The possibility that *B. caldotenax* ICDH was controlled by phosphorylation *in vivo*. was investigated. A 46,000 dalton protein was seen to become phosphorylated in crude extracts of *B. caldotenax* incubated with [$\gamma$-$^{32}$P]ATP but immunoprecipitation experiments confirmed that the protein was not ICDH. No evidence for phosphorylation of *B. caldotenax* ICDH in intact cells was obtained. However it remains possible that phosphorylation might be observed in conditions other than the ones tested.

Amino acid sequence was obtained from peptides of pure *B. caldotenax* ICDH .The sequence was used to design a probe for cloning the ICDH gene by reverse genetics. A 4.5 kb fragment of *B. caldotenax* genomic DNA was cloned into the plasmid vector pUC18. The fragment contained the complete coding region of ICDH which was sequenced together with the flanking regions of DNA. The gene for ICDH consists of an open reading frame of 1272 bp coding for a protein of 422 amino acids. The amino acid sequence of *B. caldotenax* ICDH was compared with that of *E. coli* ICDH and the sequences found to be 70% identical.

The C-terminus of a second open reading frame was identified upstream to the gene for ICDH. The translated open reading frame could be aligned with significant sequence similarity to the C-terminal end of the amino acid sequences from a large number of bacterial citrate synthases. It was concluded from this alignment that in *B. caldotenax* the gene for citrate synthase lies upstream to that of ICDH. The close proximity of these two genes suggested the possibility that they might constitute an operon.

# Chapter 1

# Introduction

## 1.1 Introduction

### 1.1.1 Isocitrate dehydrogenase

Isocitrate dehydrogenase (ICDH) catalyses the oxidative decarboxylation of isocitrate to give 2-oxoglutarate and carbon dioxide. The enzyme is found in most micro-organisms, plants and animals (Plaut, 1963). There are two types of ICDH, one dependent on NADP, the other dependent on NAD. Both types of ICDH have been found in eukaryotes (Kornberg and Pricer, 1951). The two types of ICDH are distributed differently within the cell, NAD-dependent ICDH being found exclusively within the mitochondria, having an important role in the TCA cycle (Ernster and Navazio, 1956; Sung and Hsu, 1957). The NADP-dependent form is largely found within the cytoplasm where it functions to provide reducing equivalents for biosynthesis. Studies carried out on a number of species of bacteria showed that most prokaryotes only contain the NADP-dependent form of ICDH (Ragland *et al.*, 1966).

NADP-dependent isocitrate dehydrogenase (threo-Ds-isocitrate $NADP^+$ oxidoreductase (decarboxylating) EC.1.1.1.42, hereafter ICDH) has a requirement for $Mn^{2+}$ (Adler *et al.*, 1939; Moyle, 1956). They showed that $Mn^{2+}$ could be substituted with $Mg^{2+}$ but this was less effective. NADP is the natural coenzyme for this type of ICDH, NAD is not active.

Bacterial NADP-dependent ICDHs seem to fall into two main types. For example, two isoenzymes of NADP dependent ICDH, termed IDH-I and IDH-II, have been isolated from the psychrophilic bacterium, *Vibrio* ABE-1 (Ishii *et al.*, 1987). IDH-I is a dimer of molecular weight 88,100 daltons, having a similar subunit composition to *E. coli* ICDH which is a dimer of 90,000 daltons. Most other purified bacterial ICDHs also take this dimeric form. The second type of ICDH, IDH-II is a monomeric enzyme of molecular weight 80,500 daltons; other similar monomeric forms of ICDH have been purified from other prokaryotes. These two types of ICDH are discussed further in section 1.9.

### 1.1.2 The Tricarboxylic Acid Cycle

Cells contain several central metabolic pathways into which all carbon sources are directed and from which all constituents are derived. The central metabolic pathways produce precursors for biosynthesis, generate energy in the form of ATP and produce necessary reducing power. In aerobic organisms or tissues this is achieved by complete combustion of nutrients to $CO_2$ and $H_2O$.

The Tricarboxylic acid cycle (TCA cycle) or Krebs cycle is the central metabolic pathway in most organisms serving as the terminal pathway of oxidation. In each turn of the cycle acetate, as acetyl-CoA, condenses with oxaloacetate (OAA) to form citrate. Subsequent reactions result in the stepwise oxidation of citrate to regenerate oxaloacetate with the loss of two carbon atoms as $CO_2$ (figure 1.1) (Krebs and Johnson 1937). The coenzymes NADH, NADPH and $FADH_2$ are generated in the cycle and can be used in reductive biosynthesis or to regenerate ATP via oxidative phosphorylation.

Many of the intermediates of the cycle are used as precursors of macromolecular constituents of the cell (Roberts *et al.*, 1955; Gilvarg and Davies, 1956). For example OAA is used to synthesize aspartate which in turn supplies the amino acids methionine, lysine, threonine and isoleucine together with the pyrimidines of nucleic acids. Therefore during growth, intermediates of the TCA cycle are constantly being removed for biosynthetic purposes. Removal of these intermediates would prevent regeneration of OAA and hence cause breakdown of the cycle. If growth is to continue these intermediates must be replenished, therefore anaplerotic pathways operate to maintain the levels of intermediates of the central pathways (Kornberg, 1966).

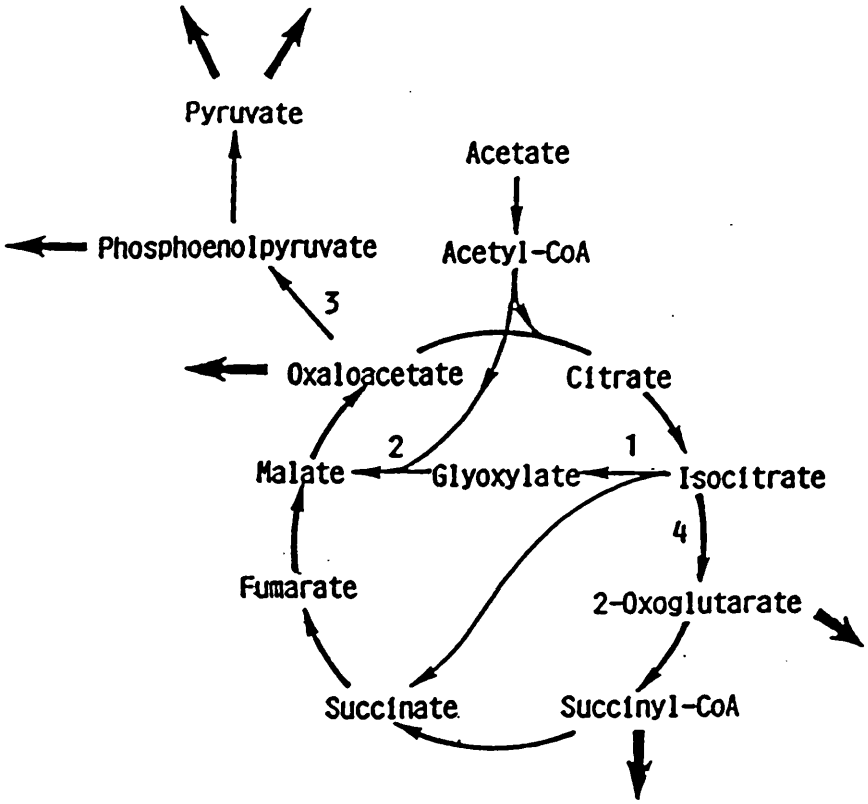



**Figure 1.1 The glyoxylate bypass and the TCA cycle**

The reactions numbered 1-4 are catalysed by the enzymes isocitrate lyase, malate synthase-A, phosphoenolpyruvate carboxykinase, and isocitrate dehydrogenase respectively. The heavy arrows indicate fluxes to biosynthesis.

### 1.1.3 The Glyoxylate Bypass

Operation of the TCA cycle results in the loss of both carbon atoms of the acetyl unit as $CO_2$, and by itself would allow no net accumulation of carbon. However *E. coli* is able to grow on acetate or fatty acids as the sole carbon source. This is achieved by diverting flux from the two $CO_2$ evolving steps of the TCA cycle through the glyoxylate bypass (figure 1.1).

The glyoxylate bypass is an anaplerotic sequence which serves to replace the intermediates of the TCA cycle which are drawn away for biosynthetic purposes. During growth on acetate or fatty acids as sole carbon source the two unique enzymes of the glyoxylate bypass isocitrate lyase (ICL) and malate synthaseA (MSA) are induced (Kornberg, 1966; Vanderwinkel and DeVliegher, 1968). There is a second malate synthase induced during growth on glycolate and compounds which are metabolised to glycolate as sole carbon source (Falmange *et al.*, 1965 ).

ICL (threo-Ds-isocitrate glyoxylate-lyase EC.4.1.3.1.) was first observed in sonic extracts of *Pseudomonas aeruginosa* which catalysed the reversible formation of succinate and glyoxylate on the addition of citrate or cis-aconitate (Campbell *et al.*, 1953). This result was later confirmed using several bacteria and fungi, and Ds(+)-isocitrate was established as the true substrate (Olsen, 1954; Saz, 1954; Smith and Gunsalus, 1954). The second enzyme MSA (EC.4.1.3.2) catalyses the condensation of glyoxylate with acetyl-CoA to generate malate (Wong and Ajl, 1956). Later experiments using labelled acetate showed that ICL and MSA act together to give the overall reaction shown below (Kornberg and Madsen, 1958; Kornberg and Quayle, 1958; Kornberg *et al.*, 1960).

**acetylCoA + isocitrate + $H_2O$ = succinate + malate + CoA**

As a result of the activity of the glyoxylate bypass there is the net gain of one $C_4$-dicarboxylic acid, a TCA cycle intermediate. Since the intermediates of the TCA cycle are interconvertible and OAA can be converted to phosphoenolpyruvate (PEP) by PEP carboxykinase, the bypass can generate all the precursors required for biosynthesis.

Flux through ICDH is essential to produce 2-oxoglutarate and succinyl-CoA for biosynthesis as well as energy and reducing power. Therefore when the glyoxylate bypass is in operation a branch point is created at the level of isocitrate. Under these conditions there is competition between the TCA cycle enzyme ICDH and the glyoxylate bypass enzyme ICL for available isocitrate. In *E. coli* growing on acetate the maximum catalytic activity of ICDH is potentially much greater than that of ICL. In addition the Km of ICDH for isocitrate is much lower than that of ICL (Ashworth and Kornberg, 1963; Bautista *et al.*, 1979). Therefore to allow flux through the glyoxylate bypass to occur there must be a method of either activating ICL, inhibiting ICDH or both. The control of this branch point has been most extensively studied in *E. coli.*

## 1.2 Control of the branchpoint between ICL and ICDH in *E. coli*

There is no evidence to suggest that ICL from *E. coli* is allosterically controlled. Likewise ICDH from *E. coli* does not seem to be controlled by any allosteric inhibitors or activators (Ragland *et al.*, 1966). It has been suggested that ICDH is subject to concerted inhibition by glyoxylate plus OAA. This inhibition is kinetically complex. Inhibition is partly caused by the unstable compound oxalomalate which forms non-enzymically from the condensation of glyoxylate and OAA. Oxalomalate is a potent competitive inhibitor of ICDH but decomposes spontaneously to form 4-hydroxy-2-oxoglutarate, a much less potent inhibitor of ICDH (Nimmo, 1986). Several groups have reported the condensation of OAA and glyoxylate to be too slow to account for inhibition of ICDH by oxalomalate (Shiio and Ozaki, 1968; Marr and Weber, 1968; Johanson and Reeves, 1977).

Inhibition is also caused by the slow binding of OAA and glyoxylate to the ICDH isocitrate binding site (Nimmo, 1986). However this is not thought to be physiologically significant as the intracellular concentrations of these inhibitors are probably too low (Nimmo, 1986). Moreover, glyoxylate and OAA might be expected to activate ICDH rather than inhibit it as these are end products of the glyoxylate bypass.

Holms and Bennett (1971) whilst studying the levels of TCA cycle enzymes in *E. coli* ML308 during growth on different carbon sources, provided the first evidence that ICDH may be controlled by a reversible modification. They showed that specific activities of ICDH, malate dehydrogenase (MDH) and 2-oxoglutarate dehydrogenase, in crude extracts remained constant when growth on limiting glycerol had ceased. However after cessation of growth on limiting glucose, although the activities of MDH and 2-oxoglutarate dehydrogenase remained constant, the activity of ICDH fell to 20% of its original value over a 2 hour period, recovering to 75% over the next 2hours. The reason for this observation was that *E. coli* excretes acetate during growth on glucose but not on glycerol. After cessation of growth on glucose the enzymes of the glyoxylate bypass were induced and the acetate was oxidised. The specific activity of ICDH decreased during this period of adaptation to use of acetate but increased once all available acetate had been utilised.

The specific activity of ICDH in cells growing on acetate as carbon source was 30% of the corresponding value for cells growing on glucose or glycerol. When cells were removed from the acetate medium, or on addition of compounds such as pyruvate which made the operation of the glyoxylate bypass unnecessary, a 3-4 fold increase in ICDH activity was observed. The specific activity of ICDH decreased again on restoration to an acetate medium or when the added carbon source, e.g. pyruvate had been exhausted. Changes in activity were rapid, independent of protein synthesis and not caused by low molecular weight effectors. Bennett and Holms (1975) concluded that a reversible covalent modification might be involved. In addition they showed that this mechanism was not

unique to *E. coli* but could be observed in at least one strain each of *Salmonella typhimurium, Klebsiella aerogenes* and *Serratia marcescens.*

## 1.3 Phosphorylation in the control of ICDH activity

### 1.3.1 Early studies

Phosphorylation was first implicated in the control of ICDH activity by Garnak and Reeves (1979 a, b). *E. coli* was grown on limiting glucose in a low phosphate medium. At the beginning of stationary phase $^{32}P$ and acetate were added to the culture. A 51,000 dalton radiolabelled protein was extracted from these cells which co-purified with ICDH and was found to be immunologically identical to ICDH. The radioactive band corresponded with ICDH activity in native gels suggesting the protein was phospho-ICDH. In retrospect, the material was probably a mixture of phosphorylated and dephosphorylated forms. The phosphorylated residue was found to be serine by using anion-exchange chromatography followed by acid hydrolysis.

Borthwick *et al.* (1984 a) purified active ICDH from cells grown on glycerol and inactive and partially active ICDH from cells grown on acetate. These three proteins had identical mobilities on SDS polyacrylamide gels corresponding to a subunit molecular weight of 45,000 daltons. Non-denaturing gel electrophoresis, however, did show differences between the three proteins. Active ICDH gave a single band when stained either for protein or for activity. Partially active ICDH showed two bands when stained for protein. The higher mobility band did not appear on gels stained for ICDH activity. Protein staining of inactive ICDH showed one band of the same mobility as the higher mobility band of the partially active ICDH. It was shown that these differences in mobility were due to charge and not size differences. In addition alkali-labile phosphate measurements showed that active and inactive ICDH correspond to dephosphorylated and phosphorylated forms respectively, differing in one phosphate/ subunit.

### 1.3.2 Phosphorylation of ICDH *in vivo*

Phosphorylation of ICDH was established to be of physiological significance by Borthwick *et al.* (1984 b). These workers correlated changes in the phosphorylation state of ICDH with changes in the activity of the enzyme.

When pyruvate was added to cells growing on acetate, in the presence of $^{32}Pi$, ICDH activity rose 4 fold over 20-30 min, declining to its basal level after 50 min. Denatured cell pellets were run on SDS gels. A $^{32}P$ containing band of protein of the same molecular weight as ICDH was identified. Addition of pyruvate resulted in a loss of $^{32}P$ from this band but there was an increase in $^{32}P$ in this band after 40-60 min. This observation was not due to changes in the amount of ICDH present which remained constant throughout the experiment. It was evident that there is an inverse correlation between the phosphorylation state of ICDH and its activity in intact cells.

Cell extracts were prepared during the same experiment and incubated in the presence of purified ICDH kinase/phosphatase, under conditions promoting dephosphorylation. For samples with a low initial ICDH activity (e.g. before addition of pyruvate) this treatment caused an increase in ICDH activity to the value seen in extracts after addition of pyruvate. It was concluded therefore that the reversible inactivation of ICDH observed *in vivo* is solely due to reversible phosphorylation. Borthwick *et al.*, (1984 b) isolated chymotryptic peptides from ICDH labelled *in vivo* and *in vitro*. The same serine residue was found to be phosphorylated both *in vivo* and *in vitro*.

This work confirmed that changes in the activity of ICDH could be interpreted in terms of changes in its phosphorylation state and thus must reflect control of the activities that interconvert the phosphorylated and dephosphorylated forms of ICDH.

## 1.4 Isocitrate dehydrogenase kinase/phosphatase

### 1.4.1 The bifunctional nature of ICDH kinase/phosphatase

The phosphorylation and dephosphorylation of ICDH are carried out by a single bifunctional kinase/phosphatase enzyme. The first indication that this enzyme was bifunctional came during the development of a purification protocol for this enzyme. LaPorte and Koshland (1982) found that the two activities were eluted together from a variety of chromatographic media. The purified protein gave a single band with SDS electrophoresis and catalysed both reactions efficiently.

The bifunctional nature of the kinase/phosphatase was confirmed when the locus encoding the protein was cloned. The gene encoding ICDH kinase/phosphatase (*aceK*) was cloned by complementation of an *ace*K mutation (LaPorte and Chung, 1985) which restored the host's ability to grow on acetate. The clone expressed both ICDH kinase and ICDH phosphatase activities and was shown to encode a polypeptide with the same molecular weight (65,000 daltons) as the purified protein.

The nucleotide sequence of the gene for ICDH kinase/phosphatase has been established independently by two groups (Klumpp *et al.*, 1988 and Cortay *et al.*, 1988) which confirmed that the kinase and phosphatase were expressed from the same gene.

A number of workers have studied the organisation of the active site (or sites) in ICDH kinase/phosphatase and there is now a considerable amount of evidence suggesting that there is only a single active site within ICDH kinase/phosphatase. The activity of ICDH kinase/phosphatase has an unusual requirement for either ATP or ADP (LaPorte and Koshland, 1982). Examination of the sequence of ICDH kinase/phosphatase showed that it contained only a single region which matched the consensus for ATP binding sites (Klumpp *et al.*, 1988). This site contains an invariant lysine residue which has been found in the ATP binding site of every protein kinase sequence to date. Stueland *et al.* (1989)

carried out site directed mutagenesis converting this lysine to a methionine residue; the allele produced was refered to as *aceK*(lys>met). This lys>met mutation inhibited both ICDH kinase and phosphatase activities by at least 500 fold. The possibility of proteolytic degradation was ruled out by Western blotting, the protein was present at wild type levels but was inactive. A single mutation had abolished both kinase and phosphatase activities indicating that the kinase and phosphatase may share the same ATP binding site and therefore probably the same active site.

Affinity labelling of ICDH kinase/phosphatase with the ATP analogue [$^{32}$P]azido-ATP also gave evidence for a single ATP binding site. This analogue inactivated both activities in parallel presumably because of its covalent attachment at, or near, the active site. Proteolysis of the labelled protein followed by chromatography yielded a single peak of radioactivity again confirming the existence of only a single ATP binding site (Varela and Nimmo 1988).

Random mutagenesis of *aceK* resulted in several mutants which retained their kinase activity but not their phosphatase activity (Stueland *et al.*, 1987). It was discovered that these mutants suffer a drastic loss in their affinity for phospho-ICDH. These mutations had the same effect on the kinase. The mutant kinase, unlike the wild type enzyme, was not subject to product inhibition by phospho-ICDH. As a result of the mutation both the kinase and the phosphatase had simultaneously lost their ability to bind phospho-ICDH suggesting that the two reactions occur at the same active site.

Stueland *et al.* (1987) showed that ICDH kinase/phosphatase had ATPase activity, catalysing the hydrolysis of ATP even in the absence of ICDH. In addition they found that this activity was greater than that of the phosphatase and kinase and was retained even when the enzyme was complexed with either ICDH or phospho-ICDH.

In an attempt to combine all these pieces of evidence with regards to the mechanism of ICDH kinase/phosphatase LaPorte *et al.* (1989) put forward a working model for this protein. The model proposes that ICDH kinase/phosphatase has a single active site and that the phosphatase reaction results from the kinase back reaction tightly coupled to hydrolysis of ATP. The phosphatase reaction would require the formation of a ternary complex involving ICDH kinase/phosphatase, ADP and phospho-ICDH. The phosphate would then be transferred from phospho-ICDH to ADP and then to water the net result being dephosphorylation (figure 1.2). This model is only speculative but it satisfactorily explains all the evidence produced to date.

### 1.4.2 Molecular organisation and control of the gene for ICDH kinase/phosphatase and enzymes of the glyoxylate bypass

In *E. coli* the genes encoding the enzymes of the glyoxylate bypass, malate synthase A (*aceB* ) and isocitrate lyase (*aceA* ) together with the gene for ICDH kinase/phosphatase (*aceK*), in this order, comprise an operon called the glyoxylate bypass operon (Malloy and Nunn, 1982; LaPorte *et al.*, 1985). This operon is located at 90 min on the *E. coli* chromosomal map.

Expression of the ace operon is negatively controlled at the transcriptional level by two genes *iclR* and *fadR* (Malloy and Nunn, 1982) which code for repressors. The gene for *iclR* has been cloned (Negre *et al.*, 1991) and overexpressed and the repressor has been purified to homogeneity (Cortay *et al.*, 1991). Studies using the purified IclR protein showed it to bind to the promoter region of the *ace* operon. The *fadR* gene has also been cloned and is known to participate in the regulation of fatty acid degradation (*fad* ) regulon (Simons *et al.*, 1980).

It has been suggested by Malloy and Nunn (1982) that the iclR and fadR repressors can act

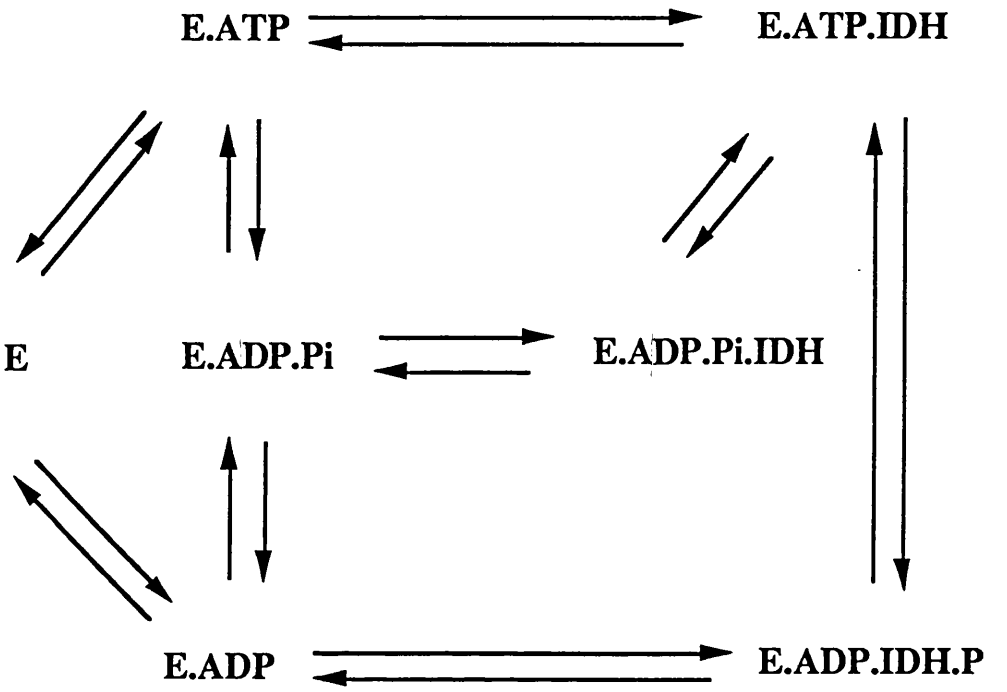



**Figure 1.2 Model for the mechanism of ICDH kinase phosphatase**

The model proposes that the ICDH kinase and ICDH phosphatase reactions are catalysed at the same active site and that the ICDH phosphatase reaction results from the ICDH kinase reaction tightly coupled to ATP hydrolysis. Taken from LaPorte *et al.*(1989).

independently to cause partial repression of the *ace* operon or together to cause complete repression. The operon could therefore be activated to different levels depending on the carbon source.

Expression of the glyoxylate bypass is induced during growth on acetate, however addition of acetate to *E. coli* growing on glucose did not induce ICL (Kornberg, 1963). This suggests that neither acetate nor acetylCoA act directly as inducers. Kornberg (1966) proposed that PEP or a metabolite close to PEP was more likely to be the inducer. In support of this view experiments with purified IclR and the promoter /operator region of the ace operon showed reduced binding in the presence of PEP but not in the presence of acetate, acetylCoA, pyruvate or OAA (Cortay *et al.*, 1991).

The mechanism of control of the *ace* operon is complex and the metabolites which regulate its expression have yet to be identified. Further research in this area is required before the regulatory mechanism is fully elucidated.

### 1.4.3 Biochemical regulation of ICDH kinase/phosphatase activity

*In vitro* and *in vivo* studies have identified a large number of effectors which can alter the activity of ICDH kinase and/or phosphatase, (LaPorte and Koshland, 1983; Nimmo and Nimmo, 1984). These effectors and their actions are summarised in figure 1.3. Isocitrate, 3-phosphoglycerate, pyruvate, AMP, ADP, oxaloacetate, 2-oxoglutarate and PEP activate ICDH phosphatase and inhibit ICDH kinase, whereas NADP, citrate, fructose-6-phosphate and glyoxylate inhibit the kinase having little effect on the phosphatase. Considering the intracellular concentrations of these metabolites (Lowry *et al.*, 1971; Bautista *et al.*, 1979; Morikawa *et al.*, 1980; El-Mansi *et al.*, 1985) the effectors most likely to be significant *in vivo* are isocitrate, PEP, 3-phosphoglycerate, AMP, ADP and NADPH (Nimmo and Nimmo, 1984).

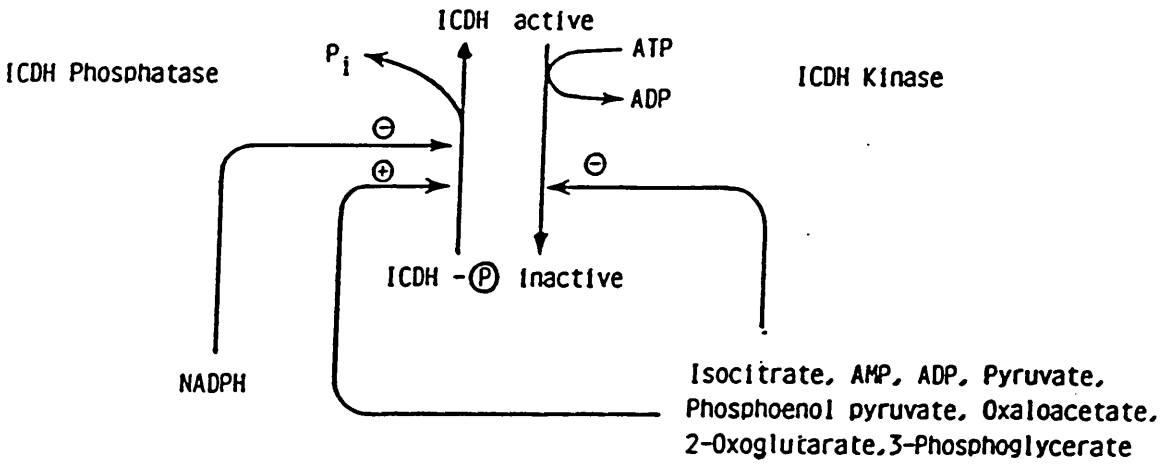



**Figure 1.3 Control of ICDH kinase/phosphatase**

Taken from Nimmo (1984). + and - represent stimulatory and inhibitory effects respectively.

The metabolite isocitrate plays a key role in controlling the phosphorylation state of ICDH. The Km of ICDH for isocitrate has been estimated in intact cells to be 5μM whereas that of ICL was estimated to be >260μM (Nimmo *et al* ., 1988), much higher than the usual intracellular concentration of isocitrate (Bautista *et al.*, 1979; El-Mansi *et al.* , 1985). It was proposed by Nimmo (1984) that during growth on acetate phosphorylation renders the ICDH rate limiting in the TCA cycle, resulting in an increase in the intracellular concentration of isocitrate. This increase in the isocitrate concentration facilitates flux through the glyoxylate bypass. A similar mechanism to explain how phosphorylation affects the division of flux between the TCA cycle and glyoxylate bypass was also proposed by Walsh and Koshland (1985).

There is a substantial amount of evidence, both experimental and theoretical, in support of these proposed mechanisms. Support for the role of isocitrate was provided by El-Mansi *et al.*, (1985; 1986). These workers showed the intracellular concentration of isocitrate in *E.coli* to be much higher during growth on acetate than during growth on glucose or glycerol. Moreover the addition of pyruvate to cells growing on acetate caused a transient 2-fold increase in the intracellular concentration of isocitrate. The role of phosphorylation seems to be to maintain the intracellular concentration of isocitrate at a level such that it can sustain the necessary flux through the glyoxylate bypass to produce biosynthetic precursors. The other effectors of ICDH kinase/phosphatase (figure 1.3) can also be explained in terms of metabolic control.OAA, pyruvate, 3-phosphoglycerate, 2-oxoglutarate and PEP are all precursors of biosynthesis. One would expect high levels of these to promote flux through the TCA cycle rather than the glyoxylate bypass. All these effectors are capable of completely inhibiting the kinase and activating the phosphatase (Nimmo and Nimmo, 1984). High concentrations of AMP and ADP represent a decrease in the energy charge of the cell and therefore also favour flux through the TCA cycle, activating the phosphatase and inhibiting the kinase. In addition NADPH acts to inhibit the phosphatase (Nimmo and Nimmo, 1984). NADPH is one of the end products of the TCA cycle and this effect may be thought of as feedback inhibition.

Further amplification of the sensitivity of the ICDH phosphorylation cycle is the result of "zero-order ultrasensitivity" (LaPorte and Koshland, 1983) which refers to the situation where one or both converter enzymes are saturated with their substrates. The phosphorylation system can also respond to changes in the ICDH concentration *in vivo* (LaPorte *et al.*, 1985).

In conclusion the system acts to integrate a variety of information in the form of metabolite concentrations. This information is used to adjust the phosphorylation state of ICDH so as to optimise the division of flux between the TCA cycle and glyoxylate bypass.

## 1.5 Mechanism of inactivation of ICDH by phosphorylation

### 1.5.1 Inactivation is a result of the negative charge of the phosphate

Many eukaryotic enzymes regulated by phosphorylation are allosteric proteins and phosphorylation often results in changes in the affinity of the target enzyme for a substrate or allosteric factor rather than changes in Vmax. In contrast ICDH is not an allosteric protein and the phosphorylated form is almost totally inactive. The unusual nature of this phosphorylation event has led a number of groups to investigate molecular mechanisms for the way in which phosphorylation causes inactivation of ICDH.

ICDH kinase transfers the γ-phosphoryl group of ATP to the serine 113 residue of ICDH and this causes complete loss of enzyme activity when measured under Vmax conditions. Thorsness and Koshland (1987) showed that the inactivation of ICDH by phosphorylation was a result of introduction of the negative charge of the phosphate. They determined the complete nucleotide sequence of the gene for ICDH and then carried out site directed mutagenesis of the serine residue at position 113. Substitution of serine with aspartate resulted in complete inactivation of ICDH suggesting that it was the introduction of a negative charge in this position, when phosphorylated, which causes loss of activity.

### 1.5.2 Phosphorylation inactivates ICDH by preventing isocitrate binding

Two proposed mechanisms for the inactivation of ICDH by phosphorylation have been suggested. Garland and Nimmo (1984) proposed that phosphorylation inactivated ICDH by preventing NADP from binding. However more recent results obtained by Dean *et al.* (1989) indicate that phosphorylation inactivates ICDH by preventing isocitrate from binding. Garland and Nimmo suggested that the NADP-binding site contained one or more positive charges that interact with the phosphate group of the co-enzyme and that this charge-charge interaction could trigger a conformational change which would block NADP binding. A certain amount of evidence was available in favour of this idea. Firstly inactive ICDH was unable to bind to a Procion red-Sepharose column whereas active ICDH bound to the column and could be eluted with NADP (Borthwick *et al.*, 1984 a). It was therefore suggested that ICDH bound to the column via its NADP binding site. Garland and Nimmo also provided support for their idea from fluorescence studies which showed enhanced fluorescence of NADPH in the presence of native enzyme but not in the presence of the phosphorylated enzyme. Finally experiments by Garland and Nimmo using limited proteolysis showed that binding of NADP to the active enzyme significantly increased resistance to proteolysis, whereas it did not protect the phosphorylated form of the enzyme. The interpretations made from these experiments were that inactive enzyme cannot bind NADP and that binding of NADP and phosphorylation cause similar conformational changes.

Dean *et al.* (1989) carried out equilibrium binding studies which showed active ICDH to bind to α-ketoglutarate, NADP, NADPH and isocitrate at 1:1 ratios of enzyme to substrate monomer. In contrast phosphorylated ICDH was unable to bind isocitrate but maintained the ability to bind NADP and NADPH. These studies indicated that phosphorylation inactivates ICDH by preventing isocitrate from binding. Dean *et al.* (1989) correctly pointed out that the earlier evidence was indirect and that the failure of phospho-ICDH to bind to Procion-red Sepharose could be explained by other reasons. However, it is still true

that the binding of NADP and phosphorylation of ICDH have similar effects on the enzyme. Further evidence of this was provided by McKee and Nimmo (1989). They showed the arginine specific reagent phenylglyoxal to inactivate ICDH by reacting with one arginine per subunit. This residue was protected from attack by either the presence of NADP or phosphorylation of ICDH. A mutant (R112Q) of ICDH was constructed (G.A. Nimmo personal communication) in which the arginine residue at position 112 was replaced by glutamine. This mutant was completely resistant to attack by phenylglyoxal confirming Arg112 to be the site of attack.

### 1.5.3 Steric hindrance and electrostatic effects due to phosphorylation

As mentioned previously in section 1.5.1 variants of ICDH have been made, using site directed mutagenesis, in which Ser113 was replaced with either aspartate or glutamate (Thorsness and Koshland, 1987; Dean and Koshland, 1990). The structures of these two mutant enzymes were determined using X-ray crystallography and compared to those of active ICDH and inactive ICDH (Hurley *et al.*, 1990). Such comparisons showed the structure of the mutant enzymes to be be more similar to that of the active form of ICDH than that of the phosphorylated form. There were only slight structural shifts due to the presence of a negatively charged residue at position 113. Slight conformational changes were evident in phospho-ICDH as compared to the active, dephosphorylated form of the enzyme. However, as both mutant enzymes were also inactive it was apparent that inactivation is not a result of conformational changes.

Calculations made on the observed structures indicated that the change in electrostatic potential due to the introduction of a negative charge, either by site directed mutagenesis or phosphorylation is sufficient to inactivate ICDH. This prediction, based on structural and kinetic data, was confirmed by Dean and Koshland (1990) using a combination of site directed mutants and a substrate analogue of 2R, 3S-isocitrate (2R-malate) which lacks the

carboxyl group. The use of mutants in which Ser113 had been substituted with aspartate or glutamate enabled the importance of electrostatic effects as a result of phosphorylation to be established. Similarly substitution with bulky residues such as tyrosine allowed for analysis of the steric effects of the phosphate group. Measurements of kinetic parameters of these mutant proteins were made in conjunction with the use of the isocitrate analogue, 2R-malate. The results indicated that the inactivation of ICDH by phosphorylation results from electrostatic repulsion and steric hindrance between the phosphoryl moiety and the γ-carboxyl group of isocitrate and to a lesser extent from the loss of the hydrogen bond between the γ–carboxyl group of isocitrate and serine113.

## 1.6 Other molecular mechanisms of phosphorylation

The regulation of enzymic activity by protein phosphorylation results from structural changes at the active site. There are known to be at least three different molecular mechanisms through which this control is mediated (Reviewed by Barford, 1991).

a) Control by allosteric interactions; studies on glycogen phosphorylase showed phosphorylation of this enzyme to occur at a site distinct from the active site which results in long range conformational changes causing the enzyme's activity to increase (Sprang *et al.*, 1988).

b) Control by direct interaction; the molecular mechanism of control of bacterial ICDH by phosphorylation falls into this category. As described in previous sections inactivation of the enzyme by phosphorylation at the active site results in the direct inhibition of substrate binding.

c) Control by phosphorylation of pseudo-substrate auto-inhibitory domains; this mechanism was proposed to explain the activation of pp70 ribosomal S6 protein kinase (Banerjee *et al.*, 1990). The model proposes that the enzyme has a low basal activity as a result of a pseudo-substrate binding to the catalytic site. Phosphorylation of the pseudo-substrate domain causes it to dissociate from the active site thus activating the enzyme.

## 1.7 The structure of *E. coli* ICDH

X-ray crystallography has been used to determine the structure of *E. coli* ICDH (Hurley *et al.*, 1989). The structure was found to consist of three domains, a large α+ß domain a small α /ß domain and an α/ß clasp domain involving both subunits. Two pockets line the cleft between the large and small domains.

With the exception of the recently determined structure of *Thermus thermophilus* isopropylmalate dehydrogenase, the *E. coli* ICDH structure was found to be topologically different from all other known dehydrogenase structures. The best known dehydrogenase structure is that of the lactate dehydrogenase-like class which includes lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. The structure of the catalytic domains of these dehydrogenases vary, however the structure of their nucleotide binding domains are similar. This structure is termed the Rossmann or LDH fold after the enzyme it was first described for (Rossman *et al.*, 1974) (figure 1.4). ICDH was found to contain a nucleotide binding fold which had not previously been characterised.
The phosphorylated residue ser113 was found to lie in the front pocket on the protein's surface, presumably accessible to ICDH kinase /phosphatase. The position of the residue within the pocket was compatible with there being a direct phosphoserine-substrate interaction. Within this pocket there were a number of conserved residues including arg119, arg129, arg153, tyr160 and lys230. The conservation of these positive charges suggest a binding site for a negatively charged substrate and maybe a means of stabilising the oxyanion intermediate believed to occur in the ICDH reaction.

Comparisons of the amino acid sequence of ICDH with the known amino acid sequences of other proteins showed it to only have significant homology to isopropylmalate dehydrogenase (IMDH) (Thorsness and Koshland, 1987). The structure of *T.thermophilus*

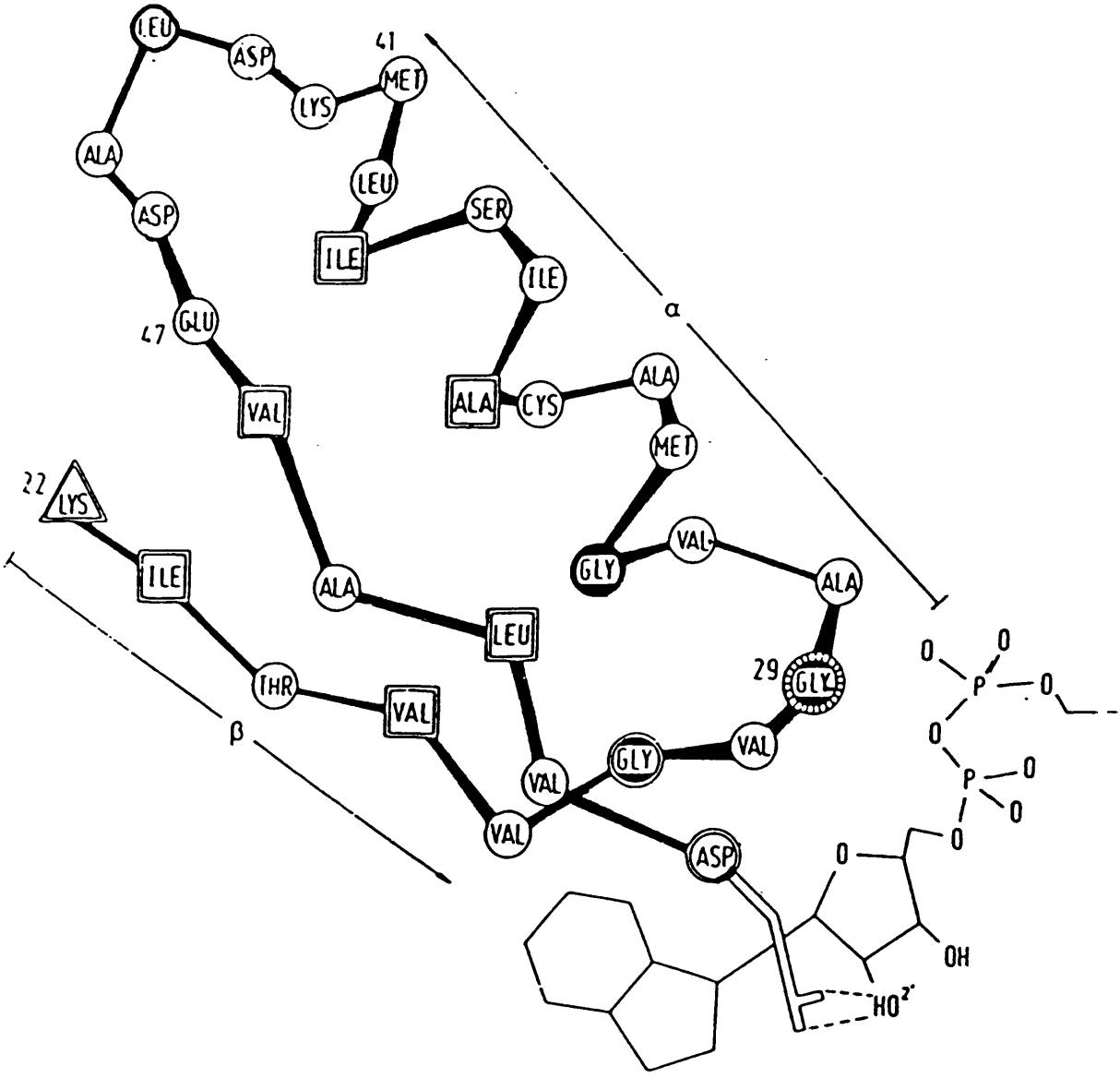



**Figure 1.4 Schematic diagram of the LDH fold**

An $NAD^+$ molecule is shown in the position in which it is bound to the Spiny dogfish M-lactate dehydrogenase (LDH). From Wierenga *et al.*,(1986).

□= basic or hydrophobic amino acids.

Δ= small and hydrophobic amino acids.

IMDH has recently been determined (Imada *et al.*, 1991) and was found to have a similar nucleotide binding fold to that of ICDH.

## 1.8 Protein phosphorylation in prokaryotes

The control of ICDH by phosphorylation in *E. coli* was the first example of prokaryotic phosphorylation to be well characterised. It is now widely accepted that phosphorylation plays an important role as a regulatory control mechanism in prokaryotes, however this has not always been the case. For many years it was thought that protein phosphorylation did not occur in prokaryotic cells (e.g. Rubin and Rosen, 1975). Protein kinase activity had been detected in cells infected with bacteriophage T7 however kinase activity in uninfected cells was negligible. In addition U-V irradiation of the phage genome prevented appearance of the protein kinase whereas irradiation of the host genome did not. It was therefore concluded that the kinase activity was a phage gene product (Rahmsdorf *et al.*, 1974; Zillig *et al.*, 1972). However in 1978, Wang and Koshland used pulse labelling experiments to demonstrate the existence of at least four $^{32}P$ containing proteins in *Salmonella typhimurium* . These proteins were labelled *in vitro* by the addition of [$\gamma$-$^{32}P$] ATP to crude cell extracts; $^{32}P$ was incorporated as either phosphoserine or phosphothreonine residues.

Likewise Manai and Cozzone (1979) observed similar results in *E. coli* . Cortay *et al.* (1986) showed it was possible to resolve over 100 phosphorylated proteins from *E. coli*, much more than observed in previous studies, although few of these have been identified. Similar studies in other micro-organisms, such as *Salmonella typhimurium* (Ferro-Luzzi Ames and Nikaido, 1981) and *Clostridium sphenoides* (Antranikian, 1985 a) gave similar results although in these cases fewer phosphorylated proteins were detected than in *E. coli* . Contrary to original belief protein phosphorylation in prokaryotes is now believed to be a common occurrence.

Evidence has been obtained from prokaryotic studies for examples of phosphorylation in metabolic control (*e.g.* the regulation of citrate lyase ligase (Antranikian, 1985 b) ) and in the control of transport processes (the regulation of the phosphotransferase system in Streptococci (Reizer, 1983) ). There is also evidence that phosphorylation controls gene expression *e.g.* the transcription of the gene for glutamine synthase, *glnA* (Ninfa and Magasanik, 1986).

Glutamine synthetase is the central enzyme in bacterial nitrogen metabolism and under limiting conditions mediates the entry of ammonia into anabolic metabolism. When cells are starved of nitrogen expression of the gene for glutamine synthetase (*glnA* ) is induced. This response to nitrogen starvation is one of a number of examples of a prokaryotic response system which uses a phospho-transfer network to convey an environmental signal to a range of response elements. These response elements control cell motility and regulation of gene expression. Other examples are reviewed in Stock *et al* . (1990).

In all these examples two families of signal transduction proteins are employed to produce a response. One of these proteins is a histidine protein kinase (HPK) which uses ATP to phosphorylate itself at a histidine residue. The phosphoryl group is then transferred from the HPK to an aspartic acid side chain of the second protein, known as a response regulator which can control a wide variety of cellular activities including gene expression.

The full implications of protein phosphorylation in prokaryotes are as yet unclear as so few target proteins have been identified. However it is clear that phosphorylation functions as an important regulatory control mechanism.

## 1.9 The control of ICDH in other prokaryotes

As described in previous sections the control of ICDH by phosphorylation in *E. coli* has

been intensively studied and both the molecular details and physiological role are well established. It is likely that this mechanism is not unique to *E. coli* but operates in other prokaryotes. As a result of the early work of Bennett and Holms (1975) (section 1.2) it was thought likely that ICDH was controlled in a similar manner in other enteric bacteria including *Klebsiella aerogenes, Salmonella typhimurium* and *Serratia marsescens.*

ICDH has been purified from a large number of prokaryotic species including species of *Bacillus, Thermus* and cyanobacteria. ICDH from all these species was of a comparable size to *E. coli* ICDH and had the same subunit composition. However this is not the case in all prokaryotes. Studies on the micro-organism *Acinetobacter calcoaceticus* showed it to contain two isoenzymes of NADP linked ICDH. The two forms were termed isoenzyme-I and isoenzyme-II (Self and Weitzman, 1972). The two isoenzymes were originally separated by ion exchange chromatography or zonal ultracentrifugation. More recently f.p.l.c. MonoQ chromatography has been utilised in separating the two forms.

The two isoenzymes differ in the pH dependence of their activity, kinetic parameters, stability to heat or urea and molecular size. Isoenzyme-I has a molecular weight under 100,000 daltons resembling the NADP linked ICDH from *E. coli* . Isoenzyme-II resembles the NAD linked ICDH of eukaryotes in that it is a much larger enzyme of approximate molecular weight 300,000 daltons. In addition isoenzyme-II is allosterically controlled as is the NAD linked ICDH of higher organisms. The activity of the eukaryotic enzyme is dependent on the intracellular isocitrate concentration and enzyme activity is stimulated by AMP or ADP. Likewise the activity of *A. calcoaceticus* isoenzyme-II is also stimulated by AMP or ADP. A high concentration of AMP and ADP reflect a low energy charge within the cell and one might expect high levels of these to promote activity of the TCA cycle. In addition isoenzyme-II activity is increased by low concentrations of glyoxylate or pyruvate. Kinetic studies showed this stimulation to result from an increase in Vmax and a decrease in the apparent Km for both substrates. ICDH competes with the glyoxylate bypass enzyme, ICL, for available isocitrate and a balance has to be achieved at this branch

point. The stimulation of ICDH by glyoxylate, a product of the glyoxylate bypass, may help to maintain the balance by partitioning isocitrate appropriately between the two pathways. There is some evidence to suggest that isoenzyme-II has two distinct regulatory sites, one for adenylate regulation and one for regulation by glyoxylate and pyruvate. Interaction of these sites with their specific effectors is thought to cause conformational changes leading to an increase in enzyme activity (Self *et al* ., 1973).

Reeves *et al.* (1986) showed ICDH of *A. calcoaceticus* to exhibit unique behaviour during growth on acetate. These workers reported the specific activity of NADP dependent ICDH to increase whilst growing on acetate. This is in contrast to other bacterial ICDH's studied which show a decrease in activity under these conditions. As mentioned previously *A. calcoaceticus* contains two isoenzymes of ICDH. During growth on acetate an increase in the relative proportion of isoenzyme-II and a corresponding decrease in the amount of isoenzyme-I was observed. The increase in the allosterically controlled enzyme might have implications in the control of the branchpoint between the TCA cycle and the glyoxylate bypass, however, precisely how regulation is achieved is unsure.

Isoenzymes of NADP-dependent ICDH have been identified in other prokaryotes. Studies of the bacterium *Vibrio* ABE-I showed this micro-organism to contain two isoenzymes of ICDH termed IDH-I and IDH-II (Ishii *et al.*, 1987). IDH-I is a dimeric form of the enzyme with a subunit molecular weight of approximately 50,000 daltons; this form is similar to that of *E. coli* ICDH. The second form of ICDH, IDH-II, is a monomer of subunit molecular weight approximately 80,000 daltons. The N-terminal amino acid sequence of the two forms have been determined and show no similarity, the two forms are immunologically distinct. The two isoenzymes were found to be differentially regulated *in vivo* under varying growth conditions. IDH-I was induced during growth on acetate whereas levels of IDH-II remained almost unchanged. The biological significance of this induction is uncertain but again may have implications in the control of the branch point.

ICDH has recently been purified from *Streptomyces coelicolor* (R.D. Taylor personal communication) and also from *Rhodomicrobium vannielii* (Leyland and Kelly, 1991). Both these enzymes are monomeric proteins of approximate molecular weight 80,000 daltons. On the basis of this information and on the immunological properties and N-terminal sequence of these enzymes it was proposed that these two types of ICDH could be grouped, with IDH-II from *Vibrio spp.* ABE-1, into a seperate class of enzymes (R.D. Taylor personal communication). It was proposed to call this class of enzymes the type II IDHs.

## 1.10 Thermophilic micro-organisms

### 1.10.1 Introduction

Most organisms which have been studied in great depth are adapted to growth at moderate temperatures and operate within a relatively narrow temperature range. In recent years there has been increasing interest in thermophilic micro-organisms which are adapted to environments of extreme temperature, such as thermal springs.

Thermophiles have been classified according to their maximum and optimum growth temperatures. The classification is somewhat imprecise but in general organisms which have optimum growth temperatures between 50-85°C are termed moderate thermophiles. Micro-organisms with optimum growth temperatures above 85°C are termed extreme thermophiles, some archaebacteria have been isolated with optimum growth temperatures as high as 110°C. (Wiegel and Ljungdahl, 1986; Stetler, 1982).
In order to survive at these temperatures the cellular constituents of thermophiles such as enzymes, nucleic acids and lipids and organised structures of this material, such as ribosomes and membranes also function at these high temperatures. How they are able to do this, in many cases, is as yet unknown.

### 1.10.2 Advantages of thermophiles

Thermophiles are industrially important micro-organisms that offer a number of advantages over mesophiles for use in biotechnology processes. Because of their ability to grow at high temperatures their enzymes have a greater thermostability than those of mesophiles. In addition they are often extremely stable towards denaturing agents (Kristjansson, 1989). It is generally accepted that the increased thermostability of the enzymes is the result of an increase in the number of hydrophobic bonds, ionic interactions and disulphide bridges which stabilise all proteins. A considerable number of such enzymes have been purified from thermophiles and are commercially available. Examples of these are the DNA polymerases obtained from *Thermus aquaticus* (*Taq* polymerase) and more recently from *Pyrococcus*. DNA polymerase is employed in several molecular biology techniques, including nucleotide sequencing and the polymerase chain reaction (PCR). The extreme thermostability of these enzymes allows them to withstand the high temperatures often required for these procedures.

In addition to being a source of thermostable enzymes thermophiles have other advantages. When micro-organisms are grown on an industrial scale a considerable amount of cost is incurred by cooling processes for maintenance of the correct growth temperature. If thermophiles are used in place of mesophiles then these costs will be reduced. One such example of an industrial processes which uses thermophiles is starch hydrolysis for the production of high fructose syrup.

### 1.10.3 *Bacillus caldotenax*

*Bacillus caldotenax* was the micro-organism used in this study. First isolated in 1969 by Epstein and Grossowicz it is a moderate thermophile having an optimum growth

temperature of between 55-60°C. A slender gram positive bacillus, it is 4-6μm long, 0.2μm wide and actively motile. Studies of this micro-organism by Epstein and Grossowicz showed it to be very similar to the moderate thermophile *Bacillus stearothermophilus,* differing only in its ability to hydrolyse starch.

*B. stearothermophilus* has been studied in considerable depth by many researchers. A wide variety of enzymes have been isolated from this organism including isocitrate dehydrogenase (Howard and Becker, 1970). In addition other aspects of *B. stearothermophilus* have been researched such as physiology and genetics, while comparatively little research has been carried out on *B. caldotenax* . Researchers in the past have found the growth requirements of *B. caldotenax* to be less fastidious than those of *B.stearothermophilus* . For this reason *B. caldotenax* was used in this study in preference to *B. stearothermophilus*. Although some aspects of thermophilic micro-organisms are fairly well documented very little is known about the regulation of their metabolism or how it compares to that of mesophiles.

## 1.11 Aims of this study

As explained in section 1.10 thermophiles are industrially important micro-organisms yet relatively little is understood about the regulation of their metabolism. In order to make any rational approaches to improving microbial biotechnological processes involving thermophiles it will be necessary to make use of or manipulate the regulation of enzyme activities.

The regulation of the TCA cycle enzyme ICDH and the control of isocitrate metabolism in *E. coli* have been well established. The aim of this project was to carry out a comparative study using the moderate thermophile *B. caldotenax.* It was thought that *B. caldotenax*

would be a good model system for studying the control of central metabolic pathways and for the isolation of key genes in thermophiles.

# Chapter 2

# Materials and methods

## 2.1 Materials

### 2.1.1 Chemicals and biochemicals

General chemicals of analytical reagent grade were obtained from BDH Ltd., Poole, U.K.; Boehringer Mannheim, Lewes, U.K.; Formachem Ltd, Strathaven, U.K.; FSA Laboratory Supplies, Loughborough, U.K.; Koch-Light Ltd, Haverhill, U.K.; May and Baker, Dagenham, U.K..

Ampicillin, dithiothreitol (DTT), ethidium bromide, Ficoll, Mops buffer , benzamidine hydrochloride, bovine serum albumin (for Bradford's protein determination), Coomassie Brilliant Blue G250, DL-isocitrate (trisodium salt), Tris buffer and Triton X-100 were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K..

Acrylamide, ammonium sulphate (enzyme grade), HCl (Aristar), 2-mercaptoethanol, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylenediamine, ATP, and NADH were obtained from Boehringer Mannheim, Lewes, U.K..

Amberlite MB3, bromophenol blue, caesium chloride and SDS were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K..

Xylene cyanol was obtained from IBI Ltd., Cambridge, U.K..

Agarose, 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal), III), isopropyl-β-thiogalactoside (IPTG), phenol (ultrapure), urea (ultrapure) and 1kb ladder were obtained from Gibco, BRL Ltd., Paisley, U.K..

Bactotryptone, yeast extract and bactoagar (agar) were obtained from Difco, Detroit, USA. Oxoid No.1 agar and trypticase soy broth were obtained from Oxoid Ltd., London, U.K..

Oligonucleotides were synthesised on an Applied Biosystems Model 280A DNA synthesiser using phosphoramidate chemistry by Dr. Veer Math (Department Of Biochemistry).

[$\gamma$-$^{32}$P]ATP and [$\alpha$-$^{35}$S]-dATP (code SJ.304) were obtained from Amersham International plc., Amersham, U.K..

The Sequenase version 2.0 sequencing kit was obtained from United States

Biochemical Corporation (distributed by Cambridge BioScience, Cambridge, U.K.).

### 2.1.2 Enzymes and proteins.

The following enzymes were obtained from Boehringer Mannheim, Lewes, UK.

rabbit muscle aldolase (EC 4.1.2.13)

pig muscle lactate dehydrogenase (EC 1.1.1.27)

pig heart malate dehydrogenase (EC 1.1.1.37)

rabbit muscle pyruvate kinase (EC 2.7.1.40)

lysozyme (EC 3.2.1.17)

calf intestine alkaline phosphatase

A kit for molecular weight determination and the following enzymes and proteins were obtained from Sigma Chemical Co., Poole, UK.

bovine albumin

egg albumin

rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)

bovine erythrocyte carbonic anhydrase (EC 4.2.1.1)

bovine pancreas trypsinogen (EC 3.4.21.4)

soybean trypsin inhibitor

bovine milk α-lactalbumin

*Escherichia coli* β-galactosidase (EC 3.2.1.23)

rabbit muscle phosphorylase b (EC 2.4.1.1)

ribonuclease A (RNase A)

Normal goat serum and peroxidase conjugate goat anti rabbit IgG were from the Scottish Antibody Production Unit Ltd., Carluke, Lanarks, U.K..

*Staphylococcus aureus* V8 protease was obtained from Miles, Slough, U.K..

All restriction enzymes, T4 DNA ligase, and T4 Polynucleotide kinase were obtained from BRL, Gibco Ltd., Paisley, UK..

### 2.1.3 Chromatography media

Sephadex G-50 was supplied by Pharmacia, Milton Keynes, UK.

Procion-red Sepharose was a gift from Dr H.G. Nimmo.

### 2.1.4 Pre-packed media

Pre-packed Mono Q, and Superose 12 columns were obtained from Pharmacia and utilised on a Pharmacia f.p.l.c. system. Genpak FAX and μ Bondapak C18 reverse phase columns were purchased from Waters Chromatography, Watford, U.K. and attached to a Beckman System Gold h.p.l.c. apparatus ( Beckman Instruments Inc., High Wycombe, U.K.).

## 2.2 General biochemical methods

### 2.2.1 pH measurements

All pH measurements were made with a Radiometer pH probe calibrated at room temperature using standards of pH 7.0 and pH 4.0 prepared from tablets obtained from the manufacturer.

### 2.2.2 Conductivity measurements

Conductivity measurements were made on a Radiometer conductivity meter, type CDM2e (Radiometer, Copenhagen, Denmark).

### 2.2.3 Glassware and plastics

Glassware was washed in Haemo-sol solutions (Alfred Cox (Surgical) Ltd., U.K.), rinsed with tap water and then distilled water and dried in an oven. Plastic tips for micropipettes and Eppendorf tubes were taken from a newly opened bag.

### 2.2.4 Distilled water

Glass distilled water stored in polythene containers was used in all experiments.

### 2.2.5 Protein estimations

Protein concentrations were determined by the method of Bradford (1976). This method is

based on the binding of Coomassie Brilliant Blue to the protein. The reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G250 in 50ml of 95% (v/v) ethanol and 85% (w/v) orthophosphoric acid. The reagent was made up to 1 litre with distilled water and filtered. For each of the assays, a standard curve in the range 0–25μg protein was obtained using a 1mg/ml BSA solution which was made up assuming that a 1mg/ml BSA solution has an absorbance of 0.62 at 280nm. To each sample, 2.5ml of Bradford Reagent were added, the samples vortexed and allowed to stand for 2 min. The absorbances were recorded at 595nm. The amount of protein present in the unknown samples was estimated from the standard curve. Protein concentrations of the fractions eluted from columns were monitored at 280nm.

### 2.2.6 Dialysis

Dialysis membranes (Scientific Instruments Centre Ltd., London), were boiled for 5 min in 1% (w/v) EDTA, pH7.0, stored in 20% ethanol and rinsed in distilled water prior to use.

### 2.2.7 Preparation of chromatographic media

All columns were poured at room temperature and packed at higher flow rates than would be used during a run, then moved to a 4°C cold room. Columns were stored in 0.02% (w/v) sodium azide and equilibrated with starting buffer before use.

### 2.2.8 Spectrophotometric determination of nucleic acid concentrations

Nucleic acid concentrations were determined spectrophotometrically at 260nm (Sambrook *et al.*, 1989). In a 1 cm path length quartz cuvette an absorbance of 1.0 corresponds to 50 μg/ml for double stranded DNA, 40 μg/ml for single stranded DNA and approximately 20 μg/ml for single stranded oligonucleotides.

### 2.2.9 Sterilisation

Solutions for the preparation of growth media and solutions used in manipulation of nucleic acids were autoclaved in a B&T Autoclave 225E (Laboratory Thermal Equipment U.K.) at 15 p.s.i. for 25 min, except carbon sources which were autoclaved at 5 p.s.i. for 50 min. Because of their probable heat lability, the following compounds were sterilised by filtration

through 0.22 μm pore-sized Millex G.V. filters (Millipore Ltd., U.K.) into sterile bottles: ampicillin, methionine and IPTG.

### 2.2.10 Gel drying and autoradiography

Polyacrylamide gels were dried onto Whatman 3MM paper using a Biorad Laboratories Gel Drier model 1125 connected to an Aquavac Junior multi-purpose vacuum unit (Uniscience Ltd., London).

$^{32}P$ in polyacrylamide gels or on filters was detected by exposure to FujiRX film using intensifying screens at -70°C. $^{35}S$ was detected by exposure to film in the absence of intensifying screens at room temperature. Films were developed by a Kodak X-OMAT processor Model ME-3.

## 2.3 Microbiological techniques:

### 2.3.1 Bacterial strains and plasmids

The *E. coli* strains are derivatives of *E. coli* K12. The bacterial strains used in this study are shown below;

| Bacterial strain | Relevant characteristics | Reference /source |
|---|---|---|
| *E. coli* DS941 | *rec* F*lac* Iq*lac* ZdelM15 | Gift from Prof. D. Sherratt (Institute of Genetics, Glasgow University) |
| *E. coli* XL1-blue | *supE hsdR lac*-F'*proAB*$^{+}$lacI$^{q}$*lac*ZΔM15 | Bullock *et al.* (1987) |

*Bacillus caldotenax* was obtained from The Public Health Laboratory Service, Centre for Applied Microbiology and Research (P.H.L.S. C.A.M.R.), Porton, Wilts.

The plasmid pUC18 was obtained from Pharmacia and the plasmid pBluescript ™ II KS$^{+}$ was

obtained from Stratagene Ltd. (La Jolla, CA, USA).

### 2.3.2 Growth Media

Unless otherwise stated, all media were sterilised by autoclaving at 15 p.s.i. for 25 min.

a) *Bacillus caldotenax* growth media:

| | | |
|---|---|---|
| Solid growth media (per litre) | trypticase soya broth | 30g |
| | Difco agar | 15g |

| | | |
|---|---|---|
| Liquid culture media (per litre) | $NH_4Cl$ | 2.14g |
| | $KH_2PO_4$ | 0.3g |
| | yeast extract | 1.0g |
| | tryptone | 1.0g |

The remaining constituents were added by filtration, post sterilisation.

| | |
|---|---|
| $MgCl_2$ | 0.03g |
| $CaCl_2.6H_2O$ | 0.005g |
| $FeSO_4.7H_2O$ | 0.004g |
| Kuhns salts | 1.5ml |
| methionine | 0.15g |
| biotin | 0.0015g |

In addition 5ml of a 50% solution of a carbon source were added to the media, this was generally glutamate. The pH of the media was adjusted to pH7 with $NaOH/H_3PO_4$. The growth temperature was 60°C.

b) *Escherichia coli* growth media:

| | | |
|---|---|---|
| L-Broth (per litre) | bactotryptone | 10g |
| | yeast extract | 5g |
| | NaCl | 10g |
| | (+5ml 20% (w/v) glucose) | |
| | pH7.5 with NaOH | |
| L-Agar (per litre) | As for L-broth, +15g Difco-agar. | |
| 2xTY Medium (per litre) | bacto-tryptone | 16g |
| | yeast extract | 10g |
| | NaCl | 5g |

### 2.3.3 Selection supplements

The following supplements were added to rich media to select and identify recombinant organisms:

a) antibiotics

Ampicillin (Amp) was used at a final concentration of 50 µg/ml. A stock solution of 25 mg/ml was filter sterilised and stored at -20°C. Hot L-agar was cooled to 55°C before ampicillin was added. L-Amp plates were stable for at least 4 weeks if stored at 4°C.

b) chromogenic substrates

X-gal and IPTG were used at a final concentration of 20 ng/ml. A stock solution of IPTG of 20 mg/ml was filter sterilised and stored at -20°C. A stock solution of X-gal (20 mg/ml) was made up in DMF and stored at -20°C.

### 2.3.4 Growth of bacteria

a) *Bacillus caldotenax*

For general purposes cells were grown on a large scale using an 8 litre fermenter at the Public Health Laboratory Service, Centre for Applied Microbiology and Research (P.H.L.S. C.A.M.R.), Porton, Salisbury. For the purpose of phosphorylation studies cells were grown on a small scale in 250ml conical shake flasks on an orbital shaker at 60°C.

b) *Escherichia coli*

10ml of media in a 20ml universal bottle was inoculated from a loop of a single colony from a plate or from 10µl of a glycerol stock. 100ml of media in a 250ml conical flask was inoculated with 500µl of an overnight 10ml L-broth culture. Cultures were grown on an orbital shaker at 37°C.

### 2.3.5 Measurement of growth

Bacterial cell density was measured as an apparent absorption at either 420nm or 600nm. The measurements were carried out in 1 cm light path cuvettes using a Unicam SP500 spectrophotometer (Pye Unicam Instruments Ltd., U.K.) equipped with a Gilford photoelectric detector and digital read out. If necessary, the culture samples were diluted 1:10 to give an absorbance of less than 0.5.

### 2.3.6 Harvesting of bacteria

Cells were harvested by centrifugation at 6,000g for 15 min at 4°C (MSE 18 with an 8x250ml rotor).

### 2.3.7 Disruption of bacteria

a) Ultrasonic Disruption

A sample of culture (2.5ml) was placed in a 2-dram vial within a brass holder (Holms and Bennett, 1971) surrounded by an ice/water slurry. The sample was ultra-sonicated (Dawe Soniprobe type 1130A from Dawe Instruments Ltd., London) for six 30 sec periods alternating with five 30 sec cooling periods.

b) French pressure cell disruption

Bacterial cell pellets were gradually resuspended in 2 volumes of 50mM potassium phosphate pH7, 1mM EDTA, 2mM $MgCl_2$, 1mM benzamidine, 1mM DTT at 4°C and disrupted by 2 passages through a French pressure cell (Cat. No. 4-3398A, American Instruments Company, Maryland, U.S.A.) at a pressure of 98 MPa (14,300 p.s.i.). The pressure cell, capacity 40ml, was cooled on ice before use.

## 2.4 Enzyme assays

### 2.4.1 Equipment

Unless otherwise stated all spectrophotometric assays were performed at 50°C using a Gilford Unicam SP 500 spectrophotometer equipped with a Gilford photoelectric detector and recorder. Quartz cells with a path length of 1cm were used. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the disappearance of 1µmol of substrate, or the appearance of 1µmol of product / min. For the purpose of kinetic measurements a Perkin-Elmer LS-5 luminescence spectrometer was used with an excitation wavelength of 340nm and a detection wavelength of 460nm (slit width 10nm for both).

### 2.4.2 Assay for isocitrate dehydrogenase

ICDH activity was assayed by a method based on that of Reeves *et al.* (1972). The assay contained in a total volume of 1ml, 0.15 M Tris/HCl buffer pH7.5, 0.5mM $MnCl_2$, 0.4mM $NADP^+$, 2.5mM DL-isocitric acid and enzyme suitably diluted. The reaction was initiated by the addition of the enzyme and the reduction of $NADP^+$ was monitored at 340nm.

### 2.4.3 Assay for isocitrate dehydrogenase kinase/phosphatase

ICDH kinase/phosphatase activity was measured essentially by the method of Nimmo *et al.* (1984). The incubation was contained in a total volume of 90µl consisting of, 25µl 50mM Mops-NaOH pH7.3, 45µl 50mM Bicine/NaOH pH 9 containing 2mM DTT, 9µl 50mM ATP, 1µl 1M $MgCl_2$. A suitable concentration of purified ICDH from either *E. coli* or *B.*

*caldotenax* and *E. coli* ICDH kinase/phosphatase were also present in the assay mixture. Incubations were carried out at 37°C the reactions being initiated by the addition of the ATP solution. Samples were removed over a time course to assess ICDH activity (section 2.4.2 above) hence any change in ICDH activity was monitored.

## 2.5 Polyacrylamide gel electrophoresis of proteins

### 2.5.1 Denaturing electrophoresis

Protein samples were analysed by discontinuous slab gel electrophoresis in the presence of SDS according to Laemmli (1970). Glass plates were separated by 1.5mm spacers and sealed with 1.0% (w/v) agarose. Gels were prepared from the volumes of stock solutions shown in table 2.1. The acrylamide, Tris buffer and distilled water were mixed and degassed; SDS, ammonium persulphate and TEMED were added and the gel was poured into the cast immediately. Isopropanol was layered onto the separating gel and rinsed off upon polymerisation. The stacking gel was then made up and poured around a well-forming template. The wells were rinsed with distilled water after polymerisation. The electrophoresis buffer consisted of 25mM Tris/192mM glycine, pH8.8, 0.1% (w/v) SDS. Electrophoresis was carried out at a constant current of 50mA.

Protein samples were mixed with at least an equal volume of sample buffer, consisting of 50mM Tris/HCl pH6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 1% (v/v) 2-mercaptoethanol. For the purpose of phosphorylation experiments, where larger sample volumes were involved, 5x concentrated sample buffer was used.

### 2.5.2 Protein staining

Protein was routinely localised on gels by staining with Coomassie blue. The Coomassie reagent was 0.1% Coomassie brilliant blue G250 in 10% (v/v) glacial acetic acid, 50% (v/v) methanol and destain reagent was 10% acetic acid, 10% methanol. These procedures were carried out at 40°C. When a more sensitive detection method was required gels were silver

**Table 2.1 Preparation of polyacrylamide gels**

Quantities and stock solutions used:

| stock solution | stacking gel (ml) | separating gel (ml) | |
|---|---|---|---|
| | | **10%** | **15%** |
| 30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide | 1.5 | 10 | 15 |
| 1.5M Tris/HCl pH8.8 | - | 7.5 | 7.5 |
| 0.5M Tris/HCl pH6.8 | 3.75 | - | - |
| $H_2O$ | 9.45 | 12.05 | 7.05 |
| 10% (w/v) SDS | 0.15 | 0.3 | 0.3 |
| 10% ammonium persulphate (freshly prepared) | 0.15 | 0.15 | 0.15 |
| TEMED | 0.01 | 0.01 | 0.01 |

stained. The silver reagent was 0.8% (w/v) $AgNO_3$, 0.08% (w/v) NaOH, in 200 mM $(NH_4)OH$. The developer reagent was 0.02% (v/v) formaldehyde, in 0.24 mM sodium citrate. The method was adapted from Wray *et al.* (1981). Gels were first soaked in 50% (v/v) methanol for at least 8 hours, then incubated for 8 minutes in freshly prepared silver reagent. After washing with distilled $H_2O$ for one hour the gel was developed with fresh developer until protein bands appeared. The reaction was then terminated by washing with distilled $H_2O$ and the gels stored in 10% (v/v) acetic acid, 10% (v/v) methanol.

## 2.6 Purification buffers

The buffers used in the purification of ICDH from *B. caldotenax* were as follows:

buffer A 50mM $KH_2PO_4$, 1mM EDTA pH7

buffer B 10mM $KH_2PO_4$, 1mM EDTA, 5mM potassium citrate, 5mM $MgCl_2$, 10% glycerol, pH7

The pH of these buffers was adjusted with KOH

## 2.7 Peptide mapping (Cleveland Mapping).

Peptides were generated from ICDH by the method of Cleveland (1977). Homogenous ICDH was run on a 10% polyacrylamide gel by the system of Laemmli (section 2.5.1), stained with Coomassie Brilliant Blue (section 2.5.2) and rapidly destained. The bands of protein were excised from the gel and rinsed several times in 0.125M Tris/HCl pH 6.8 containing 1mM DTT and 0.1% SDS. The gel chips containing ICDH were loaded onto a 15% polyacrylamide gel and overlayered with 20µl of buffer (0.125M Tris/HCl pH 6.8, 1mM DTT, 0.1% SDS, 20% glycerol) followed by 50ng of protease in a volume of 20µl. The gel was run at 45mA until the dye front was nearing the end of the stacking gel, the current was then switched off for 30 min to allow digestion to occur. After this time the gel was run to completion. Peptides for protein sequence analysis were electroblotted (section 2.9.3). Otherwise peptides were visualised by silver staining (section 2.5.2).

## 2.8 Immunoblotting techniques

The method is based on that described by Towbin *et al.* (1970) as modified by Batteigner *et al.* (1982).

Stock solutions:

| | |
|---|---|
| Buffer A (transfer buffer) | 20mM Tris/HCl pH 7.2<br>150mM NaCl<br>0.5% (w/v) tween 20 |
| Buffer B (blocking buffer) | 20mM Tris/HCl pH 7.2<br>150mM NaCl |
| Buffer C (chloronapthol stain) | 50ml 10mM Tris/HCl pH 7.4<br>30mg chloronapthol in 10ml methanol<br>150µl 4% (v/v) $H_2O_2$ |

$H_2O_2$ was mixed immediately before use.

Experimental procedure:

SDS PAGE was carried out as in section 2.5.1. When electrophoresis was complete the proteins were transferred to 0.45M nitrocellulose paper (Adderman and Co., Kingston-upon-Thames) using a Bio-Rad trans-blot cell containing bufferA with 16.6% methanol. The current was run at 350 mA for 4 hours, after which the nitrocellulose paper was placed in buffer B containing 5% normal donkey serum at 4°C for at least 16 hours. The nitrocellulose paper was then rinsed twice in buffer B. The paper was then soaked in Buffer A containing 5% (v/v) normal donkey serum and 0.5% (v/v) antiserum at 25°C for 90 min, after which the nitrocellulose paper was washed 4x12 min in Buffer A then 1x12 min in Buffer B. The nitrocellulose paper was then soaked in Buffer B containing 5% (v/v) normal donkey serum and 0.05% (v/v) peroxidase conjugate donkey anti rabbit IgG at 23°C for 90 min, washed 5x12 min in Buffer B and developed by soaking in Buffer C at 23°C. The final product was then washed in distilled water dried and stored.

## 2.9 Protein sequencing

### 2.9.1 Preparation of intact *B. caldotenax* ICDH for protein sequencing

Approximately 1 nmol of homogenous *B. caldotenax* ICDH in a volume of 100µl 0.5% (w/v) ammonium bicarbonate (pH7.5) was loaded onto a Waters bondapak C18 reverse phase column (section 2.1.4). The column was equilibrated with milliQ grade distilled water containing 0.15% TFA at a flow rate of 1ml/min. The protein was eluted with h.p.l.c. grade acetonitrile containing 0.1% TFA, a linear gradient was run over 45 min and the eluant monitored at 280 nm. ICDH was eluted as a sharp peak at 77% acetonitrile and collected in a volume of 1ml. Sequencing was carried out as described in section 2.9.4.

### 2.9.2 Preparation of peptides for protein sequencing

Peptides were generated from 120µg of purified protein by the method of Cleveland (section 2.6). Digestion of the protein was carried out using V8 protease, the peptides were resolved in a 15% polyacrylamide gel which was electroblotted.

### 2.9.3 Electroblotting

For the purpose of sequencing all reagents used were of the highest available purity, solutions and buffers were made using milliQ grade water. A 15% polyacrylamide gel containing peptides (section 2.6 )was soaked for 5min in transfer buffer (10mM CAPS pH11, 10% (v/v) methanol). A piece of Problott™ (Applied Biosystems) membrane wetted in 100% methanol, and soaked in transfer buffer was placed on top of the gel. The gel and membrane were sandwiched between two pieces of 3MM paper and two abrasive pads, also soaked in transfer buffer, and the whole was supported in a cassette. The cassette was placed in a Bio-Rad trans-blot cell with transfer buffer. Blotting was carried out for 1hour at 50V. The Problott membrane was washed in milliQ water, dipped in 100% methanol and stained for 1min with Coomassie Brilliant Blue R-250 (CBBR 0.1%, 50% methanol, 50% water). The blot was destained in several changes of destain (50% methanol, 50% water), rinsed in water and air dried. Protein staining bands were excised from the blot and used for sequencing.

### 2.9.4 Liquid-phase sequencing

Electroblotted peptides of *B. caldotenax* ICDH and a sample of intact protein were sequenced on an Applied Biosystems model 4774 pulsed liquid phase sequencer with on-line detection of amino acid thiohydantoins by a model 120A analyser. The instrument was operated by Drs. M. Cusack and G.B. Curry, Department of Geology, University of Glasgow.

## 2.10 Molecular weight determinations

### 2.10.1 Subunit Mr

SDS PAGE was used to estimate the subunit Mr of the purified protein. A Sigma Chemical Co. molecular weight marker kit was used to calculate subunit Mr. The proteins used to produce standard curves of Rf against log Mr are listed below:

| Protein | Subunit Mr (daltons) |
|---|---|
| bovine albumin | 66 000 |
| egg albumin | 45 000 |
| rabbit muscle glyceraldehyde-3-phosphate | 36 000 |
| bovine erythrocyte carbonic anhydrase | 29 000 |
| bovine pancreas trypsinogen | 24 000 |
| soybean trypsin inhibitor | 20 100 |
| bovine milk -lactalbumin | 14 200 |

Values for subunit molecular weights were taken from Weber and Osborn (1969) and Mousdale *et al.* (1987).

### 2.10.2 Native Mr

Gel permeation chromatography on a Superose 12 column was used to estimate the native Mr of the purified protein. This was carried out at room temperature using a Pharmacia f.p.l.c. apparatus. The column was eluted with 50 mM potassium phosphate pH 7, 0.4 mM DTT (flow rate = 0.3 ml/min, fraction size 0.3 ml). The eluate was monitored at 280nm and the column

calibrated with the following proteins :

| Protein | Native Mr (daltons) |
|---|---|
| pig heart malate dehydrogenase | 70 000 |
| pig muscle lactate dehydrogenase | 140 000 |
| rabbit muscle aldolase | 160 000 |
| rabbit muscle pyruvate kinase | 232 000 |
| horse apoferritin | 440 000 |

## 2.11 Phosphorylation studies

### 2.11.1 *In vitro* phosphorylation studies

a) Detection of changes in *B. caldotenax* ICDH activity

Changes in *B. caldotenax* ICDH activity as a result of the action of *E. coli* ICDH kinase/phosphatase activity were detected using the ICDH kinase/phosphatase assay described in section 2.4.3. A suitable concentration of *B. caldotenax* ICDH was used in the assay in place of *E. coli* ICDH.

b) Detection of $^{32}P$ incorporation into ICDH

To measure the incorporation of $^{32}P$ into ICDH the ICDH kinase/phosphatase assay (section 2.4.3 ) was used. 5mM ATP was replaced in the assay with 2mM [$\gamma$-$^{32}P$]ATP (20$\mu$Ci/$\mu$mol). Samples were removed from the assay for analysis by gel electrophoresis (section 2.5.1). Gels were stained for protein with Coomassie Brilliant Blue, dried and autoradiographed (sections 2.5.2 and 2.2.10).

### 2.11.2 Phosphorylation studies in crude extracts

a) Preparation of the crude extract

Approximately 0.5g of *B. caldotenax* cells were resuspended in 3ml 50mM $KH_2PO_4$, 1mM EDTA pH 7. Cells were broken open by sonication (section 2.3.7 a). Cell debris was precipitated by 5min centrifugation in a microfuge, to obtain a cell free extract.

b) Phosphorylation in crude extracts

[γ-32P] ATP was added to the crude extract to give a final concentration of 2mM ATP (20μCi/μmol). Incubations were carried out at 50°C and samples removed over a time course for analysis by one of the two following methods:

i) Analysis by gel electrophoresis:

20μl samples were removed and the reaction stopped by boiling for 2min with 5μl 5x normal sample buffer. Samples were analysed by SDS PAGE, stained with Coomassie Blue, dried and autoradiographed (sections 2.5 and 2.2.10).

ii) Immunoprecipitation of ICDH:

30μl samples were removed, mixed gently with 50μl anti *E. coli* ICDH antiserum and incubated on ice for 60 min. The sample was then microfuged for 2 min and the supernatant discarded. The pellet was washed in 1ml 1.5M NaCl, 2mM EDTA pH 7. The sample was microfuged for a further 2 min and the supernatant discarded. The pellet was resuspended in 20μl normal sample buffer and boiled for 2min prior to analysis by SDS PAGE followed by autoradiography (sections 2.5 and 2.2.10).

### 2.11.3 Phosphorylation *in vivo*

A 10ml culture of *B. caldotenax* was grown overnight using the liquid culture media described in section 2.3.2 and acetate as the carbon source. The culture was grown in the presence of $^{32}Pi$ with a final specific activity of 20μCi/μmol.Cells were harvested by 5min microfugation in 10 x 2.5ml microfuge tubes and washed extensively with buffer (50mM $KH_2PO_4$, 10mM pyrophosphate, 1mM EDTA) to remove free $^{32}Pi$. The cells were broken open by sonication (section 2.3.7 a).

## 2.12 General recombinant DNA techniques

### 2.12.1 Small scale plasmid purification

The method employed for small scale plasmid DNA preparation was based on the method of Birnboim and Doly (1979). 5ml of L-broth, containing the appropriate antibiotic, were inoculated with a single colony of plasmid transformed cells and grown overnight. 1.5 ml of the overnight culture was harvested in a microfuge and the cell pellet resuspended in 100 µl GTE (50mM glucose, 25mM Tris/HCl, pH8.0, 10mM EDTA). 10 µl freshly prepared lysozyme (10mg/ml) was added and incubated at room temperature for 5 min. 200 µl freshly prepared 0.2M NaOH, 1% (w/v) SDS was added, mixed gently and incubated on ice for 5 min. 150 µl ice cold 5M potassium acetate (pH4.8) was added and the lysate incubated on ice for 5-15 min. Cell debris was removed by micro-centrifugation. The supernatant was removed to a fresh microfuge tube and the plasmid DNA phenol/chloroform extracted (section 2.12.8). The supernatant was placed into a fresh tube and the DNA precipitated by the addition of 2 volumes ethanol at room temperature. After incubation at room temperature for 2 min, the plasmid DNA was recovered by micro-centrifugation at 4°C (cold room) for 5 min. The pellet was washed with 200 µl 70% (v/v) ethanol, air dried for 5-15 min and resuspended in 30 µl TE (10mM Tris/HCl, pH8.0, 1mM EDTA). A 5 µl sample was sufficient for digestion by restriction enzymes. During the digestion, 1µl boiled RNase A (10mg/ml) was added to the digest for the last 15 min of the incubation at 37°C.

### 2.12.2 Large scale plasmid purification

The method employed was based on the alkali lysis method of Birnboim and Doly (1979) and was used to purify milligram quantities of plasmid DNA.

100 ml of L-broth, containing the appropriate antibiotic, were inoculated with a single colony of plasmid transformed cells. The culture was incubated at 37°C overnight (16 hours) on an orbital shaker. The bacterial cells were harvested by 10 min centrifugation at 4000g, 4°C (MSE 18, 6x250ml rotor). The cell pellet was resuspended in 5ml GTE containing a concentration of 5mg/ml lysozyme and the suspension incubated at room temperature for 5min. 10 ml of 0.2M NaOH, 1% (w/v) SDS were added, gently mixed by swirling and incubated on ice for 10 min.

Lysis was achieved by addition of 7.5 ml of ice cold 5M potassium acetate, pH4.8, followed by rocking to mix and incubation on ice for 10 min. Bacterial cell debris was removed by centrifugation (12,000g, 15 min, 4°C, MSE 18, 8x50ml rotor). The supernatant was filtered through gauze to remove floating cell debris and nucleic acids precipitated by the addition of 0.6 volumes of isopropanol. After incubation at room temperature for 15 min, the nucleic acid was recovered by centrifugation (MSE 18 8x50 ml rotor, 12,000g, 10 min, 25°C). The pellet was washed with 70% (v/v) ethanol, air dried for 10-20 min and resuspended in 5ml 50mM Tris/HCl, pH8.0, 10mM EDTA.0.5 ml ethidium bromide, 10mg/ml., was added together with CsCl to a final concentration of 1g/ml. The gradient was then set up by centrifugation at 289,000g for 16 hours at 25°C in a Beckman Ti70.1 rotor in polycarbonate tubes. The DNA bands were usually visible, however if the bands were not visible, long wavelength U.V. light was used to visualise the plasmid band which migrates in front of the host chromosomal DNA. The plasmid band was removed by gently inserting a sterile pipette tip to just below the plasmid band. Ethidium bromide was removed by extracting several times with TE (10mM Tris/HCl, pH8.0, 1mM EDTA) saturated 1-butanol. The DNA was diluted to double the volume with distilled water, precipitated twice with ethanol (section 2.12.9) and resuspended in 0.2 ml TE. The DNA concentration and purity ($A_{260}/A_{280}$) was measured spectrophotometrically.

### 2.12.3 Preparation of *B. caldotenax* genomic DNA

Total DNA of *B. caldotenax* was prepared essentially as described by Saito and Miura (1963).

### 2.12.4 Digestion of DNA with restriction enzymes

The methods used were as described in Sambrook *et al.* (1989). Restriction digests were carried out using the BRL React buffers which were provided with each batch of enzyme. There are ten different React buffers with a range of salt concentrations, each one suitable for a range of enzymes. Analytical digests were carried out in a volume of 10 or 20 µl at 37°C. Preparative digests were carried out in larger volumes. When DNA was digested with two restriction enzymes, the endonuclease requiring the lower salt buffer was used first. After the recommended duration of digestion the salt concentration was adjusted and the second enzyme

added.

### 2.12.5 Agarose gel electrophoresis

DNA was separated at room temperature on horizontal submerged agarose gels as described by Sambrook *et al.* (1989). The Tris-borate (TBE) buffer system was employed. A 0.8% (w/v) agarose gel was used to size restriction fragments accurately in the range of 0.8-10 kb. Samples for agarose gels were prepared by addition of 0.2 volumes of 10 mM Tris/HCl pH7.2, 20% (w/v) ficoll, 0.5% (w/v) bromophenol blue and 10 mg/ml ethidium bromide. Ethidium bromide (0.5 μg/ml) was added to both gel and buffer, and stained DNA bands visualised on a long wave U.V. transilluminator (U.V. Products Inc.). Restriction fragment markers of known size were run alongside the unknown fragments, usually λ DNA digested with *Hin*dIII, or with *Eco*RI+*Hin*dIII. For restriction mapping a 1kb ladder was used.

### 2.12.6 Size fractionation of restriction fragments

In order to construct a sub-library of *B. caldotenax* genomic DNA, restriction fragments within a particular size range were selected. This selection was achieved by use of a Waters GenPak FAX column (section 2.1.4). Approximately 20μg of genomic DNA were digested with an appropriate restriction enzyme. The restriction fragments were loaded onto the column which was equilibrated in buffer A (25mM Tris/HCl, 1mM EDTA pH7.5). Restriction fragments were eluted with a gradient produced using buffer B(buffer A+1M NaCl). The gradient was run from 0-75% NaCl over 40min, kept constant for 10min, and then increased to 100% over a further 20min period.The flow rate was 0.5ml/min. Fractions of 125μl volume were collected and DNA recovered by ethanol precipitation. Test samples from the fractions were analysed by agarose gel electrophoresis (section 2.12.5) to establish which fraction contained DNA of the required size.

### 2.12.7 Recovery of DNA from agarose gels:

The DNA band of interest was visualised by ethidium bromide staining and a long wave U.V. transilluminator (U.V. Products Inc.). The desired DNA band was excised from the gel. A hole was pierced in the base of a sterile 0.5ml Eppendorf using a sterile syringe needle and then

plugged with a small amount of sterile, siliconised glass wool. The agarose gel chip containing DNA was placed into the Eppendorf which in turn was placed inside a 1.5 ml Eppendorf and microfuged for 10min at 6,500 rpm. The 1.5 ml Eppendorf was then removed and the buffer, containing DNA, retained. The smaller Eppendorf was microfuged once more collecting any remaining DNA in a fresh Eppendorf. The two DNA containing fractions were pooled and microfuged at 12,000 rpm, at 4°C for 30min to remove any traces of agarose. The supernatant was decanted and DNA recovered by ethanol precipitation. DNA obtained in this way was sufficiently pure for cloning purposes.

### 2.12.8 Phenol/chloroform extraction of nucleic acids:

Restriction digests and other solutions containing DNA were deproteinised by extracting with phenol and chloroform. The volume of the sample was measured and an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added. This mixture was vortexed, allowed to stand until the two layers separated and vortexed again. The two layers were separated by centrifugation in a microfuge for 5 min. The top layer was removed into a fresh tube and an equal volume of TE added to the bottom, phenol layer. The mixture was vortexed and the two layers separated as in the first extraction. The two aqueous , top, layers were pooled and extracted in an identical manner with an equal volume of TE-saturated chloroform/isoamyl alcohol (24:1) to remove any residual traces of phenol. The DNA was recovered from the aqueous layer by ethanol precipitation (section 2.12.9).

### 2.12.9 Ethanol precipitation

Sodium acetate was added to the DNA solution to a final concentration of 0.3M by adding 0.1 volumes of a 3M solution. 2.5 volumes of cold ethanol (-20°C) were added and mixed. The mixture was then incubated at -20°C overnight or alternatively, at -80°C for 1 hour and the precipitated DNA recovered by centrifugation in a microfuge at 4°C for 30 min or for larger volumes, centrifugation at 10,000 rpm for 30 min at 4°C (MSE 18, 8x50ml rotor). The DNA pellet was washed in 70% (v/v) ethanol and resuspended in a suitable volume of TE.

An alternative to the addition of sodium acetate was to add 0.5 volumes of 7.5M ammonium

acetate, however, when the DNA was to be treated with T4 polynucleotide kinase, sodium acetate was used since ammonium ions inhibit this enzyme.

## 2.13 Ligations

The insert DNA was digested with the appropriate restriction enzymes, purified using the Genpak FAX column (2.12.6) and ethanol precipitated (2.12.11). Plasmid vector pUC18 DNA was supplied linearised by digestion with the appropriate restriction enzymes and treated with bacterial alkaline phosphatase to yield DNA ready to use. Bluescript $KS^+$ vector was linearised by digestion with the appropriate restriction enzymes and treated with calf intestinal alkaline phosphatase to yield DNA ready to use. This treatment prevented self-ligation of the vector and resulted in a low background of false positive clones.

For ligation reactions where the insert size was similar to the vector size an equal concentration of foreign DNA to vector DNA was employed. For those reactions where the insert size was much greater than the vector size, at least a four-fold excess in concentration of foreign DNA to vector DNA was typical. Depending on the size of insert, ligation mixtures contained 25 ng vector and between 25 and 400 ng insert in a final volume between 5 and 10 µl.

Vector plus insert were pre-incubated at 45°C for 5 min to melt any cohesive termini that had reannealed. The ligations were performed overnight in 66 mM Tris/HCl pH 7.6, 6.6 mM $MgCl_2$, 0.5 mM ATP, 10 mM DTT at 16°C using 0.5 Units (Weiss *et al.*, 1968) of bacteriophage T4 DNA ligase.

## 2.14 Transformation of *E. coli* with plasmid DNA

### 2.14.1 Preparation of competent cells

A single colony of *E. coli* DS941 or *E. coli* XL1-blue from a stock minimal media plate was

used to inoculate 10 ml of 2 x YT which was incubated overnight at 37°C. 0.3 ml of overnight culture was used to inoculate 30 ml of 2 x YT which was grown at 37°C for about 2 hours to an OD at 600 nm of 0.4-0.6. The cells were cooled on ice for 10 min and gently harvested (12,000g, 5min, 4°C).

When being prepared by the $CaCl_2$ method (Cohen *et al.*, 1972) the cells were resuspended in one half of the original culture volume of ice-cold, sterile 50 mM $CaCl_2$, 10 mM Tris/HCl pH 8 and placed in an ice bath for 15 min. Following centrifugation (12,000g, 5min, 4°C) the cells were resuspended in 1/15th original culture volume 50 mM $CaCl_2$, 10 mM Tris/HCl pH 8 and mixed in the ratio 3:1 culture to glycerol and cells dispensed into chilled microfuge tubes in 200-400 µl aliquots. These cells were stored at -70°C.

For high transformation efficiency cells, the following steps were carried out (Hanahan, 1983). Cells were prepared and harvested as above but were then resuspended in 2.5 ml of ice cold TFB (10 mM MES/KOH pH 6.3, 100 mM RbCl, 45 mM $MnCl_2$, 10 mM $CoCl_2$, 3 mM hexaminecobaltic chloride) and incubated on ice for 15 min. 100 µl of DMSO were added and the cells were incubated on ice for 5 min. 100 µl of 2.25 M DTT, 40 mM potassium acetate pH 6.0 were added and the cells further incubated on ice for 10 min. Finally, 100 µl of DMSO were added, the cells were kept on ice and used on the day of preparation.

### 2.14.2 Transformation of competent cells

Transformations were carried out in sterile 1.5 ml microfuge tubes. Ligation mix refers to any DNA being used in the transforming process and usually was a ligation mixture. An aliquot of ligation mix containing up to 25 ng plasmid DNA was added to 100 µl aliquots of competent cells and the mixture was incubated on ice for at least 30 min. The DNA/cell mix was then heat shocked at 42°C for 2 min. 1 ml of L-broth was added to the tubes which were incubated without shaking at 37°C for 1 hour. The cells were then plated onto appropriately prepared antibiotic/chromogen containing LB plates and incubated overnight at 37°C.

### 2.14.3 Selection of pUC derived recombinant clones

The pUC plasmids have been constructed as cloning vectors using β-galactosidase activity as

the basis of selection (Messing, 1983; Norrander *et al.*, 1983; Yanisch-Perron *et al.*, 1985). The vector carries a segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*). Vectors of this type are used in host cells (e.g. *E. coli* DS941) that code for the carboxy-terminal portion of β-galactosidase, therefore though neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form active enzyme. A polycloning site has been inserted, in-frame, within the coding region of the plasmid which does not affect the complementation, however insertion of additional DNA into the polycloning site generally destroys the complementation. Active β-galactosidase cleaves the chromogenic substrate X-Gal to produce a blue chromophore, when transformed cells are grown in the presence of the non-metabolisable lac operon inducer IPTG. However in recombinant plasmids the ability for complementation is lost, the enzyme is inactive, and consequently the colonies appear white. False positive white colonies appear at low frequency, probably due to incorrect self-ligation of the vector (Yanisch-Perron *et al.*, 1985).

## 2.15 DNA transfer to nylon membranes (Southern blotting)

This method is based on that of Southern (1975). After electrophoresis the agarose gel was placed in 250 ml of 0.25 M HCl and soaked for 15 min. The gel was then placed in 250 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min and then into neutralising solution (1M Tris, 1.5M NaCl) for 60min. The DNA was blotted onto a nylon (Hybond-N, Amersham) membrane using 20xSSC transfer buffer (SSC = 0.15 M NaCl, 15 mM sodium citrate pH 7.3) overnight. After blotting the membrane was washed in 2 x SSC as described in detail in Sambrook *et al.*, (1989). The filters were air dried and then baked at 80°C for 2 hours.

## 2.16 $^{32}P$ labelling of the 5' terminus of the oligonucleotide

Bacteriophage T4 polynucleotide kinase can catalyze the transfer of the γ-phosphate group from ATP to the free hydroxyl group on the 5' terminus of DNA. In a total reaction volume of 10 µl the mixture contained ; 8 pmoles of purified oligonucleotide, kinase buffer (50 mM Tris/HCl

pH8, 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine), 8 pmoles ($\gamma$-$^{32}$P) ATP and 10 units of T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min by which time it had gone to completion.

## 2.17 Hybridisation of filter bound nucleic acid

The temperature of hybridisation and the salt concentration and temperature of washing solutions were dependent on the particular experiment being carried out and precise details are given in the text. Hybond-N filters were not pre-wetted, but were placed directly into a polythene bag containing prehybridisation solution (6 x SSC, 0.05% (w/v) sodium pyrophosphate, 200 µg/ml heparin, 0.05% (w/v) SDS); the volume of prehybridisation fluid was determined by filter surface area x 0.2. The bag was placed in an agitating waterbath and the filter prehybridised for at least 4 hours at 60°C. After prehybridisation the bag was opened, two thirds of the fluid were removed and labelled oligonucleotide (section 2.16) added. The bag was resealed and the hybridisation carried out for 2 hours in a shaking waterbath at the appropriate temperature.

The hybridised filters were washed in large volumes of buffer appropriate in ionic strength for different experimental conditions. The temperature of this buffer varied experimentally. After washing, the filters were autoradiographed damp (under Saran wrap) as described in section 2.2.10.

## 2.18 Screening of plasmid clones by colony hybridisation

Recombinant pUC clones were screened using a modification of the method described by Nygaard and Hall (1963). Nylon filters (Hybond-N, Amersham) were placed on duplicate agar plates containing the selective antibiotic. Bacterial colonies were crossed onto a master plate (containing antibiotic) then onto the filter containing plates. The plates were inverted and grown overnight at 37°C. Alignment marks were made on the filters. The filters were removed and

placed colony side up on piece of 3MM paper soaked in 10% SDS and left for 3 min. The filters were then removed, using blunt ended forceps, to a second piece of 3MM paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and left for 5 min. Finally, the filters were transferred, colony side up, to 3MM paper soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris/HCl pH 8 ) for 5 min. The filters were then transferred to dry filter paper and allowed to air dry, colony side up. Filters were baked at 80°C for 2 hours prior to being hybridised with a nucleic acid probe (section 2.17).

## 2.19 Nucleotide sequencing

### 2.19.1 Sequencing by the chain termination method

Sequencing was carried out using the United States Biochemical Corporation (USB) sequencing kit and protocols described in 'Step-By-Step Protocols For Sequencing With Sequenase Version 2.0' were strictly adhered to.

Sequenase Version 2.0 is a site directed mutant of bacteriophage T7 DNA polymerase which carries no 3'-5' exonuclease activity and gives a high rate of polymerisation of nucleotides (Tabor and Richardson, 1989).

a) Preparation of sequencing template

Sequencing was carried out using double stranded DNA. In order to use double stranded DNA as a template for sequencing it must be denatured. The alkaline denaturation method described in 'Step-By-Step Protocols For Sequencing With Sequenase Version 2.0' was used for this purpose. The DNA was denatured in 0.2M NaOH, 0.2mM EDTA (30min at 37°C) and the mixture neutralised by the addition of 0.1 volumes of 3M sodium acetate (pH5.5). The DNA was precipitated with 2-4 volumes ethanol and the pelleted DNA washed with 70% ethanol, air dried and resuspended in 7µl distilled water before use in sequencing.

b) Annealing primer to template:

7 µl of prepared template was annealed for 2 hours at 55°C in a mixture also containing:

1 µl primer, -40 primer (3ng/ µl) or designed oligonucleotide

2 µl 200mM Tris/HCl, pH7.5, 100mM $MgCl_2$, 250mM NaCl (5xSequenase Buffer)

The-40 primer has the sequence:

5'-GTT TTC CCA GTC ACG AC-3'.

Other primers are described inthe text.

c) Sequencing reactions:

To the annealed primer/template mix, the following were added; 1 µl DTT (100mM), 2 µl labelling mix (1.5 µM dGTP, 1.5µM dCTP, 1.5µM dTTP), 0.5 µl (5µCi) [$\alpha^{35}$S]-dATPαS at >600Ci./mmol. (Amersham SJ.304) and 1.5 units of Sequenase Version 2.0. After mixing, the labelling reaction was incubated at room temperature for 2-5 min. After this incubation, 3.5 µl of the labelling reaction were transferred to each of four microfuge tubes labelled A, C, G, and T, in which 2.5 µl of the appropriate termination mix (80 µM dATP, 80µM dCTP, 80µM dGTP, 80µM dTTP, 8µM ddNTP (appropriate analogue), 50mM NaCl) had been spotted at the bottom of the tube.The contents of the tubes were mixed well and incubated at 37°C. After a 5 min incubation at 37°C, 4 µl of stop mix (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) Bromophenol Blue, 0.05% (w/v) Xylene Cyanol FF) was added to each tube and mixed with the reaction by a brief spin in a microfuge. The sequencing reactions were then stored at -20°C. For sequencing areas which were particularly G/C rich, the labelling mix contained (3.0 µM dITP, 1.5µM dCTP, 1.5µM dTTP) and the termination mixes were (80 µM dATP, 80µM dCTP, 80µM dITP, 80µM dTTP, 8µM ddNTP (appropriate analogue), 50mM NaCl) except the ddGTP termination mix (80 µM dATP, 80µM dCTP, 160µM dITP, 80µM dTTP, 8µM ddNTP (appropriate analogue), 50mM NaCl).

### 2.19.2 Polyacrylamide gel electrophoresis

The nested set of primer extended fragments produced by the sequencing reactions described above were resolved by electrophoresis on thin polyacrylamide gels using the BRL S2

sequencing gel apparatus (BRL-Gibco).

Gels were composed of the following constituents:

| | |
|---|---|
| 40% acrylamide | 15ml. |
| (38% acrylamide, 2% bisacrylamide) | |
| 10xTBE | 10ml |
| urea | 50g |
| distilled water | 35ml |
| 10% (v/v) ammonium persulphate | 350µl |
| TEMED | 50µl |
| (N, N, N', N', tetramethylethylenediamine) | |

Gels were pre-run in TBE for at least 30 min at 50mA constant current before loading samples. Samples were prepared by heating to 75-80°C for 2 min before loading, during which the top of the gel was thoroughly cleared of any unpolymerised acrylamide or urea and the lanes formed using a sharkstooth comb. The heat-denatured samples were loaded immediately onto the gel and electrophoresis carried out at 60W (constant power), 50mA, 2000V. The duration of electrophoresis depended on the length of sequence required. DNA close to the primer extending 250bp away from the primer was obtained from gels run for 2-5 hours, or until the dye front reached the anode, while sequence from 200-400bp from the primer was obtained from gels run for 6 hours. Routinely, 5 µl of each sample was run until the dye front reached the anode and 5 µl run for 8 hours in order to obtain the maximum sequence from each reaction.

All gels were fixed in 10% (v/v) acetic acid, 10% (v/v) methanol for 30 min following electrophoresis and were then dried onto a sheet of Whatman 3MM paper and autoradiographed as described in section 2.2.10.

### 2.19.3 Preparation of oligonucleotides

The oligonucleotides used were supplied in a solution of 35% (w/w) $NH_4OH$. The

oligonucleotides were precipitated with ethanol (section 2.12.9) and resuspended in TE. The oligonucleotide concentration was measured and adjusted to a final concentration of 20nmol/ml.

## 2.20 Computer programs used in the analysis of DNA and amino acid sequences

A number of programs for the manipulation and analysis of DNA sequences of the UWGCG (University Of Wisconsin Genetics Computer Group) package (Devereux *et al.*, 1984) were run on a VAX computer. This package contains programs for the comparison of DNA sequences with those in GenBank and EMBL (European Molecular Biology laboratory, Heidelberg, Germany) databases. The following UWGCG programs were used:

SeqEd is an interactive editor which allows entering and modification of sequences.

Map displays both strands of a DNA sequence with a restriction map shown above the sequence and possible protein translations shown below.

BestFit makes an optimal alignment of the best segment of similarity between two sequences and inserts gaps if necessary (Smith and Waterman, 1981).

FastA searches for sequences similar to the query sequence in any group of sequences, using a Pearson and Lipman search (Pearson and Lipman, 1988).

TFastA Does a Pearson and Lipman search as for FastA, but first translates the query sequence in all six reading frames.

Strings finds sequences by searching sequence documentation for character patterns e.g. citrate.

LineUp is a screen editor for editing multiple sequence alignment

CodonPreference is a frame specific gene finder which tries to recognise protein coding sequences by comparison to a codon frequency table, or by their composition in the third position of each codon.

TestCode identifies protein coding sequences by plotting a measure of the non-randomness of the composition at every third base.

Translate translates nucleotide sequence into peptide sequence.

Peptidesort shows the peptides from a digest of an amino acid sequence, shows the amino acid composition of each peptide and gives a summary of the composition of the whole protein.

CodonFrequency tabulates codon usage from sequences and/or existing codon usage tables.

# Chapter 3

# The Purification and characterisation of *Bacillus caldotenax* ICDH

## 3.1 Introduction

NADP-dependent ICDH's have been isolated from a number of different micro-organisms including, *Salmonella typhimurium* (Marr and Weber, 1968), *Thiobacillus novellus* (Charles, 1970), *Bacillus stearothermophilus* (Howard and Becker, 1970) and *E.coli* (Reeves *et al.*, 1972). The methods of isolation used by these workers used salt fractionation of cell free extracts followed by chromatography on ion exchange and gel filtration media. These methods were developed before the techniques of f.p.l.c. and dye ligand chromatography became widely available for protein purification. The original methods were generally lengthy and gave relatively low yields. An improved method for the isolation of ICDH from *E. coli* was developed (Borthwick *et al.*, 1984 a) which utilised dye ligand chromatography giving higher yields and improved specific activities. Similarly, more recent purifications have been developed for ICDH from a number of thermophilic bacteria ; *Bacillus stearothermophilus* (Nagaoka *et al.*, 1977) using chromatography on blue dextran sepharose and *Thermus thermophilus* HB8 (Eguchi *et al.*, 1989).

In this chapter I report the purification of ICDH from a moderately thermophilic bacterium *Bacillus caldotenax*, using f.p.l.c. and dye ligand chromatography, and some properties of the purified enzyme.

## 3.2 Results

### 3.2.1 Procedure for Purification of ICDH from *Bacillus caldotenax*

In this procedure steps a) to c ) were carried out at 4°C.

#### a) Preparation of a cell free crude extract

5-10g of *B. caldotenax*, grown as described in section 2.3.4a), were resuspended in 25ml of buffer A, as defined in section 2.6, containing 1mM benzamidine and 1mM DTT. The cells were disrupted by two passages through a French pressure cell as described in section 2.3.7 b). Cell debris and unbroken cells were removed by centrifugation at 30,000g for 20min in a MSE 18 refrigerated centrifuge and the supernatant was decanted.

#### b) Ammonium sulphate fractionation.

Powdered ammonium sulphate was added gradually to the supernatant to give 35% saturation whilst the pH of the solution was maintained in the range 6.5-7.0 by the addition of 5M ammonia. The temperature was kept at 0°C by use of an ice-water bath. After the addition of all the ammonium sulphate the solution was stirred for 30min at 0°C and then centrifuged at 20,000g for 15min. The pellet was discarded and the supernatant fraction was brought to 50% saturation with ammonium sulphate, stirred and centrifuged as before. This second pellet was also discarded and the supernatant brought to 70% saturation, stirred and centrifuged as before. The supernatant from this final fractionation was discarded and the pellet was resuspended in 2ml of buffer A.

### c) Desalting by gel filtration on Sephadex G-50

The resuspended pellet was loaded onto a column of Sephadex G-50 (20ml bed volume) equilibrated with buffer A. Fractions of 2ml were collected. Fractions containing a significant amount of protein but no ammonium sulphate, as determined by conductivity readings (section 2.2.2) were pooled.

### d) f.p.l.c. ion-exchange chromatography on MonoQ

This and subsequent steps were carried out at room temperature. The desalted enzyme was filtered and then loaded on to a 10/10 MonoQ column equilibrated in buffer A at a flow rate of 4ml/min. The column was washed with 24ml of the same buffer and developed with a linear gradient of 50mM to 250mM $KH_2PO_4$ over 30 min. ICDH activity eluted at 70mM $KH_2PO_4$ (figure 3.1). Fractions containing significant ICDH activity were pooled and dialysed overnight into buffer B.

### e) Dye ligand chromatography on Procion-red Sepharose.

The dialysed material was applied to a column of Procion-red dye HE-3B immobilised on cross-linked Sepharose 6B which had been equilibrated in buffer B (as defined in section 2.6). The column was washed exhaustively with buffer B until the $A_{280}$ of the effluent returned to baseline. The ICDH was then eluted as a sharp peak by washing the column with buffer B containing 0.5mM $NADP^+$. A typical elution profile is shown in figure 3.2. The purity of ICDH throughout the purification procedure was monitored by polyacrylamide gel electrophoresis (section 2.5.1) (figure 3.3). A typical purification table is shown in table 3.1.

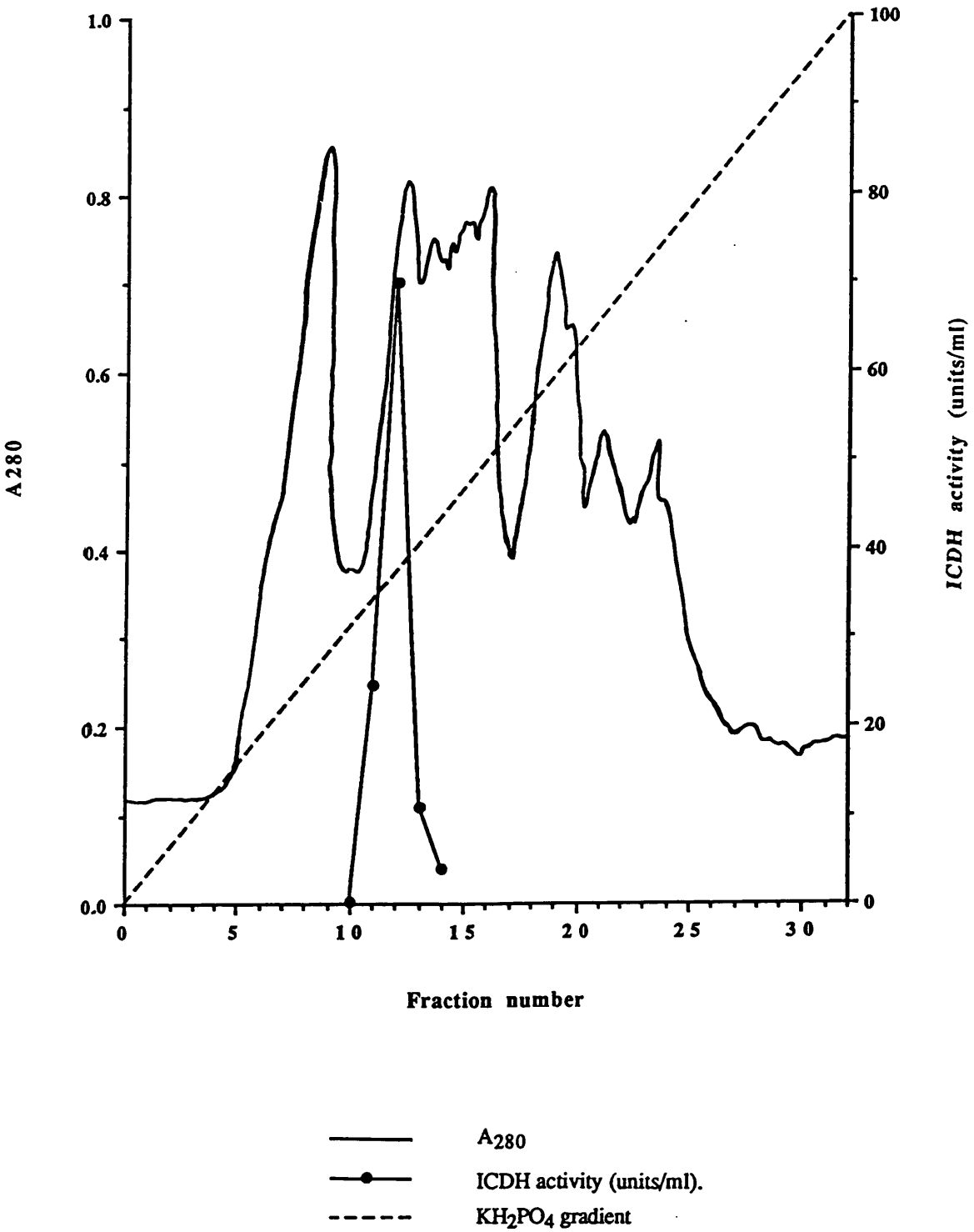



**Figure 3.1 Ion exchange chromatography on Mono Q**

Enzyme from $(NH_4)_2SO_4$ fractionation was loaded onto a Mono Q column and eluted with a phosphate gradient, as described in section 3.2.1 d). The units of the gradient are arbitrary.

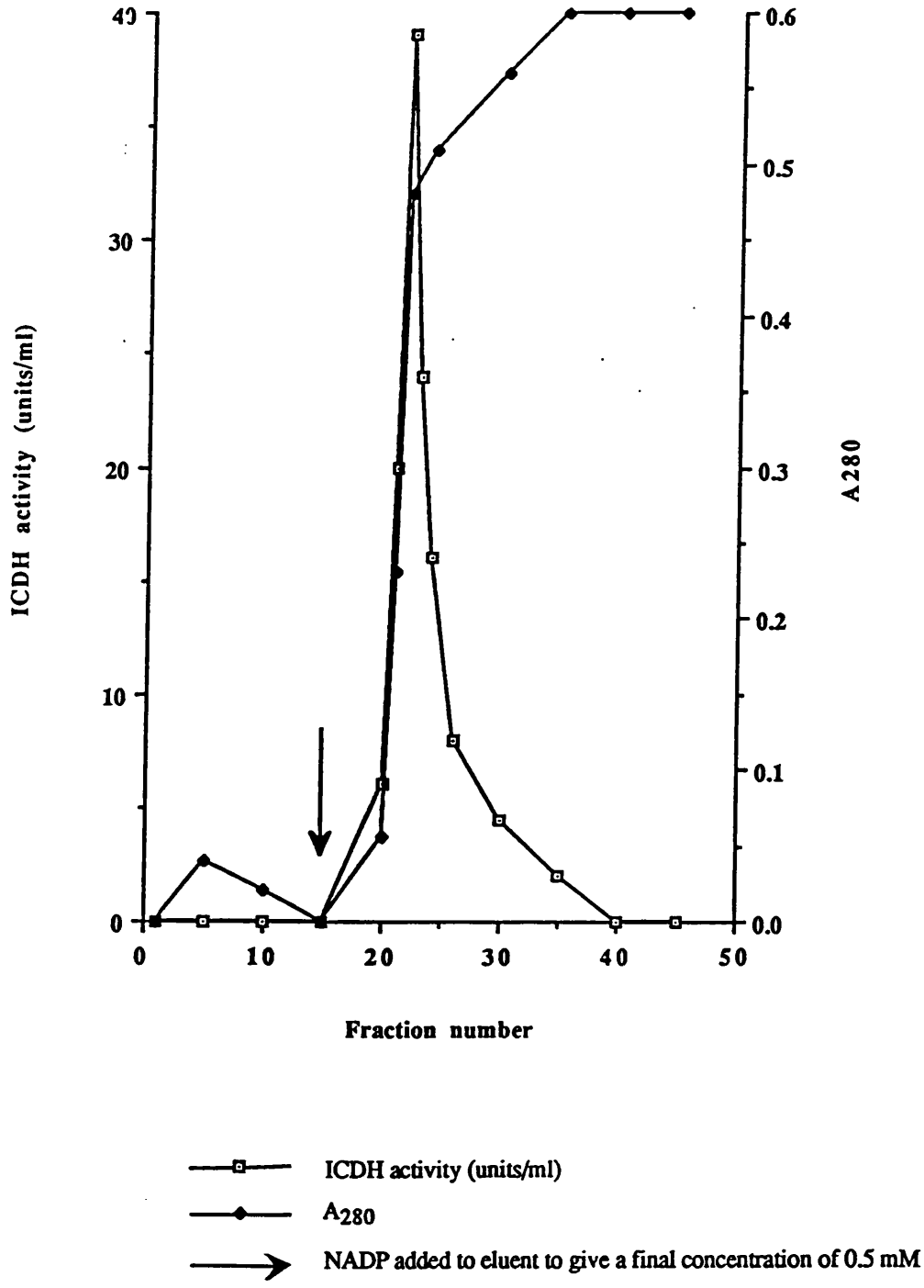



**Figure 3.2** **Dye ligand chromatography of *B.caldotenax* ICDH on Procion-red Sepharose**

Elution profile from a cross-linked Procion-red Sepharose column. Details of the procedure are described in section 3.2.1 e).

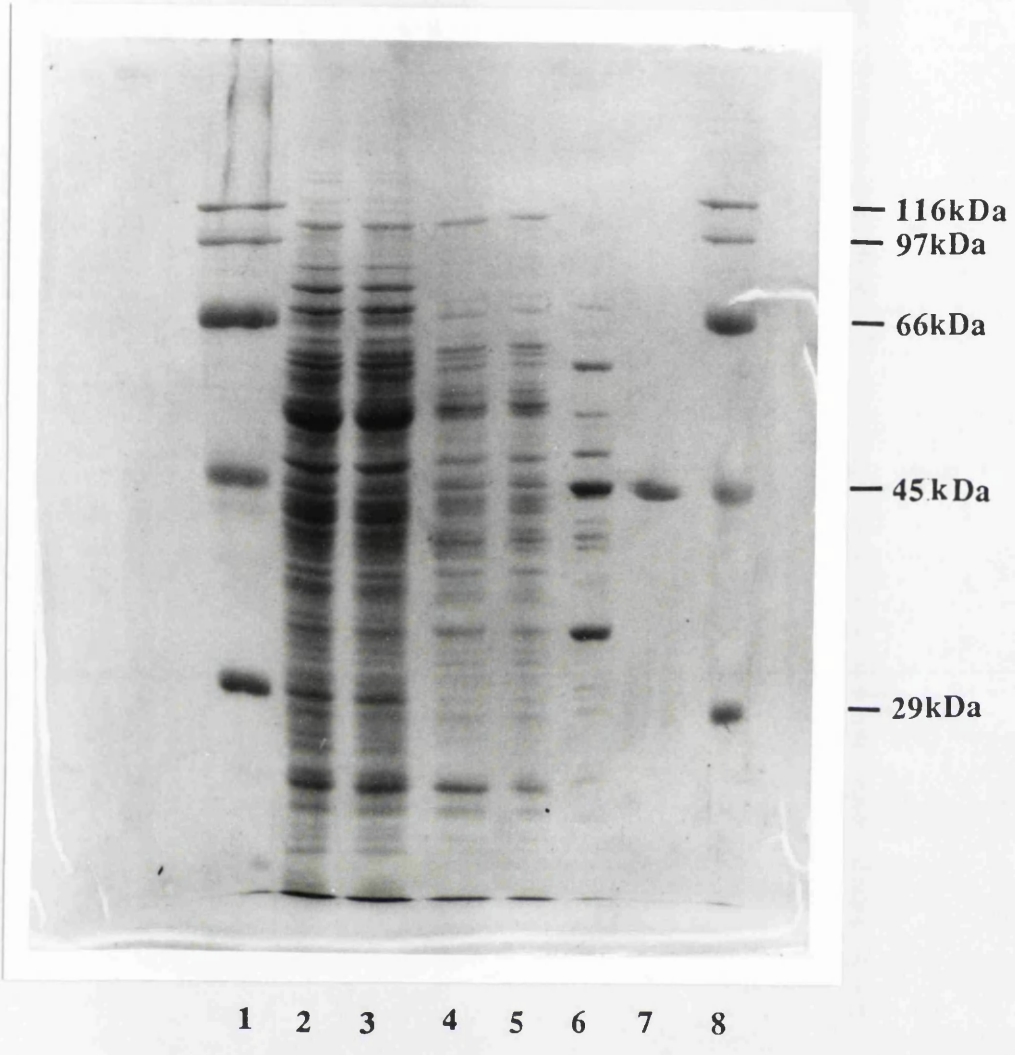



**Figure 3.3 Purification gel of *B.caldotenax* ICDH**

A 10% polyacrylamide gel was run in the presence of SDS to monitor the purification of ICDH. Marker proteins were selected from those listed in section 2.1.2.

| Track | Sample |
|---|---|
| 1 | Markers. |
| 2 | Crude extract. |
| 3 | 0-35% ammonium sulphate supernatant. |
| 4 | 35-50% ammonium sulphate supernatant. |
| 5 | 50-70% ammonium sulphate pellet. |
| 6 | Mono Q pool. |
| 7 | Procion-red Sepharose pool. |
| 8 | Markers. |

**Table 3.1 Purification of *B.caldotenax* ICDH**

The results are presented for a typical purification starting from 5g of *B.caldotenax* cells.

| step | total protein (mg) | total activity (units) | specific activity (units/mg) | yield (%) | purification (fold) |
|---|---|---|---|---|---|
| a) crude extract | 270 | 757 | 2.8 | 100 | 1 |
| b) $(NH_4)_2SO_4$ fractionation (50-70% pellet) | 35 | 418 | 12.0 | 55 | 4 |
| c) gel filtration | 25 | 300 | 12.0 | 40 | 4 |
| d) monoQ chromatography | 4 | 220 | 55 | 29 | 10 |
| e) dye ligand chromatography on Procion red Sepharose | 0.6 | 100 | 167 | 13 | 60 |

### 3.2.2 Purity and molecular weight

ICDH isolated from *B. caldotenax* was judged to be homogenous by the criterion of polyacrylamide gel electrophoresis. The purified enzyme gave a single protein staining band on a 10% polyacrylamide gel run in the presence of 0.1% (w/v) SDS (figure 3.3).

The subunit molecular weight of ICDH was estimated by SDS gel electrophoresis using the method of Laemmli (1970) as described in section 2.10.1. The electrophoretic mobility of marker proteins of known molecular weight were measured and used to construct a standard curve of electrophoretic mobility against log subunit molecular weight (figure 3.4). Comparison of the electrophoretic mobility of ICDH with these markers gave a value of 46,000 daltons for the subunit molecular weight of *B. caldotenax* ICDH.

The molecular weight of native, undissociated ICDH was estimated using a Superose 12 gel filtration column which was run on a Pharmacia f.p.l.c. system as described in section 2.10.2. Standard proteins of known molecular weight were used to calibrate the column. A standard curve was constructed of elution volume against log molecular weight (figure 3.5). The measured elution volume for ICDH corresponded to a native molecular weight of 93,000 daltons indicating that the native enzyme is a dimer.

### 3.2.3 Immunological cross-reaction.

Varying concentrations of purified *B. caldotenax* ICDH were run on a 10% polyacrylamide gel in the presence of 0.1% SDS using the method of Laemmli (1970) (section 2.5.1). The gel was Western blotted (section 2.8) using an antibody raised to *E. coli* ICDH. The *B. caldotenax* ICDH was found to cross-react with this antibody (figure 3.6) suggesting appreciable structural homology between these two types of ICDH. This usually indicates > 60% identity.

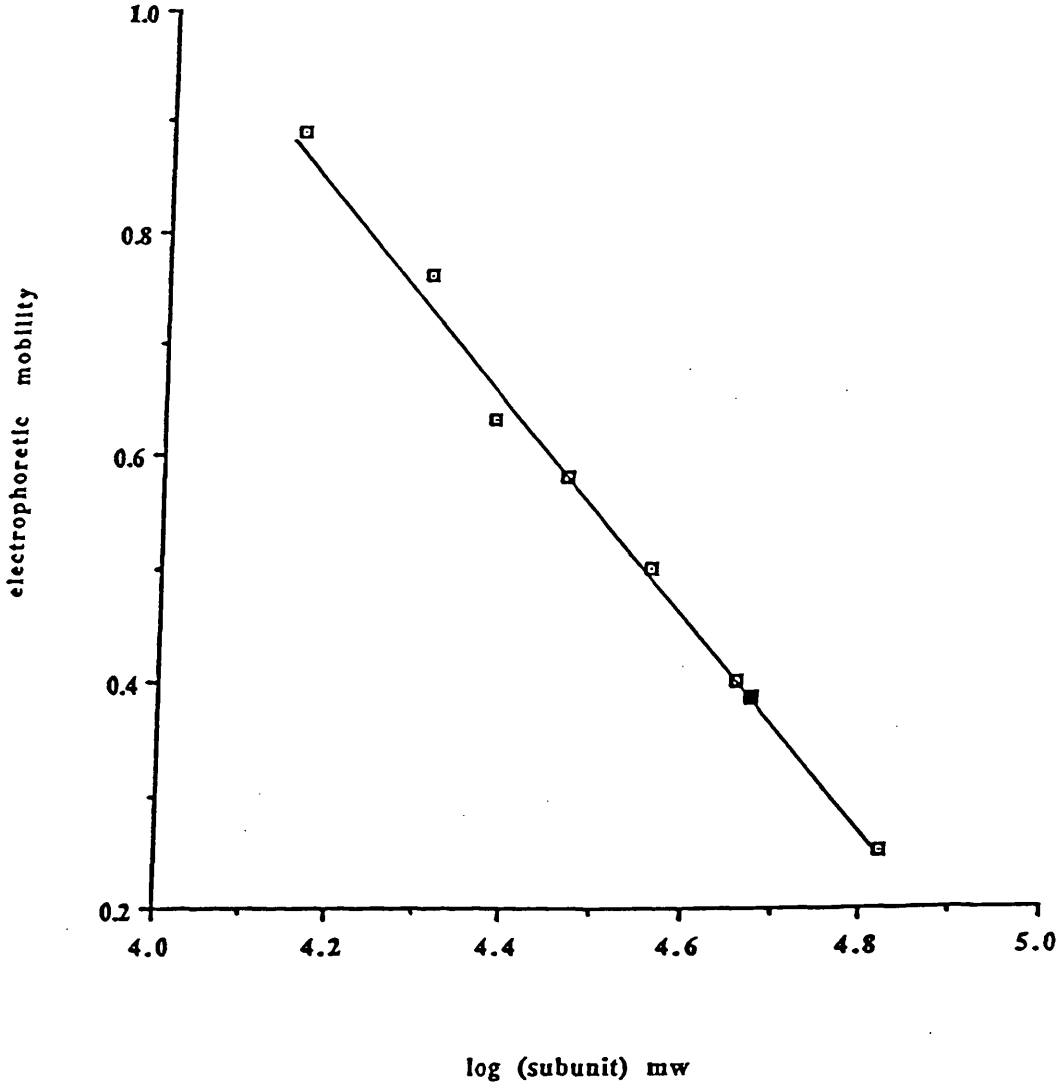



Figure 3.4 SDS PAGE standard curve

SDS PAGE (10%) was performed as described in section 2.5.1. The Rf values of standard proteins were calculated and plotted against the log Mr. The Rf of purified *B.caldotenax* ICDH is shown (■). The standard curve was fitted by eye. A list of the standard proteins used is given below.

| protein | molecular weight (Daltons) |
|---|---|
| bovine albumin | 66 000 |
| egg albumin | 45 000 |
| glyceraldehyde-3-phosphate dehydrogenase | 36 000 |
| carbonic anhydrase | 29 000 |
| trypsinogen | 24 000 |
| trypsin inhibitor | 20 000 |
| α-lactalbumin | 14 000 |

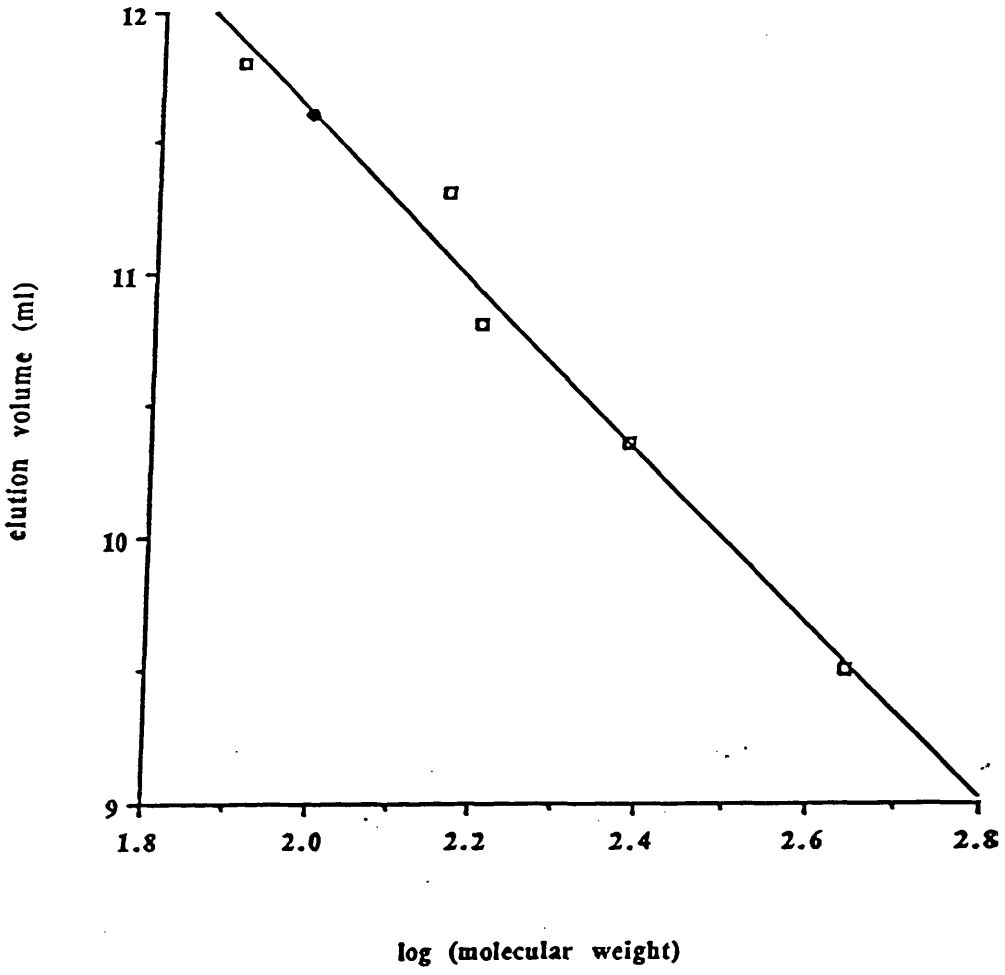



Figure 3.5 Superose 12 gel filtration standard curve

A Superose 12 gel filtration column was calibrated and run as described in section 2.10.2. Peak elution volume (ml) was plotted against log Mr. The standard curve was fitted by eye. The elution volume of purified *B.caldotenax* ICDH is shown (•). The proteins used to calibrate the column are listed below.

| Protein | molecular weight (Daltons) |
|---|---|
| pig heart malate dehydrogenase | 70 000 |
| pig muscle lactate dehydrogenase | 140 000 |
| rabbit muscle aldolase | 160 000 |
| rabbit muscle pyruvate kinase | 232 000 |
| horse apoferritin | 440 000 |

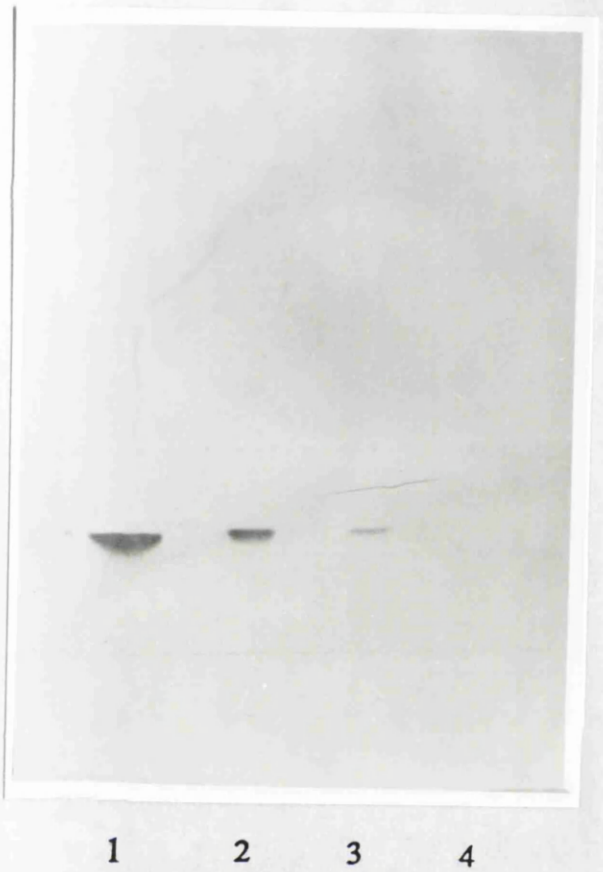



Figure 3.6 **Western blot of *B.caldotenax* ICDH using an antibody raised to *E.coli* ICDH**

Serial dilutions of *B.caldotenax* ICDH were run on a 10% polyacrylamide gel in the presence of 0.1% SDS (section 2.5.1). The gel was Western blotted using an antibody raised to *E.coli* ICDH by the method described in section 2.8.

The tracks were loaded as follows:

1) 2μg *B.caldotenax* ICDH.
2) 0.4μg *B.caldotenax* ICDH.
3) 0.08μg *B.caldotenax* ICDH.
4) 0.016μg *B.caldotenax* ICDH.

### 3.2.4 Determination of Km values for *B. caldotenax* ICDH

Km values were determined for *B.caldotenax* ICDH at 37°C. The activity of ICDH was measured, varying concentrations of one substrate with the concentration of the other being fixed and saturating. Km values were established by constructing Lineweaver-Burk plots for each substrate (figures 3.7 and 3.8). The Km values of ICDH for isocitrate and $NADP^+$ were determined to be 2.4 μM and 1.6 μM respectively.

### 3.2.5 Amino acid sequence analysis of *B. caldotenax* ICDH

#### a) N-terminal amino acid sequence analysis

*B. caldotenax* ICDH was prepared for N-terminal sequence analysis by passage down a Waters Bondapak μC18 reverse phase column as described in section 2.9.1. This served to clean the protein and transferred it to acetonitrile containing 0.1% TFA, an ideal solvent for sequencing purposes. The sample was loaded directly onto an Applied Biosystems Protein sequencer (section 2.9.4). No sequence was obtained from this sample and it was suggested that the protein was N-terminally blocked.

#### b) Amino acid sequence analysis of a V8 peptide of ICDH

Peptides were obtained from ICDH by digestion with V8 protease (section 2.9.2) and electroblotted onto PVDF membrane as described in section 2.9.3. A number of peptide bands were visible on the blot, one of which was particularly intense. This intense band was excised from the blot and used for amino acid sequence analysis on an Applied Biosystems Protein sequencer (section 2.9.4).

Two sets of sequence data were obtained simultaneously. The yields of each residue

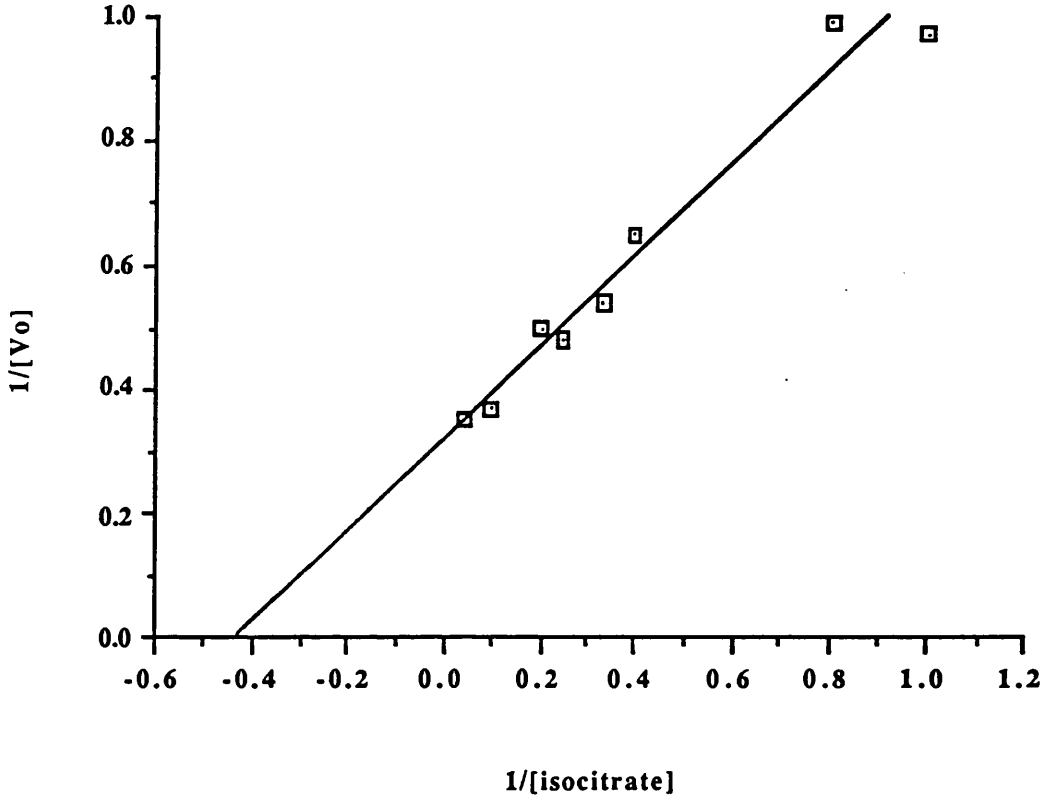



**Figure 3.7 Determination of the Km of ICDH for isocitrate**

The activity of ICDH was measured at 37°C using the assay described in section 2.4.2. The concentration of isocitrate was varied whilst that of $NADP^+$ was kept constant and in excess (0.4mM). A Lineweaver-Burk plot was constructed to determine the apparent Km for isocitrate.

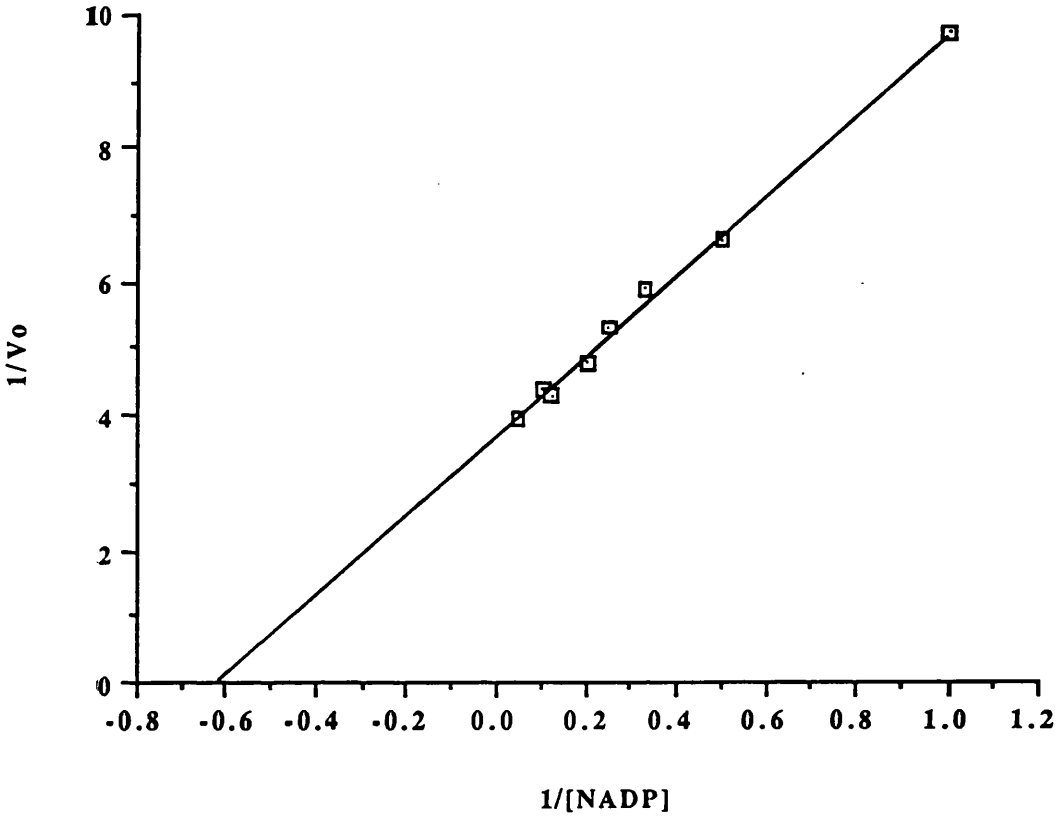



**Figure 3.8 Determination of the Km of ICDH for $NADP^+$**

The activity of ICDH was measured at 37°C using the assay described in section 2.4.2. The concentration of $NADP^+$ was varied whilst that of isocitrate was kept constant and in excess (2.5mM). A Lineweaver-Burk plot was constructed to determine the apparent Km for $NADP^+$.

**Table 3.2** **Sequence data obtained from V8 peptides of *B.caldotenax* ICDH**

| Cycle number | PTH-amino acid | pmol recovered | PTH-amino acid | pmol recovered |
|---|---|---|---|---|
| 1 | M | 62.2 | T | 40.83 |
| 2 | T | 37.52 | Q | 47.69 |
| 3 | Q | 59.63 | G | 74.80 |
| 4 | G | 97.51 | E | 37.90 |
| 5 | E | 40.61 | K | 40.11 |
| 6 | K | 49.93 | I | 66.16 |
| 7 | I | 79.67 | T | 28.16 |
| 8 | T | 30.66 | V | 44.92 |
| 9 | V | 53.91 | Q | 49.13 |
| 10 | Q | 63.99 | N | 34.19 |
| 11 | N | 40.95 | G | 61.25 |
| 12 | G | 67.3 | V | 36.48 |
| 13 | V | 42.52 | L | 45.68 |
| 14 | L | 55.84 | N | 32.96 |
| 15 | N | 42.26 | V | 37.54 |

The yields of PTH-amino acids during sequencing of V8 peptides of ICDH. Two species of peptide were sequenced simultaneously producing the two sets of data shown above. The initial amount of peptide sequencing was 100pmol.

**Met Thr Gln Gly Glu Lys Ile Thr Val Gln Asn Gly Val Leu Asn Val**

**M T Q G E K I T V Q N G V L N V**



**Figure 3.9 Amino acid sequence obtained from a V8 peptide of *B.caldotenax* ICDH**

120μg of *B.caldotenax* ICDH were digested with V8 protease by the method of Cleveland (1977) as described in section 2.9.2. Peptides were electroblotted (section 2.9.3) and the peptide band of greatest intensity sequenced (section 2.9.4).

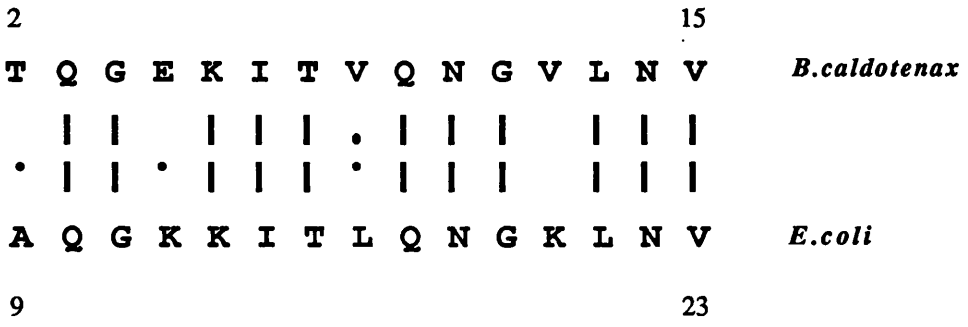



**Figure 3.10 Comparison of the amino acid sequence obtained from *B. caldotenax* ICDH with the *E. coli* ICDH amino acid sequence**

The results are shown of a Bestfit computer analysis (section 2.20) between the amino acid sequence of *B. caldotenax* ICDH and the amino acid sequence of *E. coli* ICDH (Thorsness and Koshland, 1987). The numbers relate to the position of the amino acids within the sequence as a whole. Identical matches are marked with a line ( | ). Matches were scored using the table provided with the GCG package.Matches with a score of greater than 0.5 are marked with a colon ( : ) and matches with a score of greater than or equal to 0.1 are marked with a full stop (.).

recovered from each cycle of sequencing are shown in table 3.2. The two sets of data resulted from two types of peptide being sequenced in unison. The two types of peptide had an identical sequence but one was a single residue shorter. The derived amino acid sequence is shown in figure 3.9. Sequence was obtained for the first fifteen amino acid residues. This was judged to be sufficient sequence for designing a probe for use in cloning the ICDH gene. Sequencing was therefore terminated after fifteen residues.

### 3.2.6 Comparison of *B. caldotenax* ICDH sequence with that of *E. coli*

Amino acid sequence was obtained from a peptide of *B. caldotenax* ICDH. Comparison of the sequence of *B. caldotenax* ICDH with that of *E. coli* ICDH, determined from the nucleotide sequence by Thorsness and Koshland (1987), showed considerable homology between the two (figure 3.10). Bestfit computer analysis was used to find the optimal alignment between the two sequences (section 2.20). The amino acid sequence of *B.caldotenax* ICDH lined up with 78% identity to a region very close to the N-terminus of *E. coli* ICDH (figure 3.10).

## 3.3 Discussion

This chapter reports the purification to homogeneity of an NADP-dependent ICDH from the moderate thermophile *Bacillus caldotenax*. Typical purifications gave yields of between 10 and 15%. The purified enzyme had a specific activity of approximately 150 units/mg at 50°C giving a purification factor of 60 fold over the initial extract.

The purification procedure involves $(NH_4)_2SO_4$ fractionation, anion exchange chromatography and dye ligand chromatography on Procion-red Sepharose and is very

similar to that developed by Borthwick *et al.* (1984a) for the purification of ICDH from *E.coli.*

Similar procedures for the purification of NADP-dependent ICDH from a number of thermophilic bacteria have been reported including *Bacillus stearothermophilus* (Nagaoka *et al.*, 1977), *Thermus thermophilus* (Eguchi *et al.*, 1989) and *Thermus aquaticus* ( Ramaley & Hudock, 1973). At the start of this study no purification had been reported for ICDH from the moderate thermophile *Bacillus caldotenax*. Recently Edlin and Sundaram (1989) reported a procedure for the purification of ICDH from several different micro-organisms including *B. caldotenax*. Their method of purification uses selective elution of ICDH from a Procion-red Sepharose HE3B column with a mixture of $NADP^+$ and isocitrate followed by anion exchange chromatography. The method gives ICDH in high yield but with a low specific activity (4 units/mg protein at 37°C). In contrast the purification reported here gives a relatively low yield of between 10 and 15%. I intended to purify ICDH in order to obtain pure protein for enzyme characterisation, N-terminal sequence analysis and phosphorylation studies. Although the yield was low, enough protein was obtained to satisfy these requirements.

Characterisation of the ICDH isolated from *B. caldotenax* showed that its undissociated molecular weight was 93,000 daltons (figure 3.5) and its subunit molecular weight 46,000 daltons (figure 3.4). This suggests that the native protein exists as a dimer. This subunit molecular weight is in good agreement with that obtained for *B. caldotenax* ICDH by Edlin and Sundaram (1989) of 46,000 daltons (table 3.3). *B. stearothermophilus* is a thermophile known to be extremely similar to *B. caldotenax* (Epstein and Grossowicz 1969). The subunit molecular weight of *B. stearothermophilus* has been reported as being 45,000 daltons (Howard and Becker, 1970) which also agrees with our value for *B. caldotenax* ICDH. Table 3.3 shows subunit molecular weights of ICDH from *E. coli, B. subtilis* and two extremely thermophilic *Thermus* strains for comparison. All the ICDHs documented in table 3.3 are dimers and with the exception of *T. aquaticus* and *T. thermophilus* , there is

**Table 3.3 Molecular weight estimations of various bacterial ICDH s**

| source | subunit molecular weight (Daltons) | native molecular weight (Daltons) | study |
|---|---|---|---|
| *E.coli* ML308 | 45 756 | 92 000 | Thorsness and Koshland (1987) |
| *B.subtilis* | nd | 75-80 000 | Ramaley and Hudock (1973) |
| *B.subtilis* | 47 000 | nd | Edlin and Sundaram (1989) |
| *B.stearothermophilus* | 45 000 | 92 500 | Howard and Becker (1970) |
| *B.caldotenax* | 46 000 | 93 000 | This study |
| *B.caldotenax* | 46 000 | nd | Edlin and Sundaram (1989) |
| *T.aquaticus* | 33 000 | 66 000 | Edlin and Sundaram (1989) |
| *T.thermophilus* | 57 500 | 120 000 | Eguchi *et al.*(1989) |
| *Synechocystis* sp. | 57 000 | 108 000 | Muro-Pastor and Florencio (1991) |

little variation in size. As previously mentioned in chapter 1 there are two classes of NADP-dependent ICDH, enzymes being classified according to size and composition. Class I includes dimeric forms of ICDH and class II monomeric forms of the enzyme. ICDH from *B. caldotenax* is a class I ICDH.

Km values were determined for *B. caldotenax* ICDH. These values together with the Km values of several other purified ICDH's are summarised in table 3.4. It is not possible to draw a comparison of these different Km values due to the variability of assay conditions used by different workers. However, it is evident that the Km values determined for *B. caldotenax* ICDH are of a similar order of magnitude to those established for ICDH from other species.

Amino acid sequence was obtained from *B. caldotenax* ICDH and found to align with 78% identity to a region close to the N-terminus of of *E. coli* ICDH. It seems likely from the alignment that the amino acid sequence obtained from *B. caldotenax* was in fact N-terminal sequence. Initial attempts to gain N-terminal sequence from *B. caldotenax* ICDH proved unsuccessful. This observation may have resulted from a sequencing error or it is possible that the process of electroblotting has removed impurities from ICDH which had previously prevented sequence analysis. If this sequence is N-terminal, the two species of peptide observed during sequencing may have resulted if the N-terminal methionine residue was post-translationally cleaved from some, but not all of, the ICDH.

In conclusion, *B. caldotenax* ICDH has been shown to be both similar in size and composition to that of *E. coli*. Western blotting has shown *B. caldotenax* ICDH to cross-react with an antibody raised to the *E. coli* ICDH. Finally, amino acid sequence analysis of *B. caldotenax* ICDH has demonstrated considerable sequence homology with *E. coli* ICDH. Therefore, although from two quite different organisms, these two types of ICDH exhibit remarkable similarity. A further approach in the comparison of these two enzymes would be to clone the gene for *B. caldotenax* ICDH and to determine the complete

**Table 3.4 The Km values for $NADP^+$ and isocitrate determined for various different bacterial ICDHs under different conditions**

| organism | assay conditions | Km $NADP^+$ (μM) | Km isocitrate (μM) | study |
|---|---|---|---|---|
| *E.coli* | Tris pH 7.5 $[Mn^{2+}]$ 37°C | 5 | 10 | Nimmo (1986) |
| *B.subtilis* | Hepes pH 7.4 $[Mn^{2+}]$ 25°C | 27.4 | 9.1 | Ramaley and Hudock (1973) |
| *B.stearothermophilus* | Triethanolamine buffer pH 7.5 $[Mg^{2+}]$ 50°C | 4.4 | 6 | Howard and Becker (1970) |
| *B.caldotenax* | Tris/HCl pH 7.5 $[Mn^{2+}]$ 37°C | 1.6 | 2.4 | this study |
| *T.aquaticus* | Hepes pH 7.5 $[Mn^{2+}]$ 70°C | 31 | 18.1 | Ramaley and Hudock (1973) |
| *T.thermophilus* | Hepes-Na pH 7.8 $[Mn^{2+}]$ 70°C | 6.3 | 8.8 | Eguchi *et al.* (1989) |
| *Synechocystis* (cyanobacterium) | phosphate pH 7.5 $[Mn^{2+}]$ 30°C | 6 | 5.7 | Muro-Passtor Florencio (1991) |
| *S.typhimurium* | Tris/HCl pH 7.7 $[Mn^{2+}]$ 25°C | 11.5 | 1.9 | Marr and Weber (1968) |
| *Azotobacter vinelandii* | phosphate pH 7 $[Mg^{2+}]$ 30°C | 23 | 20 | Chung and Franzen (1969) |
| *Thiobacillus novelus* | phosphate pH 7.8 $[Mn^{2+}]$ 25°C | 13 | 8.3 | Charles (1969) |

nucleotide sequence of the gene. It would be possible to deduce the entire amino acid sequence of ICDH, from the nucleotide sequence, and use this for more detailed comparison with *E. coli* ICDH. This approach has been used in the course of this study and the results are reported in chapter 5.

# Chapter 4

# Phosphorylation studies using *Bacillus caldotenax* ICDH

## 4.1 Introduction

As mentioned in chapter 1 during the growth of *E. coli* on acetate or fatty acids, as the sole carbon source, most of the precursors for biosynthesis are generated by means of the glyoxylate bypass (Kornberg, 1966). During growth under these conditions there is competition between the TCA cycle enzyme ICDH and the glyoxylate bypass enzyme ICL for available isocitrate. It has been found in *E. coli* that this competition is overcome by the partial phosphorylation and inactivation of ICDH (Garnak and Reeves, 1979a,b). Bennett and Holms (1975) reported some of the early work to suggest the existence of a covalent modification in the control of this branch point in *E. coli* . In addition they showed that this mechanism was not unique to *E. coli* but was also seen to exist in at least one strain each of *Klebsiella aerogenes, Salmonella typhimurium* and *Serratia marcescens*. In view of this, together with the fact that *B. caldotenax* ICDH has been shown to be structurally similar to *E. coli* ICDH (section 3.2.3 ), it would not be unreasonable to suggest that the ICDH of *B. caldotenax* might also be controlled by phosphorylation.

In this chapter I report results of *in vitro* phosphorylation studies which indicate that *B. caldotenax* ICDH is under the control of phosphorylation. I also report experiments in which I have looked for evidence of phosphorylation *in vivo*, although within the limits of this study I have been unable to confirm that phosphorylation is physiologically important in *B. caldotenax.*

## 4.2 Results

### 4.2.1 Inactivation of *B. caldotenax* ICDH by *E. coli* ICDH kinase

Homogenous ICDH purified from *B. caldotenax* was incubated in the presence of *E. coli* ICDH kinase/phosphatase (purified by the method of Nimmo *et al.*, 1984) in the assay described in section 2.4.3. Samples were removed from the incubation, to assay for ICDH

activity, over a time course of 0-30 min. A progressive decrease in activity was observed over this period of time (figure 4.1). Appropriate controls were carried out which showed that this decrease was only observed when *E. coli* ICDH kinase was present in the incubation. In addition no decrease in activity was observed in the absence of ATP or if ATP was substituted with 0.5mM AMP-PCP, a non-hydrolysable analogue of ATP (figure 4.1).

### 4.2.2 Phosphorylation of ICDH *in vitro*

Purified *B. caldotenax* ICDH was incubated with *E. coli* kinase in the presence of [$\gamma$-$^{32}$P] ATP as described in section 2.11.1 b). Samples were taken over a time course of 0-60 min for estimation of the activity of ICDH (section 2.11.1 a) and analysis of $^{32}$P incorporation into protein as shown by electrophoresis followed by autoradiography . Over a 60 min period a progressive decrease in ICDH activity was observed (figure 4.2) which corresponded to a progressive increase in $^{32}$P incorporation into ICDH (figure 4.3). Two control assays were performed one in the absence of *E. coli* ICDH kinase and the other in the absence of *B. caldotenax* ICDH. Samples were taken from each control assay after 60 min for estimation of $^{32}$P incorporation into protein. Neither control showed any evidence of $^{32}$P incorporation (figure 4.3). It was confirmed from these control results that phosphorylation is dependent on the presence of the kinase and that $^{32}$P is incorporated into *B. caldotenax* ICDH and not into any contaminating *E. coli* ICDH present in the *E. coli* ICDH kinase preparation.

### 4.2.3 The stoichiometry of phosphorylation of ICDH

*E. coli* ICDH is known to be phosphorylated with a stoichiometry of 1 mol of phosphate /mol of subunit (Laporte and Koshland, 1983; Nimmo *et al.*, 1984, ). The stoichiometry of

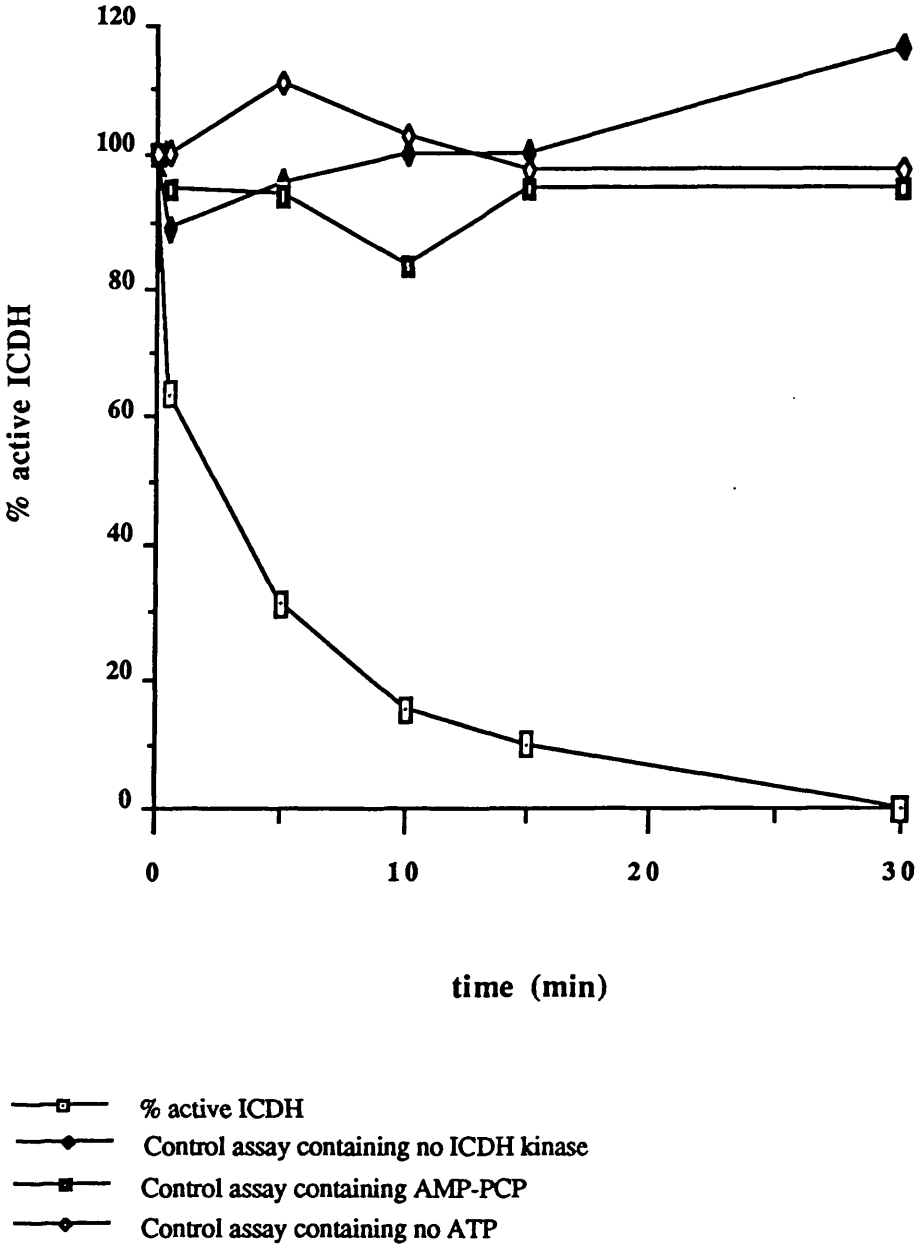



**Figure 4.1 Inactivation of *B.caldotenax* ICDH *in vitro***

Purified *B.caldotenax* ICDH was incubated with *E.coli* ICDH kinase in the assay described in section 2.11.1 a). ICDH activity was measured over a 30 min time course. Control assays were also performed 1) in the absence of *E.coli* ICDH kinase, 2) ATP was substituted with AMP-PCP and 3) in the absence of ATP.

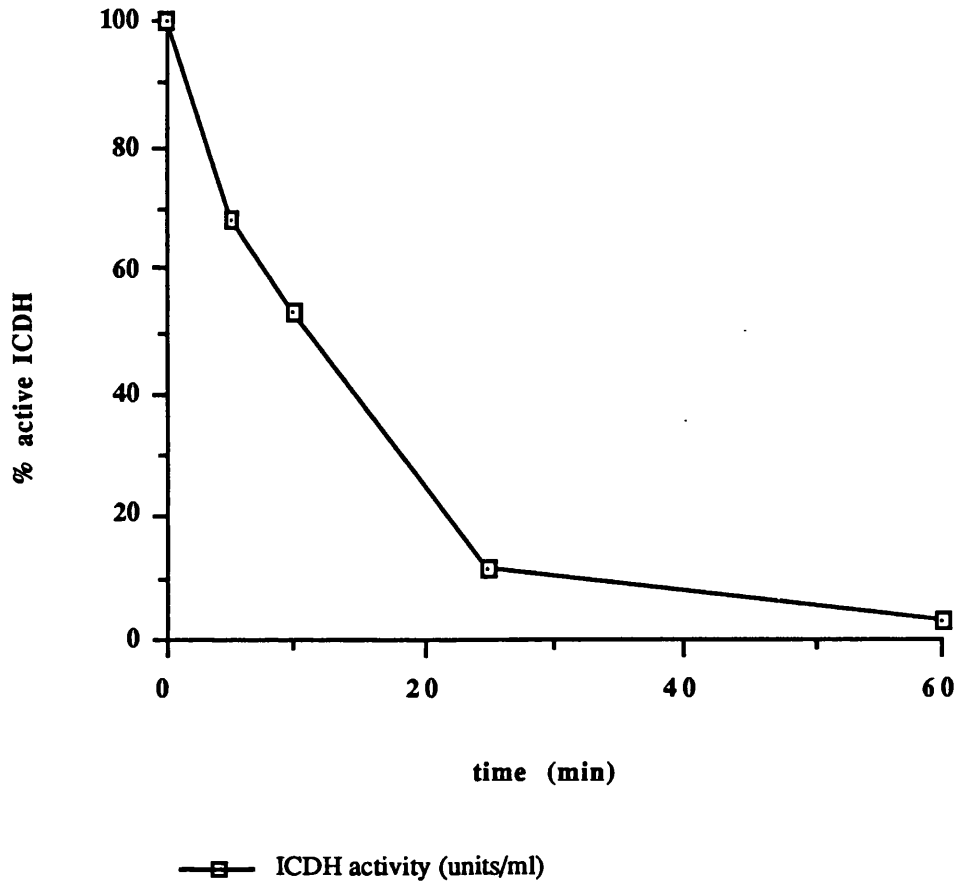



**Figure 4.2 Inactivation and phosphorylation of *B. caldotenax* ICDH *in vitro***

Purified *B. caldotenax* ICDH was incubated with *E. coli* ICDH kinase in the presence of [$\gamma$-$^{32}$P]ATP as described in section 2.11.1. The activity of ICDH and the incorporation of $^{32}$P into the protein (figure 4.3) were followed.

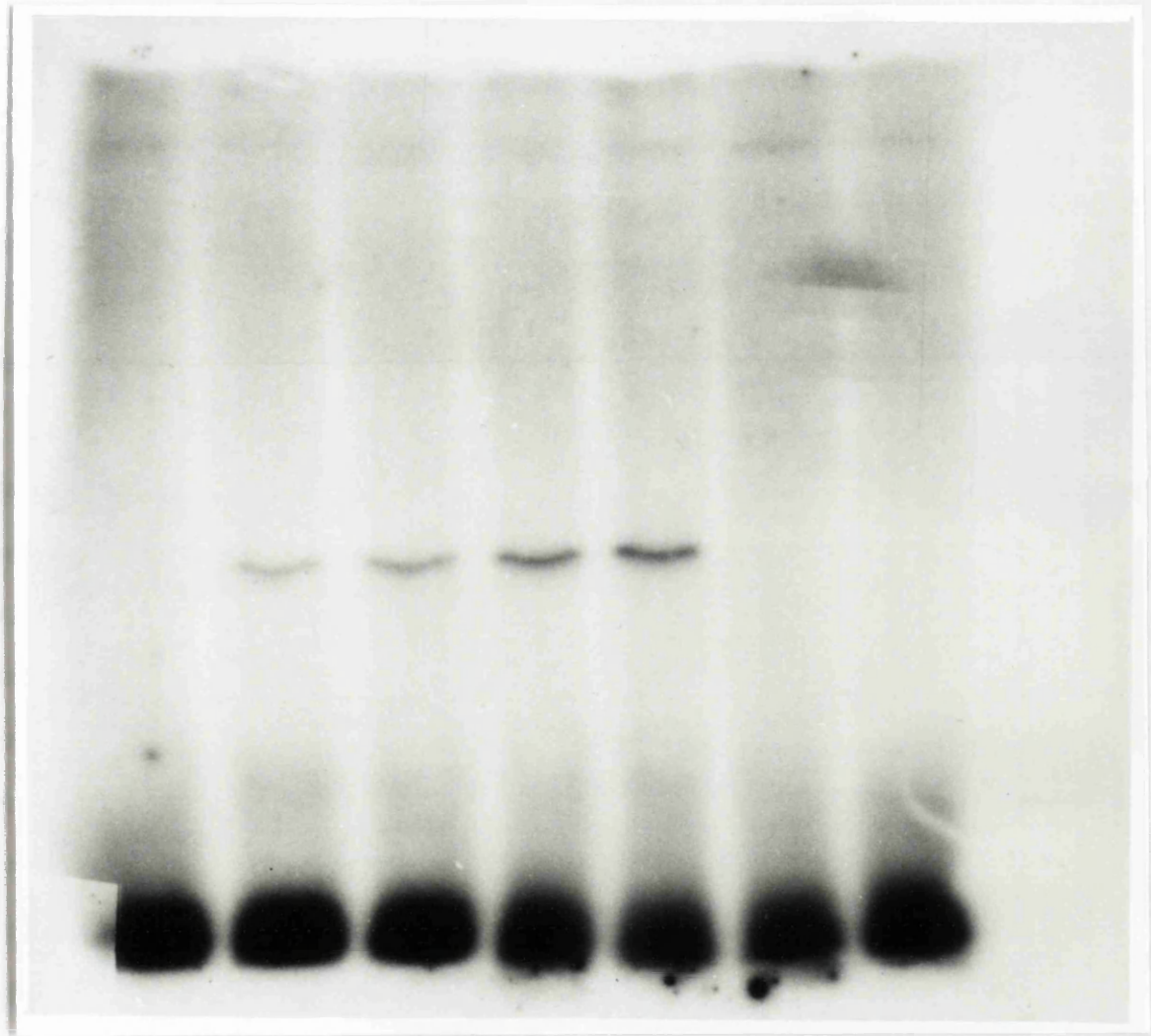
**Figure 4.3 SDS PAGE of ICDH during *in vitro* inactivation and phosphorylation**

40µl samples were removed from the incubation in figure 4.2 at the times shown. The reaction was stopped by heating the sample to 100°C for 2 min with 10µl 5x normal sample buffer. SDS PAGE of these samples was carried out as described in section 2.5.1. The gel was stained for protein, dried and autoradiographed (sections 2.5.2 and 2.2.10).

A 10% polyacrylamide gel was loaded as follows:

| Track | Time (min) | |
|---|---|---|
| 1 | 0 | |
| 2 | 5 | |
| 3 | 10 | |
| 4 | 25 | |
| 5 | 60 | |
| 6 | 60 | control containing no ICDH |
| 7 | 60 | control containing no ICDH kinase |

The dried gel was autoradiographed for 48 hours.



1 2 3 4 5 6 7

phosphorylation of *B. caldotenax* ICDH was determined by comparing the degree of phosphorylation of *B. caldotenax* ICDH with that of *E. coli* ICDH under the same conditions. Two parallel assays were performed in the presence of [$\gamma$-$^{32}$P] ATP as described in section 2.11.1 b). One of the assays contained *B. caldotenax* ICDH whilst the other contained an equal concentration of ICDH purified from *E. coli*. The enzymes were incubated in the presence of equal amounts of *E. coli* ICDH kinase. Samples were removed from both assays over a 60 min time course for estimation of $^{32}$P incorporation into ICDH as shown by gel electrophoresis followed by autoradiography (section 2.2.10). Duplicate assays were performed using non-radioactive ATP in order to estimate ICDH activity over the same time course. Control assays were carried out in the absence of ICDH and in the absence of kinase.

The results showed that *E. coli* ICDH was phosphorylated to completion within the first 5min of incubation (figures 4.4 and 4.5). No *E. coli* ICDH activity remained after 5min and no further incorporation of $^{32}$P was observed after this time (figure 4.5). In contrast the incorporation of $^{32}$P into *B. caldotenax* ICDH occurred more slowly being only complete after 60 min incubation when no ICDH activity remained (figures 4.4 and 4.5). The intensity of the autoradiograph band corresponding to fully inactivated *B. caldotenax* ICDH (i.e. the 60 min sample) was equal to that for the fully inactivated *E. coli* ICDH samples (figure 4.5). As both types of ICDH were present in equal concentrations this would suggest that the inactivation of *B. caldotenax* ICDH by phosphorylation proceeds with the same stoichiometry as phosphorylation of *E. coli* ICDH, i.e. 1mol of phosphate /mol of subunit. However it is possible that more $^{32}$P might be incorporated during a longer incubation.

### 4.2.4 Phosphorylation of ICDH in crude extracts of *B. caldotenax*

A crude extract was made from *B. caldotenax* grown on glutamate as described in section

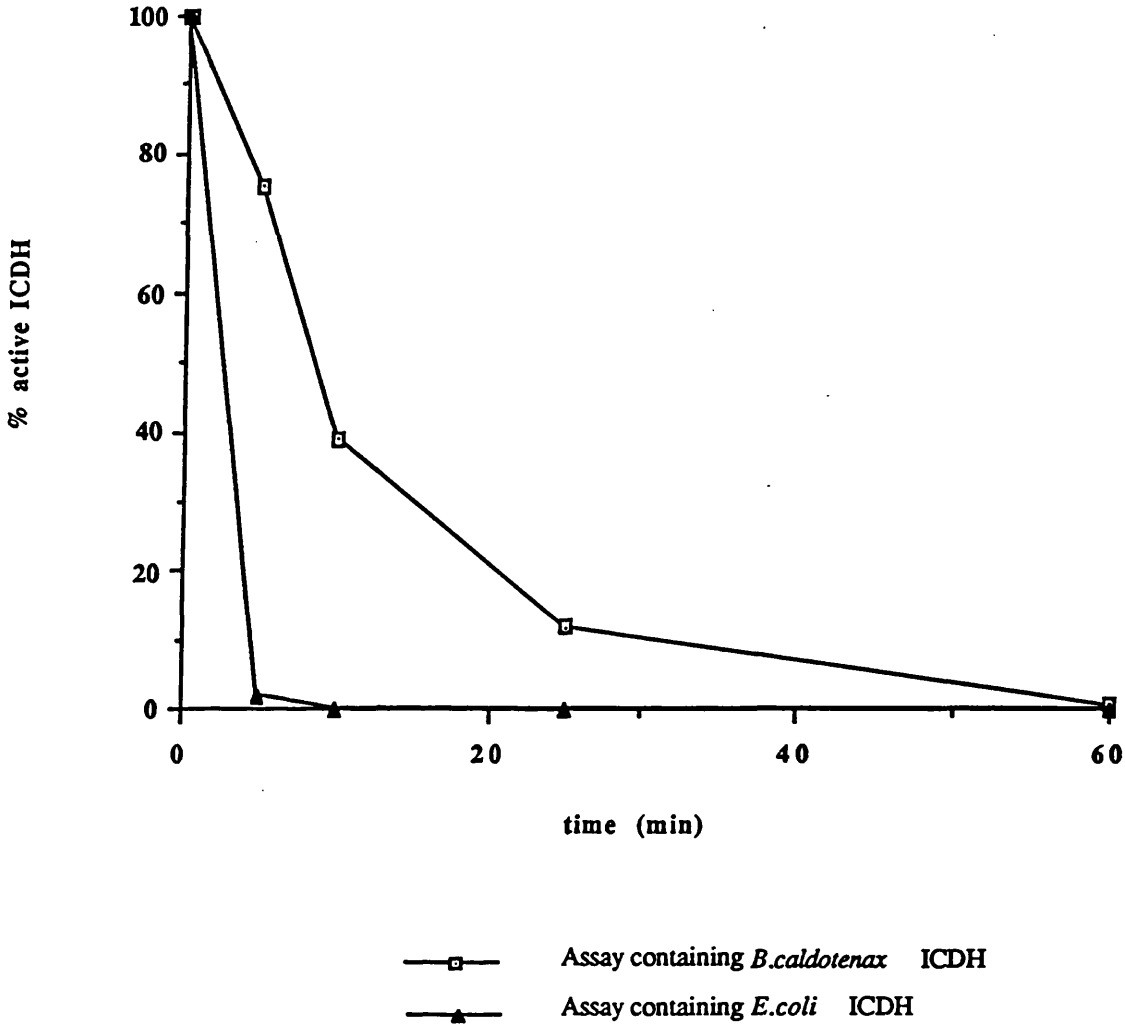



**Figure 4.4 Comparison of the inactivation of *B.caldotenax* ICDH to that of *E.coli* ICDH using *E.coli* ICDH kinase**

Two parallel incubations were performed using either *B.caldotenax* ICDH or *E.coli* ICDH in the presence of [$\gamma$-$^{32}$P]ATP. Samples were removed at the time intervals shown for analysis of ICDH activity (above) and the incorporation of $^{32}$P into ICDH (figure 4.5).

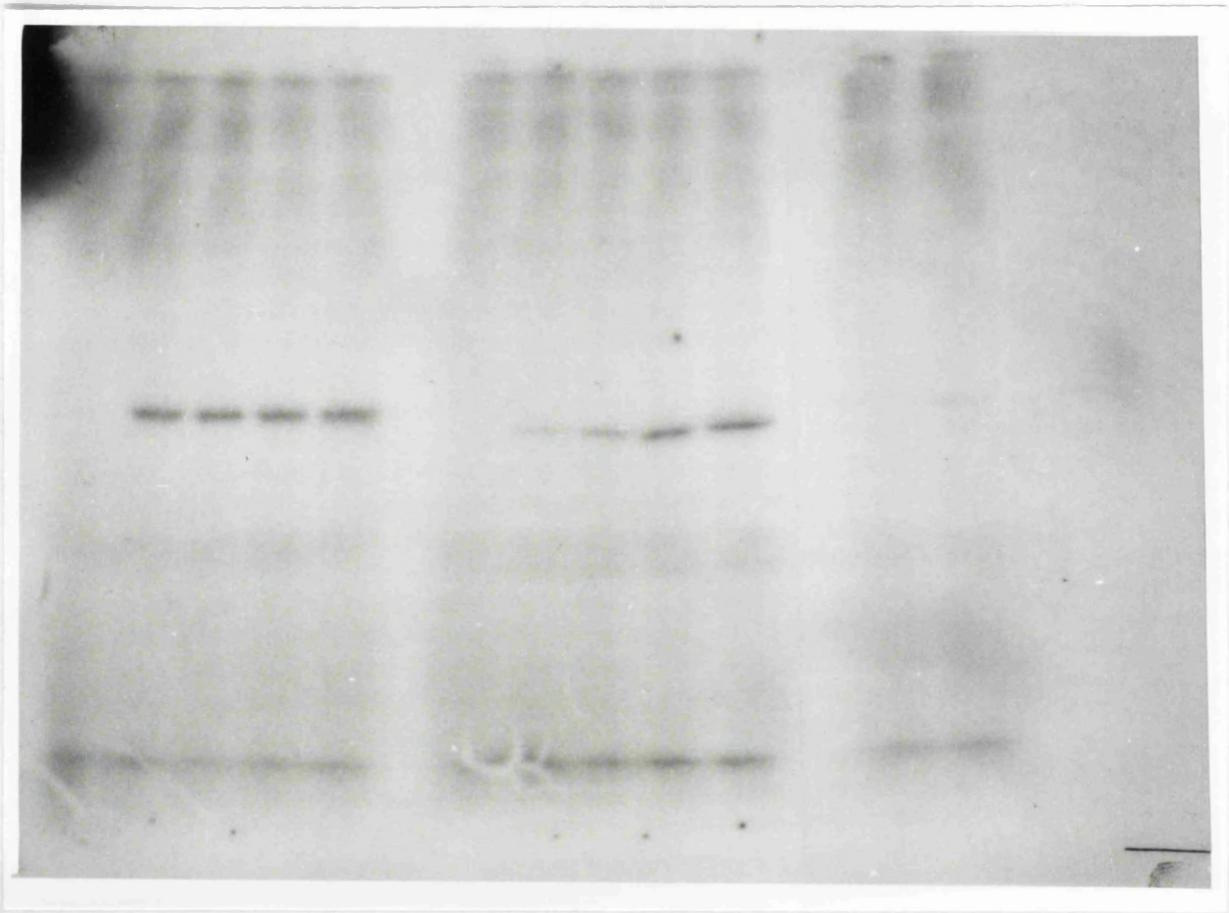
**Figure 4.5 SDS PAGE to compare the stoichiometry of phosphorylation of *E.coli* ICDH to that of *B.caldotenax* ICDH**

Two parallel incubations were performed containing purified ICDH from either *E.coli* or *B.caldotenax* . Samples (40µl) were removed from the incubations (figure 4.4) at the times indicated. The reaction was stopped by heating the sample to 100°C for 2min with 10µl 5x normal sample buffer. SDS PAGE was carried out as described in section 2.5.1. The gel was stained for protein, dried and autoradiographed (sections 2.5.2 and 2.2.10).

A 10% polyacrylamide gel was loaded as follows:

| track | time (min) | |
|---|---|---|
| 1 | 0 | *E.coli* ICDH |
| 2 | 5 | " " |
| 3 | 10 | " " |
| 4 | 25 | " " |
| 5 | 60 | " " |
| 6 | 0 | *B.caldotenax* ICDH |
| 7 | 5 | " " |
| 8 | 10 | " " |
| 9 | 25 | " " |
| 10 | 60 | " " |
| 11 | 60 | control containing no ICDH kinase |
| 12 | 60 | control containing no ICDH |

The dried gel was autoradiographed for 48 hours.



1 2 3 4 5 6 7 8 9 10 11 12

2.11.2 a) and incubated in the presence of [$\gamma$-$^{32}$P]ATP (section 2.11.2b ). Samples were removed, over a time course of 60 min, for analysis of any $^{32}$P incorporation into protein by gel electrophoresis followed by autoradiography (section 2.11.2 b)i) (figure 4.6). The autoradiograph showed incorporation of $^{32}$P into a number of proteins including a 46kDa protein i.e. a protein of the same molecular weight as *B. caldotenax* ICDH.

### 4.2.5 Immunoprecipitation of phosphorylated *B. caldotenax* ICDH

As described in section 3.2.3 cross-reaction was observed between anti-*E. coli* ICDH antibody and *B. caldotenax* ICDH. I therefore developed a procedure for immunoprecipitation of *B. caldotenax* ICDH in order to investigate if the 46kDa protein observed to be phosphorylated in crude extracts was ICDH. Trial experiments with purified *B. caldotenax* ICDH were used to define conditions for immunoprecipitation. *B. caldotenax* ICDH and *E. coli* ICDH were then both phosphorylated with *E. coli* ICDH kinase for 1hour as described in figure 4.5. After this time samples were removed for analysis by immunoprecipitation by the method described in section 2.11.2 b)ii. The results showed it was posssible to immunoprecipitate phosphorylated *B. caldotenax* ICDH from the incubation mixture using anti-*E. coli* ICDH antibody (figure 4.7).

### 4.2.6 Immunoprecipitation of *B. caldotenax* ICDH from crude extracts

Crude extracts were made from *B. caldotenax* , as described in section 2.11.2 a), from cells grown on either glutamate or acetate. Both extracts were incubated at 50°C in the presence of [$\gamma$-$^{32}$P]ATP and samples removed over a 30 min time course. Duplicate samples were taken at each time point and analysed in one of two ways;

a) Incorporation of $^{32}$P into total protein was analysed by gel electrophoresis followed by autoradiography (section 2.11.2 b)i).

**Figure 4.6 SDS PAGE of phosphorylated crude extracts made from *B.caldotenax***

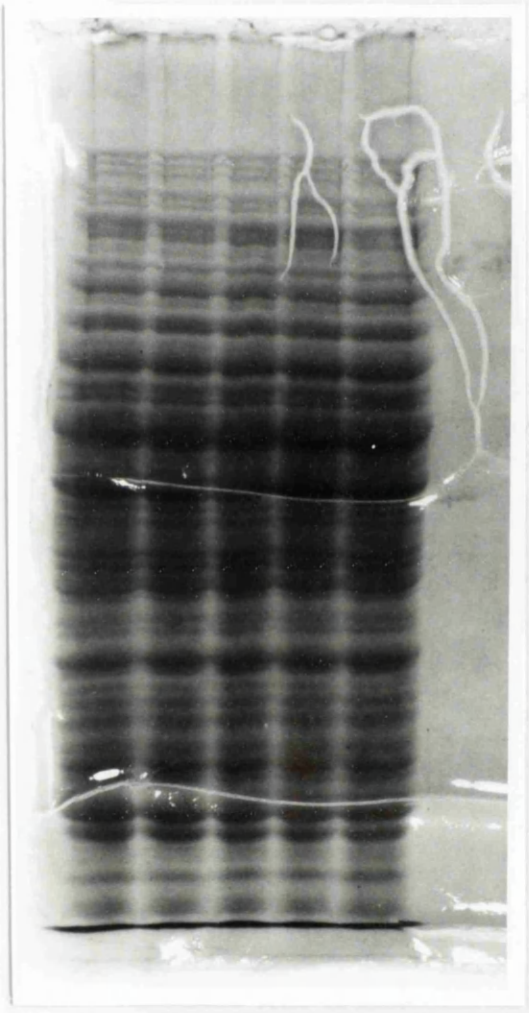
A crude extract was made from *B.caldotenax* grown on glutamate as described in section 2.11.2 a) and incubated in the presence of [$\gamma$-$^{32}$P] ATP (section 2.11.2 b). Samples were removed over a time course for analysis by gel electrophoresis (2.11.2 b i).

**A** A 10% polyacrylamide gel was loaded as follows:

| track | time (min) |
|---|---|
| 1 | 0 |
| 2 | 5 |
| 3 | 10 |
| 4 | 25 |
| 5 | 60 |
| 6 | purified *B.caldotenax* ICDH |

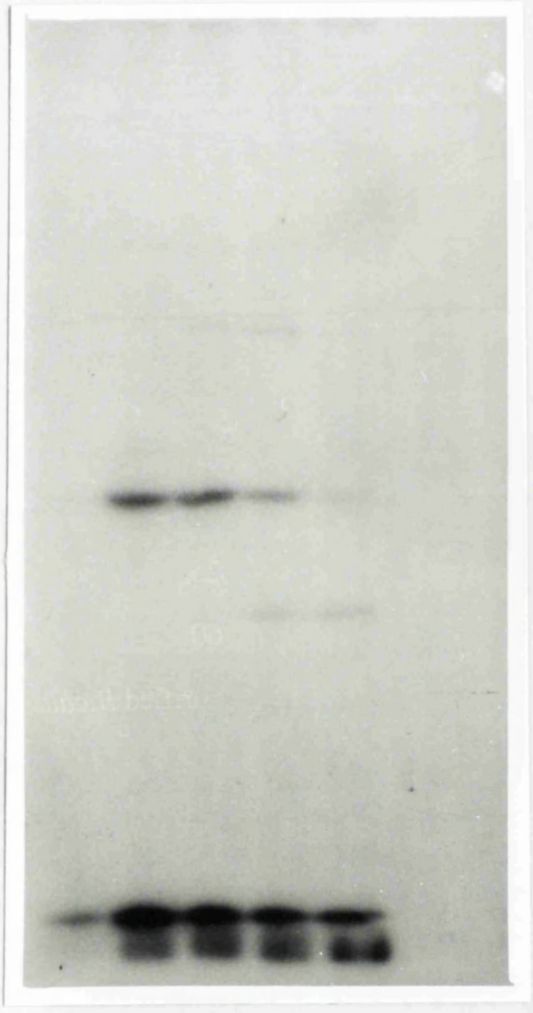
**B** 48 hour autoradiograph of the above gel

A



1 2 3 4 5 6

B




1 2 3 4 5 6

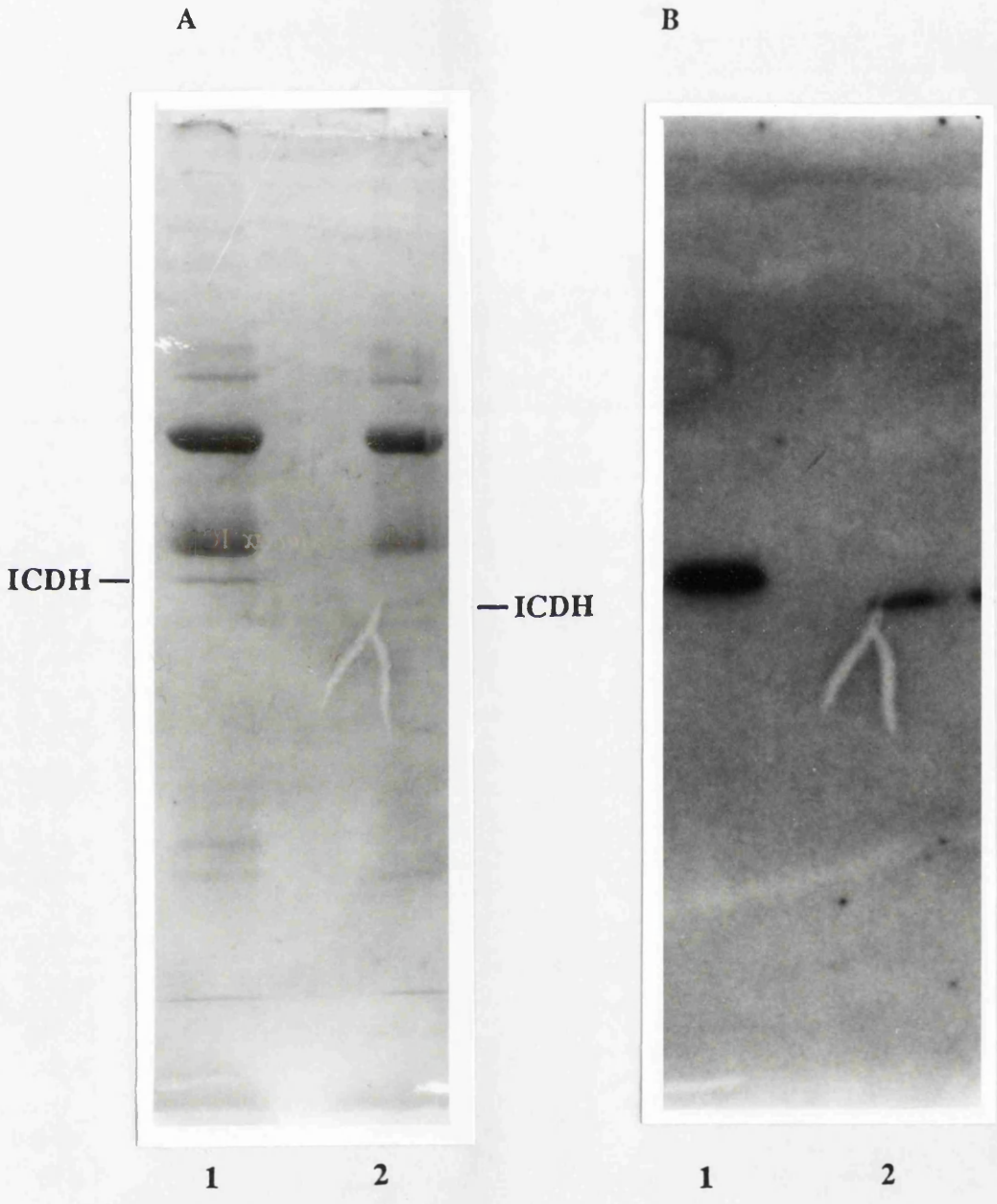
**Figure 4.7 Immunoprecipitation of *B.caldotenax* ICDH phosphorylated *in vitro***

Samples of purified *B.caldotenax* ICDH were phosphorylated with $^{32}P$ *in vitro* by the method described in 2.11.1b. ICDH was immunoprecipitated from the assay by the method described in section 2.11.2 b ii. The same experiment was also carried out using *E.coli* ICDH for comparison.

**A** A 10% polyacrylamide gel was loaded as follows:

| track | sample |
|---|---|
| 1 | immunoprecipitated *E.coli* ICDH |
| 2 | immunoprecipitated *B.caldotenax* ICDH |

**B** Autoradiograph of the above gel


A
B
ICDH
ICDH
1
2
1
2

b) Incorporation of $^{32}P$ into ICDH was estimated by immunoprecipitation of ICDH from the sample prior to gel electrophoresis and autoradiography (section 2.11.2 b)ii).

Within the first 5 min in both types of extract a 46 kDa protein was phosphorylated (figure 4.8B). A number of other proteins were also seen to incorporate $^{32}P$. The pattern of phosphorylation was similar in the two extracts. ICDH was immunoprecipitated from both extracts (figure 4.8A) but the corresponding autoradiograph showed no incorporation of $^{32}P$ into ICDH (figure 4.8B). The immunoprecipitated protein was confirmed to be ICDH by the method of Cleveland (1977 ) as described in section 2.7 (figure 4.9).

### 4.2.7 Immunoprecipitation of *B. caldotenax* ICDH from crude extracts incubated in the presence of phosphatase inhibitors

An identical experiment to that described in section 4.2.6 was carried out with phosphatase inhibitors present in the extracts. Extracts of *B. caldotenax* grown on glutamate or acetate were incubated in the presence of either 10nM okadaic acid or 25mM sodium fluoride. Aliquots were removed at 5 and 30 min intervals for analysis as described in section 4.2.6 (figure 4.10). A similar phosphorylation pattern was obtained to that when phosphatase inhibitors were absent from the incubations. As in the previous experiment no detectable $^{32}P$ was incorporated into immunoprecipitated ICDH.

### 4.2.8 Phosphorylation of *B. caldotenax* ICDH *in vivo*

A culture of *B. caldotenax* was grown on acetate in the presence of $^{32}Pi$ for 1hour (section 2.11.3). A cell free extract was made from these cells as described in section 2.11.2 a) and samples were removed for analysis of $^{32}P$ incorporation into protein by gel electrophoresis followed by autoradiography. In addition ICDH was immunoprecipitated from duplicate

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

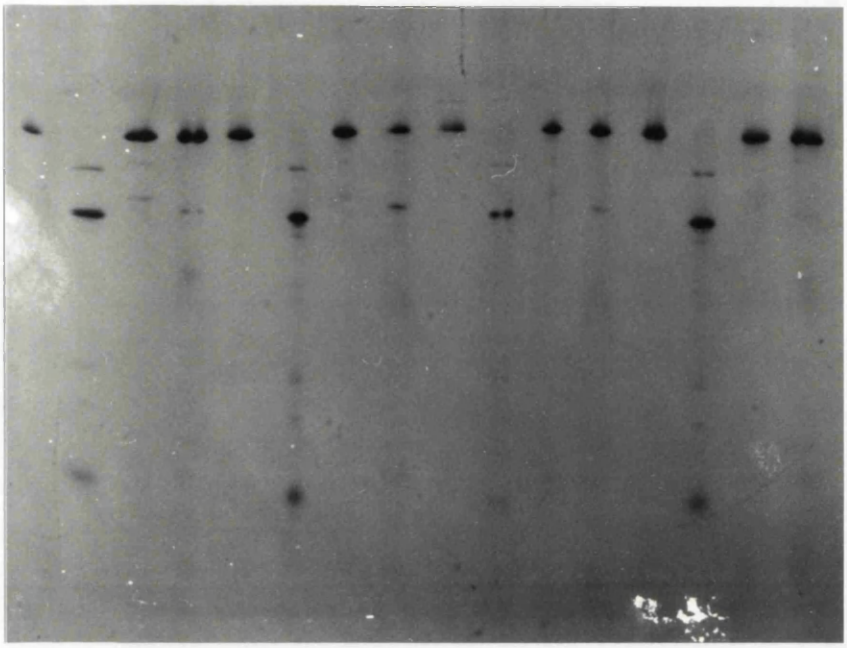


**Figure 4.8 Cleveland map to confirm immunoprecipitated protein to be ICDH**

Immunoprecipitations were performed on crude extracts of *B.caldotenax* using anti-*E.coli* ICDH antibody (section 4.2.6)and the immunoprecipitated protein resolved in a 10% poly acrylamide gel (section 2.5.1). The gel was rapidly stained and destained in Coomassie Brilliant Blue (section 2.5.2). The band of immunoprecipitated protein was excised from the gel and Cleveland mapping performed as described in section 2.7. Purified *B.caldotenax* ICDH was mapped alongside in order to compare the patterns of digestion.

track

1 purified *B.caldotenax* ICDH

2 " " digested with 50ng V8 protease

3 " " digested with 50ng trypsin

4 " " digested with 50ng chymotrypsin

5 immunoprecipitated protein (uncut)

6 immunoprecipitated protein digested with 50ng V8 protease

7 " " " 50ng trypsin

8 " " " 50ng chymotrypsin

Tracks 9 and13, 10 and 14, 11 and 15, 12 and16 are duplicates of 5, 6, 7, and 8 respectively.

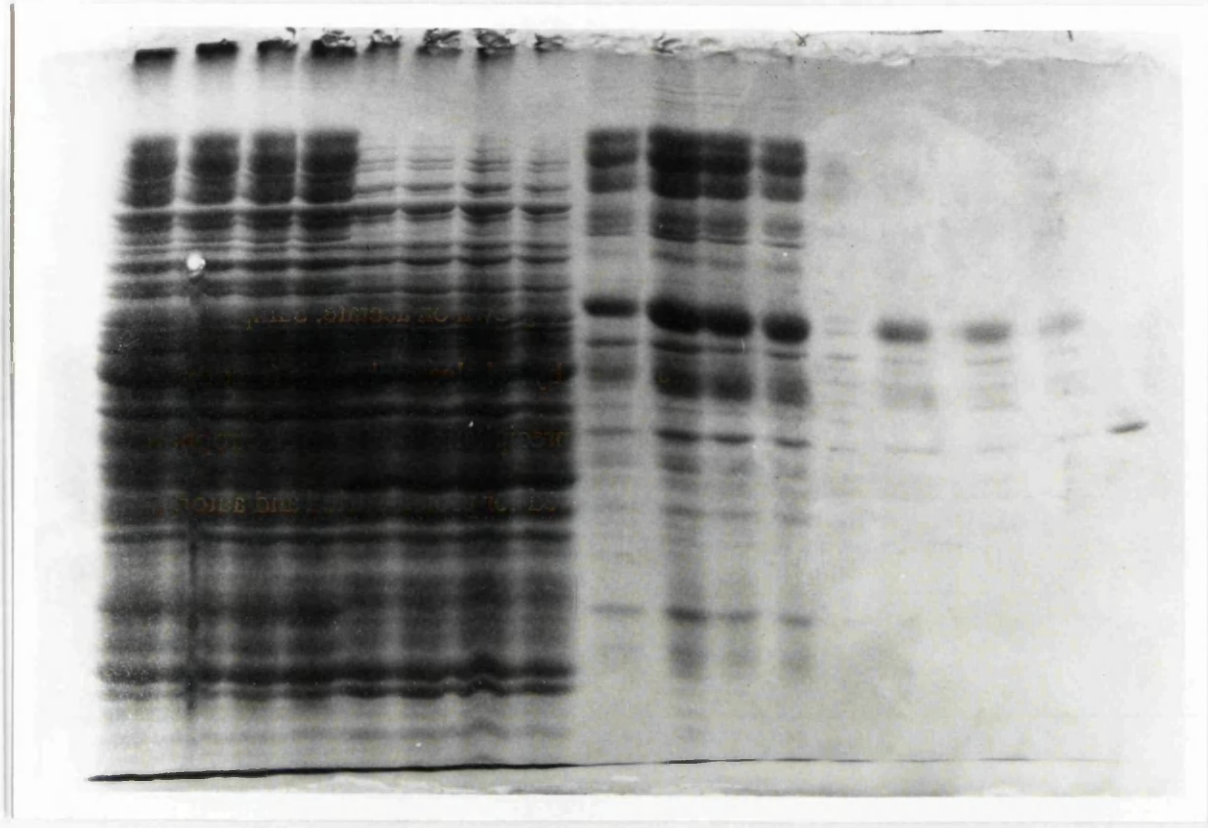
**Figure 4.9 SDS PAGE of *B.caldotenax* ICDH immunoprecipitated from crude extracts**

Two incubations were performed, one using a crude extract made from *B.caldotenax* grown on glutamate the other from *B.caldotenax* grown on acetate. Samples (20µl) were removed over a 30min time course for analysis by gel electrophoresis (section 2.11.2a). Duplicate samples (30µl) were used in immunoprecipitations prior to electrophoresis as described in section 2.11.2.b. The gel was stained for protein, dried and autoradiographed (sections 2.5.2 and 2.2.10).

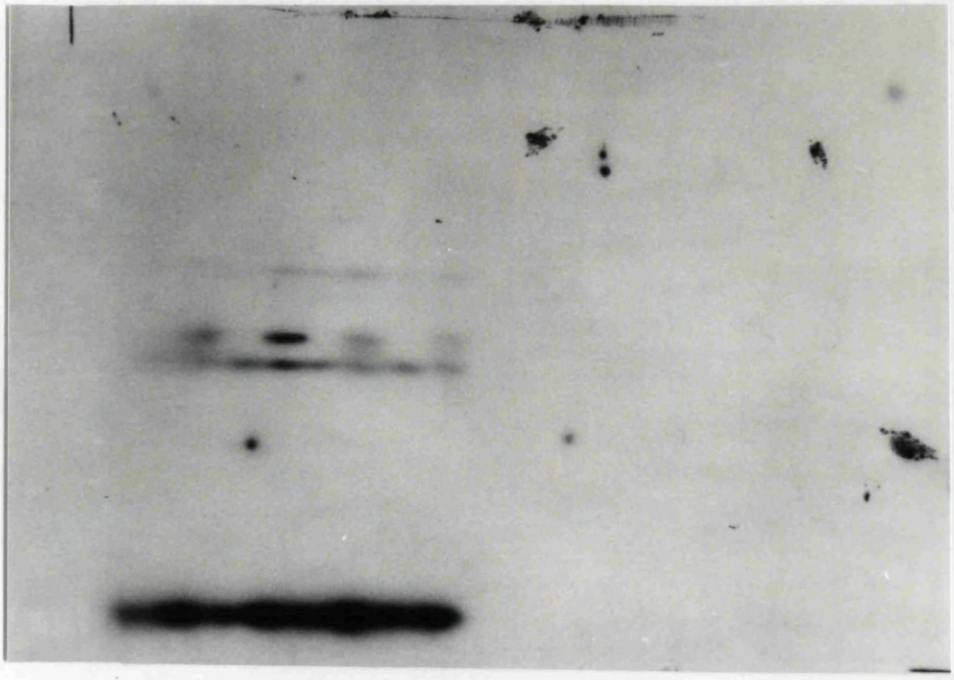
A A 10% polyacrylamide gel was loaded as follows:

| track | extract | time (min) | treatment |
|---|---|---|---|
| 1 | grown on glutamate | 0 | sample used directly for gel electrophoresis. |
| 2 | " " | 5 | |
| 3 | " " | 10 | |
| 4 | " " | 30 | |
| 5 | grown on acetate | 0 | |
| 6 | " " | 5 | |
| 7 | " " | 10 | |
| 8 | " " | 30 | |
| 9 | grown on glutamate | 0 | ICDH immunoprecipitated prior to gel electrophoresis. |
| 10 | " " | 5 | |
| 11 | " " | 10 | |
| 12 | " " | 30 | |
| 13 | grown on acetate | 0 | |
| 14 | " " | 5 | |
| 15 | " " | 10 | |
| 16 | " " | 30 | |
| 17 | Purified *B.caldotenax* ICDH | | |

B A 48 hour autoradiograph of the above gel.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

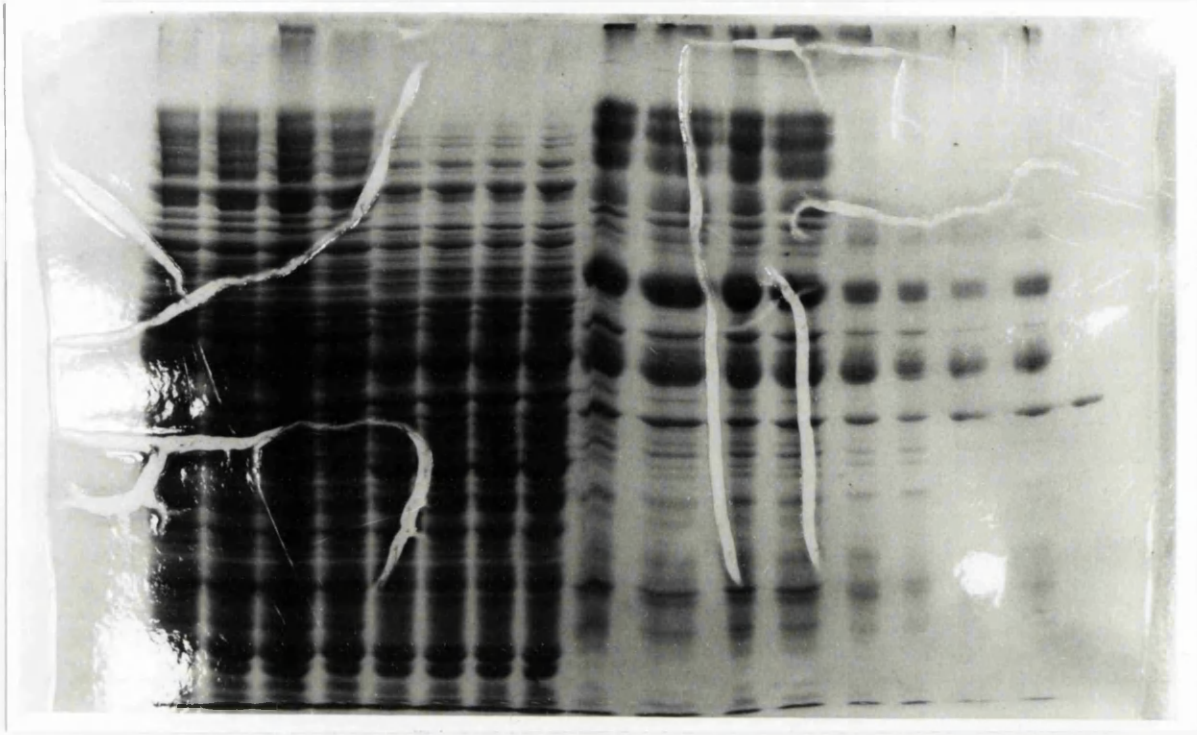
**Figure 4.10 SDS PAGE of *B.caldotenax* ICDH immunoprecipitated from crude extracts incubated in the presence of phosphatase inhibitors**

Four incubations were performed, two using crude extracts made from *B.caldotenax* grown on glutamate and two using extracts made from *B.caldotenax* grown on acetate. Each type of extract was incubated either in the presence of 25mM sodium fluoride or 10nM okadaic acid. Samples (20µl) were removed over a 30 min time course for analysis by gel electrophoresis (section 2.11.2a). Duplicate samples (30µl) were used in immunoprecipitations prior to electrophoresis as described in section 2.11.2.b. The gel was stained for protein, dried and autoradiographed (sections 2.5.2 and 2.2.10).

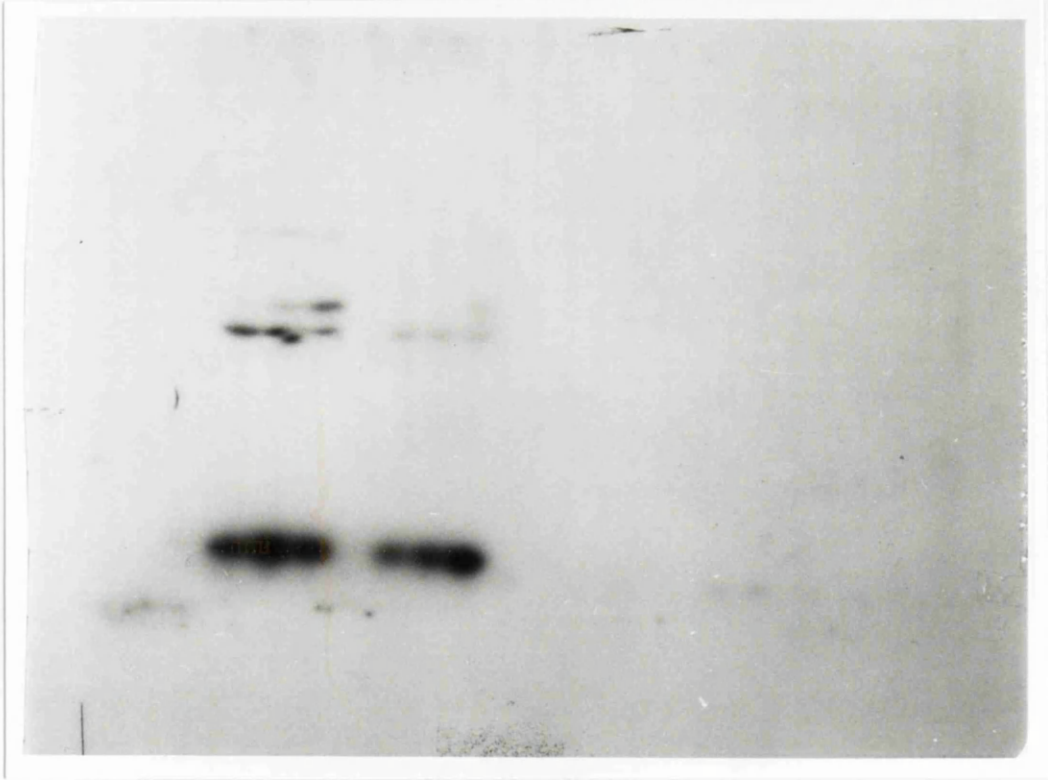
A A 10% polyacrylamide gel was loaded as follows:

| track | extract | time (min) | phosphatase inhibitor | treatment |
|---|---|---|---|---|
| 1 | grown on glutamate | 5 | okadaic acid | sample used directly for gel electrophoresis |
| 2 | " " | 30 | okadaic acid | |
| 3 | " " | 5 | NaF | |
| 4 | " " | 30 | NaF | |
| 5 | grown on acetate | 5 | okadaic acid | |
| 6 | " " | 30 | okadaic acid | |
| 7 | " " | 5 | NaF | |
| 8 | " " | 30 | NaF | |
| 9 | grown on glutamate | 5 | okadaic acid | ICDH immunoprecipitated prior to gel electrophoresis |
| 10 | " " | 30 | okadaic acid | |
| 11 | " " | 5 | NaF | |
| 12 | " " | 30 | NaF | |
| 13 | grown on acetate | 5 | okadaic acid | |
| 14 | " " | 30 | okadaic acid | |
| 15 | " " | 5 | NaF | |
| 16 | " " | 30 | NaF | |
| 17 | Purified *B.caldotenax* ICDH | | | |

B A 48 hour autoradiograph of the above gel.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

**Figure 4.11 Immunoprecipitation of *B.caldotenax* ICDH from cells grown in the presence of $^{32}Pi$**

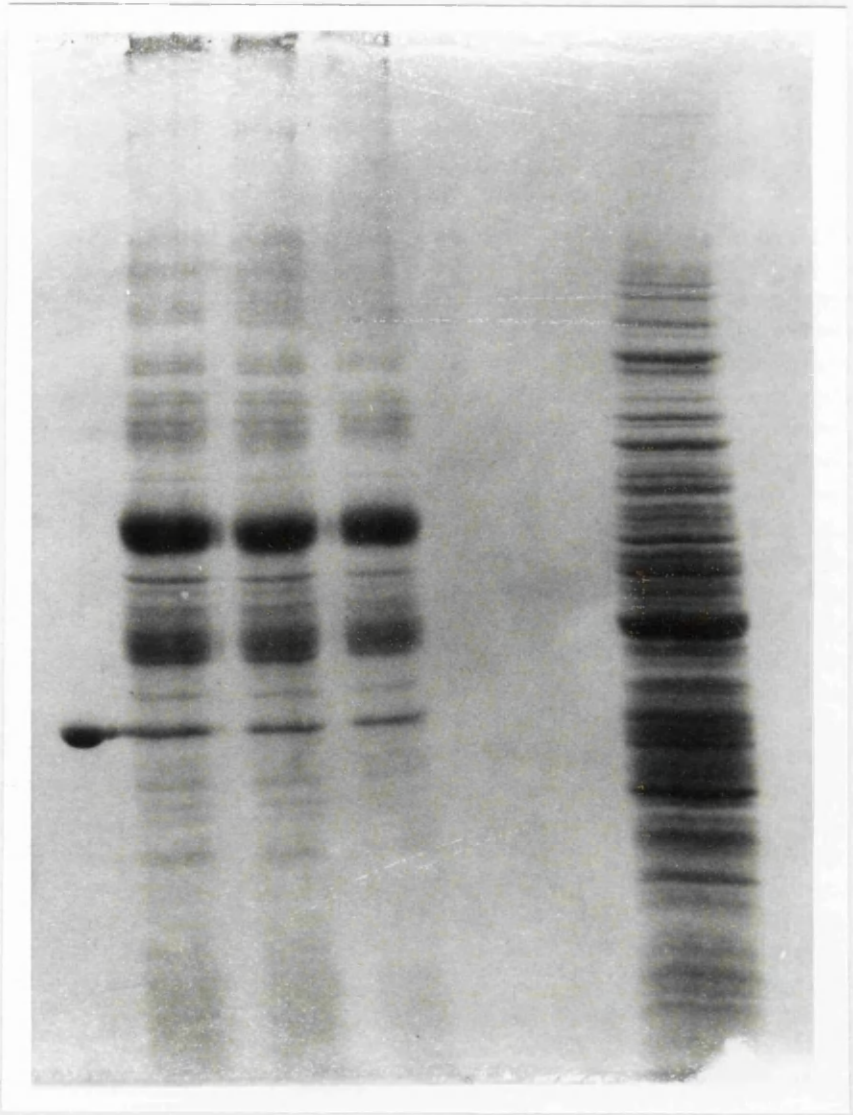
A cell free exract was prepared from *B.caldotenax* grown in the presence of $^{32}Pi$ (20μCi /μmol) as described in section 2.11.3. ICDH was immunoprecipitated from samples of the extract (section 2.11.2 b ii) for analysis by gel electrophoresis followed by autoradiography.

**A** A 10% polyacrylamide gel was loaded as follows:

| track | sample |
|---|---|
| 1 | 30μl of cell free extract denatured in normal sample buffer |
| 2 | ICDH immunoprecipitated from 25μl of extract |
| 3 | ICDH immunoprecipitated from 50μl of extract |
| 4 | ICDH immunoprecipitated from 100μl of extract |
| 5 | Purified *B.caldotenax* ICDH |

**B** 48 hour autoradiograph of the above gel

A



5 4 3 2 1

B

1 2 3 4

samples, prior to analysis of $^{32}P$ incorporation. $^{32}P$ was incorporated into a number of proteins when grown in the presence of labelled inorganic phosphate (figure 4.11) however no detectable $^{32}P$ was incorporated into immunoprecipitated ICDH.

## 4.3 Discussion

The data presented here shows that *B. caldotenax* ICDH can be phosphorylated and inactivated by *E. coli* ICDH kinase, albeit more slowly than is *E. coli* ICDH. The stoichiometry of the two phosphorylations seems to be the same namely 1 mol phosphate /mol subunit. The slow rate of phosphorylation of the *B. caldotenax* enzyme may reflect differences in the sequence and/or conformation between it and *E. coli* ICDH. Phosphorylation was carried out at 37°C, well below the optimum growth temperature of *B. caldotenax*. The conformation of *B. caldotenax* ICDH may be dependent on temperature which could explain the slow rate of phosphorylation observed.

There are two possible explanations for the results obtained in these *in vitro* phosphorylation studies. The acquisition of the complete nucleotide sequence of the *B. caldotenax* gene for ICDH is reported in chapter 5. The amino acid sequence was deduced from this which allowed comparison of the sequence with that of *E. coli* ICDH. As a result of this comparison we now know that there is immense similarity between the amino acid sequences of *E. coli* and *B. caldotenax* ICDH's (approximately 70% identity). In consequence there would be expected to be much structural homology, as was originally suggested by immunological cross-reaction between *B. caldotenax* ICDH and an antibody raised to *E. coli* ICDH (section 3.2.3). The phosphorylation observed *in vitro* might be explained by the fact that *B. caldotenax* ICDH is so similar to that of *E. coli* it merely acts as a suitable substrate for the *E. coli* ICDH kinase and therefore becomes phosphorylated. Although the phosphorylation is genuine and results in a decrease in activity it is possible that the phosphorylation is not used as a control mechanism *in vivo* and is not of any

physiological significance. This would explain why it was not possible to show evidence of phosphorylation *in vivo*.

Alternatively the sequence of ICDH is highly conserved between the two genera *Escherichia* and *Bacillus* , the sequence of amino acids at the phosphorylation site having 100% identity (chapter 5). It could be argued that the reason for this extreme level of conservation is to maintain the ability to be controlled by phosphorylation, as in *E. coli*. It was reported by Kuhn *et al.* (1980) that the activity of ICL in *B. caldotenax* was seen to increase during growth on acetate as sole carbon source. It would not therefore be unreasonable to expect some form of control mechanism at the branch point between the TCA cycle and the glyoxylate bypass in this micro-organism.

The results from *in vitro* phosphorylation studies kept open the possibility that *B. caldotenax* ICDH might be, like that of *E. coli* , under the control of phosphorylation. I therefore investigated whether phosphorylation occured in intact cells. A 46kDa protein was shown to be phosphorylated in crude extracts of *B. caldotenax*. Immunoprecipitation of ICDH from such extracts demonstrated that the phosphorylated protein was not ICDH but another protein of the same subunit molecular weight. The experiments carried out in the presence of phosphatase inhibitors go some way to eliminating the possibility that the action of phosphatases in the crude extract was preventing us from seeing ICDH phosphorylation. However in retrospect it might have been better to phosphorylate ICDH *in vitro*, add this to the extract, and look for loss of $^{32}P$.

It was of course possible that ICDH could be phosphorylated in intact cells but not in crude extracts. This possibility was investigated by immunoprecipitating ICDH from $^{32}P$ labelled cells, however this experiment gave no evidence of phosphorylation.

The rate of turn over of isocitrate and other metabolites in the cell is likely to be very high. In addition, in *E. coli* it has been reported that anaerobiosis can cause activation of ICDH in

cells grown on acetate (Holms and Nimmo, 1982). Any process which alters the cell's environment prior to stopping metabolism might result in the dephosphorylation of ICDH. The method chosen to investigate phosphorylation *in vivo* was a relatively lengthy process and it is possible that if *B. caldotenax* ICDH was phosphorylated *in vivo* it may have reverted to the dephosphorylated state during these manipulations. This was shown not to be the case when similar experiments were carried out with *E. coli* ICDH (Borthwick *et al.*,1984 b) although these experiments employed different conditions. Within the limits of this study it would seem likely that we have not chosen quite the correct experimental conditions in which to observe phosphorylation. The negative data that we have obtained here do not prove that phosphorylation does not occur *in vivo* only that we did not see it.

# Chapter 5

# Cloning and sequencing the gene for *Bacillus caldotenax* ICDH

## 5.1 Introduction

Only a small number of genes have been cloned from the thermophile *B. caldotenax* including those genes for lactate dehydrogenase (Zulli *et al.*, 1987), isopropylmalate dehydrogenase (Sekiguchi *et al.*, 1986a), and tyrosyl tRNA synthetase (Jones *et al.*, 1986). Therefore the characterisation of any gene encoding an enzyme of central metabolism from this micro-organism would be important.

The gene which codes for ICDH in *E. coli* has been cloned and its complete nucleotide sequence determined (Thorsness and Koshland, 1987). Similarities between the *E. coli* and *B. caldotenax* ICDHs were previously reported in chapter 3 as a result of immunological cross-reaction and comparisons of some amino acid sequence. A much more detailed comparison of these two enzymes could be carried out if the nucleotide sequence of the *B. caldotenax icd* gene were to be determined and the complete amino acid sequence inferred from this.

In this chapter I report the use of the amino acid sequence of *B. caldotenax* ICDH (see section 3.2.5) to design an oligonucleotide probe which has been used to clone the gene for *B. caldotenax* ICDH by reverse genetics. The complete nucleotide sequence of the ICDH coding region was determined and compared to that of the *E. coli icd* gene. Some analysis of the DNA peripheral to the gene has also been performed.

## 5.2 Results

### 5.2.1 Design of a probe for cloning the *B. caldotenax* ICDH gene

The amino acid sequence derived from *B. caldotenax* ICDH (figure 3.9) has been used to design an oligonucleotide probe for use in cloning the gene for this enzyme. The sequences of very few genes of *B. caldotenax* are known and no codon preference table has been constructed for this micro-organism. *Bacillus stearothermophilus* is a moderately thermophilic bacillus very similar to *B. caldotenax*, differing only in its ability to hydrolise starch ( Epstein and Grossowicz, 1969). Unlike *B. caldotenax*, *B.stearothermophilus* has been extensively studied and sequence data has been obtained for a number of genes. A codon preference table had been constructed for *B.stearothermophilus* (Hawkins *et al.*, 1990) using sequence obtained from seven different genes. Zulli *et al.* (1987) compared codon usage within the *B.stearothermophilus* and *B. caldotenax* genes for LDH and showed there was considerable similarity in the codon preference of these two micro-organisms. Therefore the codon usage table of Hawkins was used to design a probe for cloning the *icd* gene of *B. caldotenax* (figure 5.1). The designed probe is a 35mer deoxyoligonucleotide which is the reverse of the coding strand.

### 5.2.2 Southern blot analysis of digests of *B. caldotenax* genomic DNA

Samples of *B. caldotenax* genomic DNA were digested with a series of restriction enzymes and the fragments resolved in agarose gels (sections 2.12.4 and 2.12.5) (figure 5.2). The DNA was transferred to nylon filters (section 2.15) and probed with the 35mer oligonucleotide which was end labelled with $\gamma$-$^{32}$P (sections 2.16 and 2.17). Probed filters were washed under increasingly stringent conditions of temperature and ionic strength, air dried and autoradiographed for 24 hours. Adjusting the temperature and salt concentration of the washes enabled optimum conditions to be established, under which the probe was

amino acid sequence **M T Q G E K I T V Q N G V L N V**

coding strand **5'-ATG ACG CAA GGC GAA AAA ATC ACG GTC CAA AAC GGC GTC CTC AAC GTC -3'**

probe **5' - AC GCC GTT TTG GAC CGT GAT TTT TTC GCC TTG CAT - 3'**



**Figure 5.1 Design of an oligonucleotide probe for cloning the ICDH gene of *B. caldotenax***

The 35 mer oligonucleotide probe was derived using the codon usage table of *B. stearothermophilus* compiled by Hawkins *et al.* (1987) together with the amino acid sequence obtained from *B. caldotenax* ICDH shown above. The deduced sequence of the coding strand is also shown, the probe is the reverse of the coding strand.

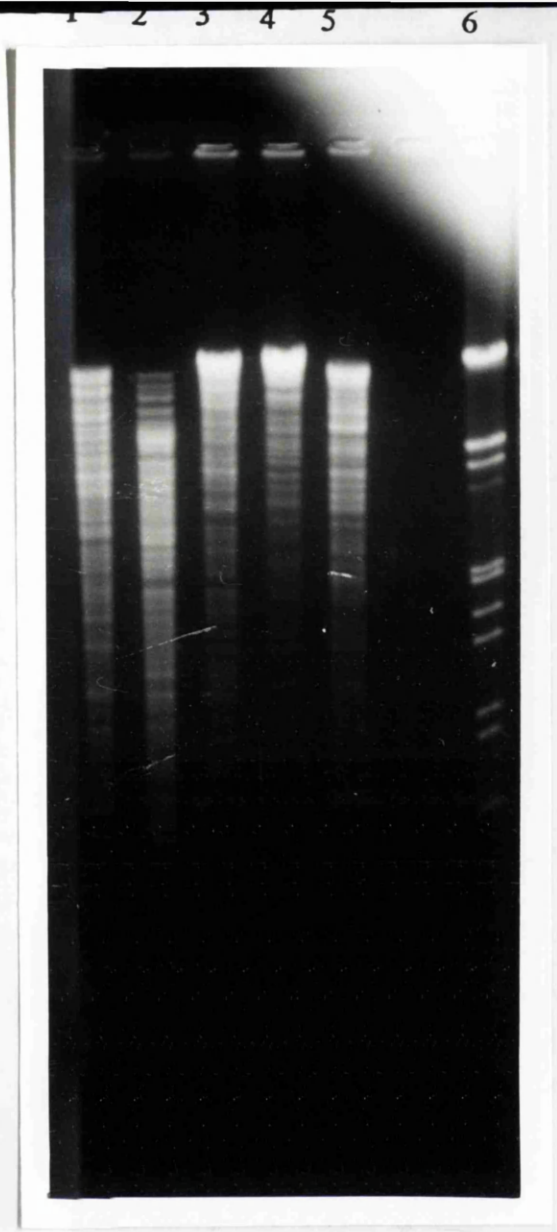



**Figure 5.2 Agarose gel electrophoresis of restriction digests of *B.caldotenax* genomic DNA**

A gel was run as described in section 5.2.2. 5µg samples of DNA were digested (section 2.12.4) with the following enzymes.

| track | restriction enzyme |
|---|---|
| 1 | *Sal* I |
| 2 | *Cla* I |
| 3 | *Eco* RI |
| 4 | *Bam* HI |
| 5 | *Hin* dIII |
| 6 | markers λ/*Hin* dIII +*Eco*RI |

seen to hybridise to a single restriction fragment in each digest (figure 5.3). Optimum conditions were found to be at an ionic strength of 1xSSC and a temperature of 55°C.

### 5.2.3 Choice of an appropriate restriction fragment and vector

The sizes of the hybridising fragments, seen in figure 5.3, are summarised in table 5.1. In choosing an appropriate size of restriction fragment to clone a number of criteria were employed. The fragment must be sufficiently large that is likely to contain the complete coding sequence for ICDH together with some DNA peripheral to the gene. It was reported that the subunit molecular weight of *B. caldotenax* ICDH is 46kDa (section 3.2.2). Using this value it is possible to estimate the size of the gene for ICDH to be approximately 1.4 kb. Conversely the fragment must be small enough to allow for its successful purification and be of an appropriate size to insert into a cloning vector. Applying these criteria any of the hybridising fragments would have been suitable. It was decided to use the *Sal* I digest which gave a fragment of approximately 4.2 kb. It was thought that while this fragment was large enough to be likely to contain the entire coding region for ICDH it was nevertheless sufficiently small for purification and ligation to be straightforward.

For cloning a relatively small piece of DNA, as in the *Sal* I digest, a plasmid vector should be suitable and simple. It was decided to use the plasmid vector pUC18. This vector contains a selectable antibiotic marker, for ampicillin resistance, and it possesses IPTG-inducible histochemical selection when used in an appropriate host (section 2.14.3). This property allows recombinants to be easily identified. The polylinker of pUC18 contains a number of restriction sites including a *Sal* I site, the restriction enzyme chosen above.

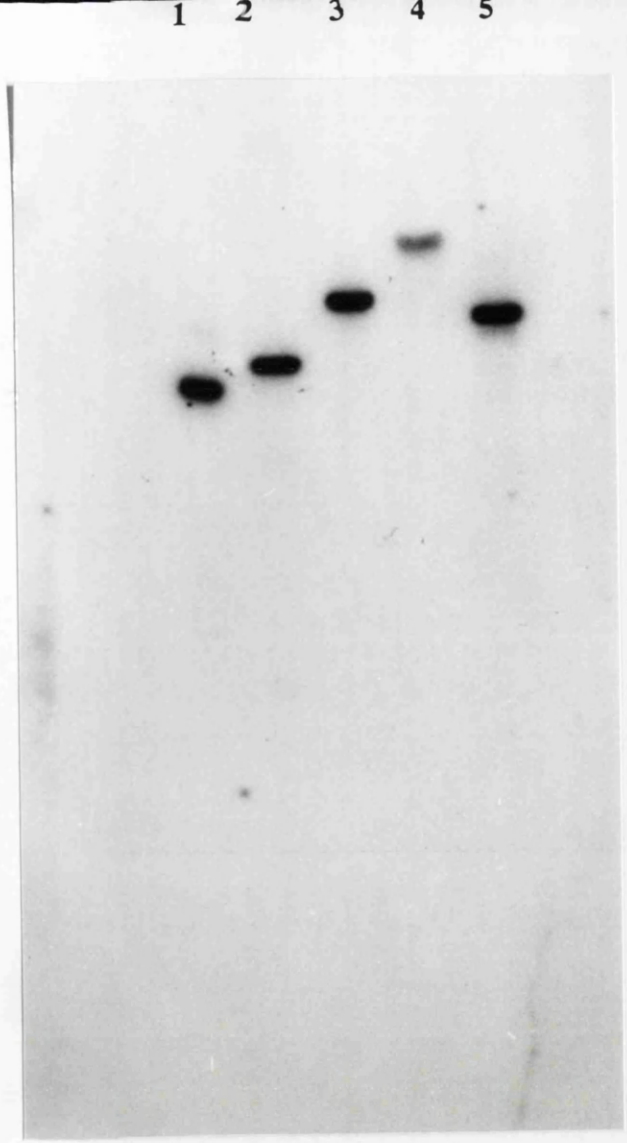



**Figure 5.3 Autoradiograph of a Southern blot of digested *B.caldotenax* genomic DNA probed with labelled oligonucleotide**

DNA was digested with a range of restriction enzymes and the fragments separated in an agarose gel (figure 5.2). The restriction fragments were transferred to a nylon filter by the method of Southern (section 2.15). The filter was probed using the oligonucleotide shown in figure 5.1 and washed with 1xSSC at 55°C (as described in section 2.17).

| track | restriction enzyme |
|---|---|
| 1 | *Sal* I |
| 2 | *Cla* I |
| 3 | *Eco* RI |
| 4 | *Bam* HI |
| 5 | *Hin* dIII |

**Table 5.1 Size of hybridising restriction fragments obtained from digests of *B.caldotenax* genomic DNA**

| Restriction enzyme | Fragment size (kb) |
|---|---|
| *Sal* I | 4.2 |
| *Cla* I | 4.4 |
| *Eco* RI | 6.3 |
| *Bam* HI | 9.3 |
| *Hin* dIII | 6.5 |

### 5.2.4 Construction of a *B. caldotenax* genomic sub-library in pUC18

#### a) Size fractionation of *Sal* I restriction fragments of genomic DNA

The oligonucleotide probe designed to hybridise to the *B. caldotenax icd* gene was shown to hybridise specifically to a 4.2 kb *Sal* I restriction fragment of *B. caldotenax* genomic DNA (figure 5.3). By size selecting for fragments of approximately 4.2 kb and constructing a library with these it was possible to enrich for the presence of the *icd* gene.

Originally attempts were made to size select fragments using the conventional methods of low melting point agarose and electroelution. These methods repeatedly gave unsatisfactory low yields and difficulties were experienced with ligations. Successful size fractionation of *Sal* I digested genomic DNA was achieved using a Waters Gen-pak FAX column capable of producing high resolution of DNA restriction fragments. *B. caldotenax* genomic DNA was digested with *Sal* I as described in section 2.12.4 and fragments resolved using the Gen-pak FAX column (section 2.12.6). Restriction fragments were eluted from the column with a gradient of increasing NaCl concentration; a typical elution profile is shown in figure 5.4. Fractions were collected and DNA precipitated by the addition of ethanol. Precipitated DNA was resuspended in TE buffer and test samples analysed by gel electrophoresis (section 2.12.5) (figure 5.5) to establish which fraction contained DNA of the required size. DNA was transferred to nylon membranes, probed, washed and autoradiographed as with genomic digests in section 5.2.2. This process identified precisely the fraction containing DNA of interest (figure 5.5). Once identified remaining DNA from this fraction was used in subsequent ligations to construct a sub-library of *B. caldotenax* genomic DNA.

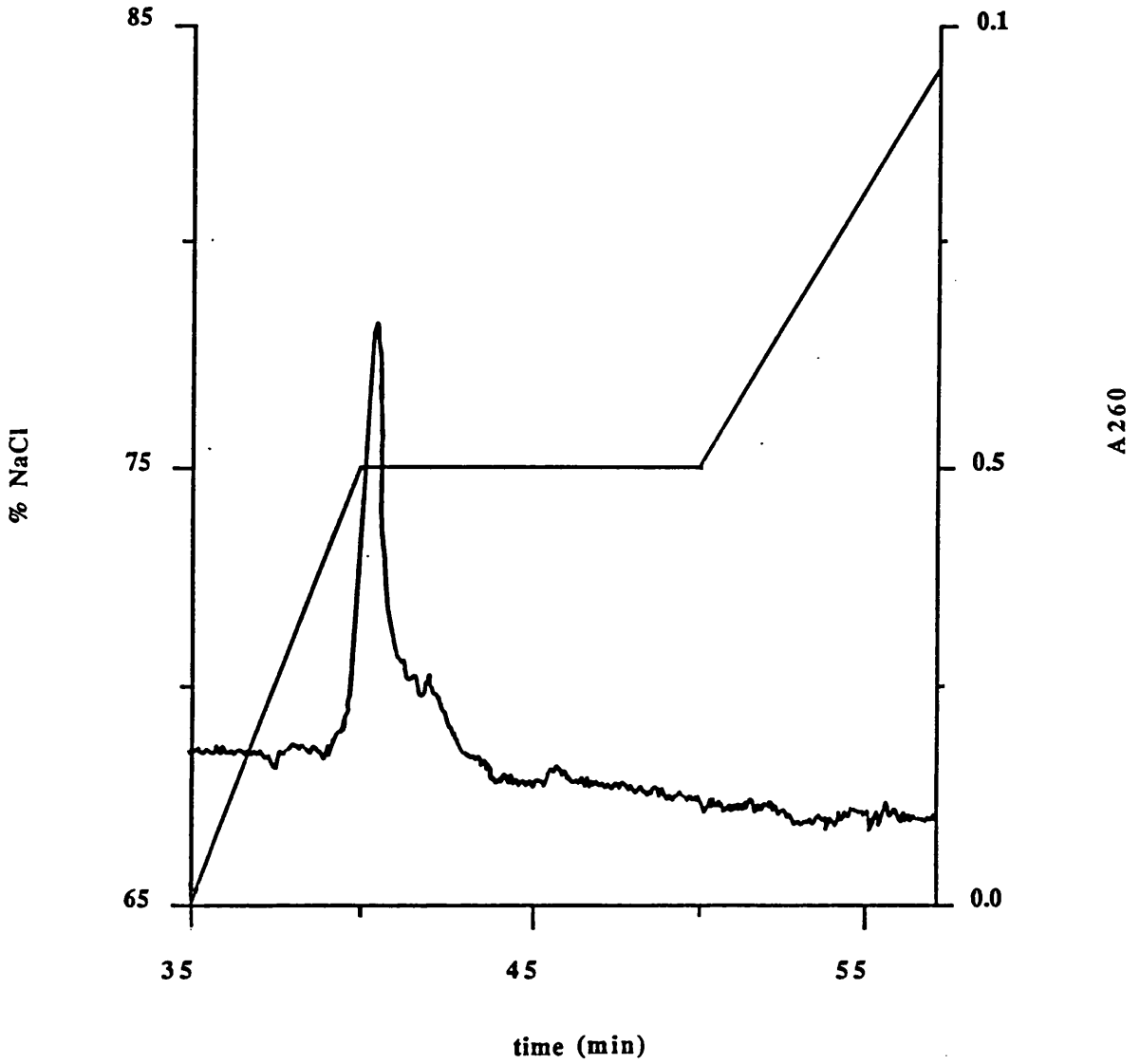



**Figure 5.4** **h.p.l.c. purification of restriction fragments generated by a *SalI* digest of *B.caldotenax* genomic DNA**

A Waters Genpak FAX column was equilibrated in 25mM Tris/HCL,1mM EDTA pH7.5 and a gradient developed with 1M NaCl. The flow rate was 0.5 ml/min and 125µl fractions were collected across the peak.

Gradient: 0-75% (v/v) NaCl in 40 min.
75-75% (v/v) NaCl in 10 min.
75-100 % (v/v) NaCl in 20 min.

**Figure 5.5 Agarose gel electrophoresis and Southern blotting of *Sal* I restriction fragments purified by h.p.l.c.**

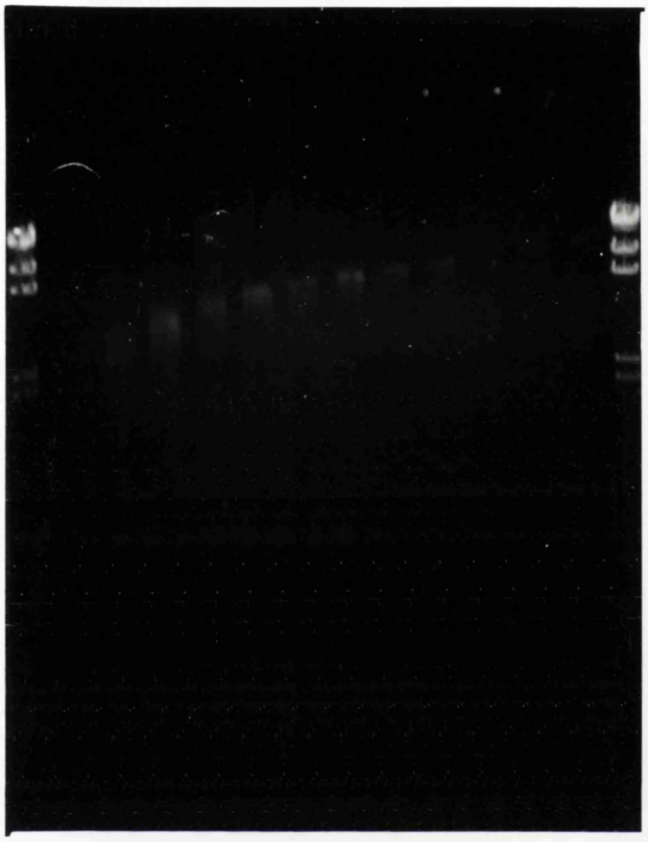
**A** Fractions were collected across the h.p.l.c. peak (figure 5.4) and the DNA recovered by ethanol precipitation. DNA was resuspended in TE buffer (10mM Tris,1mM EDTA pH8) and analysed by agarose gel electrophoresis (section 2.12.5.)

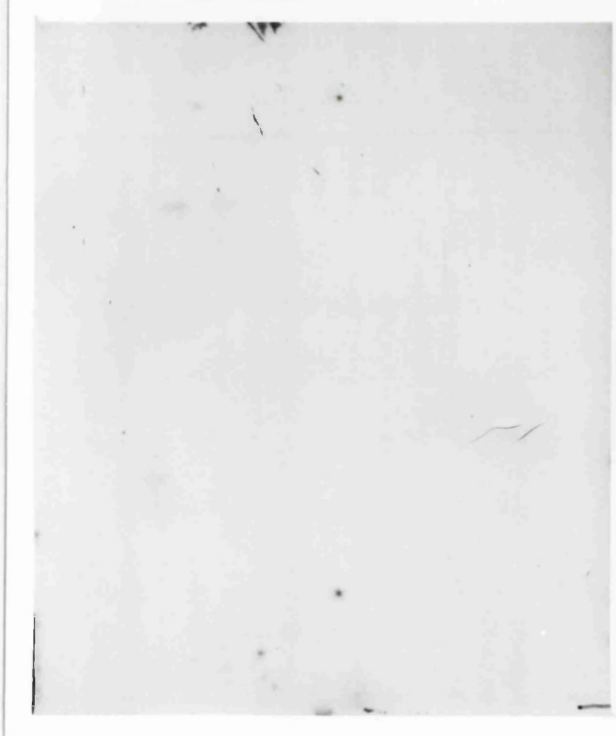
**B** The gel in figure 5.5 A was Southern blotted (section 2.15) and probed using labelled oligonucleotide (figure 5.1). The autoradiograph showed which fraction contained the DNA of interest.

λ/*Hin* dIII

fractions collected across the h.p.l.c. peak (figure 5.4)

λ/*Hin* dIII



### b) Ligation of *Sal*I fragments into pUC18

Purified *Sal* I fragments, prepared as described in section 5.2.6 a), were ligated into the plasmid vector pUC18 as described in section 2.13. This process was facilitated by the purchase of pUC18 ready cut with *Sal* I and treated with bacterial alkaline phosphatase. Appropriate control ligations were performed and agarose gel electrophoresis was used to confirm that ligations were successful.

### c) Transformation of *E. coli* DS941 with plasmid DNA

Competent cells were produced by the method of Hanahan (1983) as described in section 2.14.1 and transformed with plasmid containing insert by the method described in section 2.14.2. Suitable transformation controls were also performed. White colonies were likely to contain insert; these were picked and screened for the presence of the *B. caldotenax icd* gene by colony hybridisation.

## 5.2.5 Screening recombinants for the presence of the *B. caldotenax* gene for ICDH by colony hybridisation

Forty recombinant clones were screened by the method described in section 2.18, using oligonucleotide probe labelled as in section 2.16. Hybridisation reactions were carried out using the optimum conditions established during Southern blot analysis (section 5.2.2). Under these conditions the probe was seen to hybridise strongly to DNA from two recombinants (figure 5.6). Plasmid DNA was prepared from these two recombinants (section 2.12.1), digested with *Sal* I and analysed by gel electrophoresis (section 2.12.5). Digestion of both recombinant plasmids with *Sal* I produced a 2.7kb vector fragment and a 4.5 kb insert fragment (figure 5.7A); the recombinants appeared to be identical. The use of 1kb DNA Ladder as a marker allowed the size of the insert to be determined accurately to

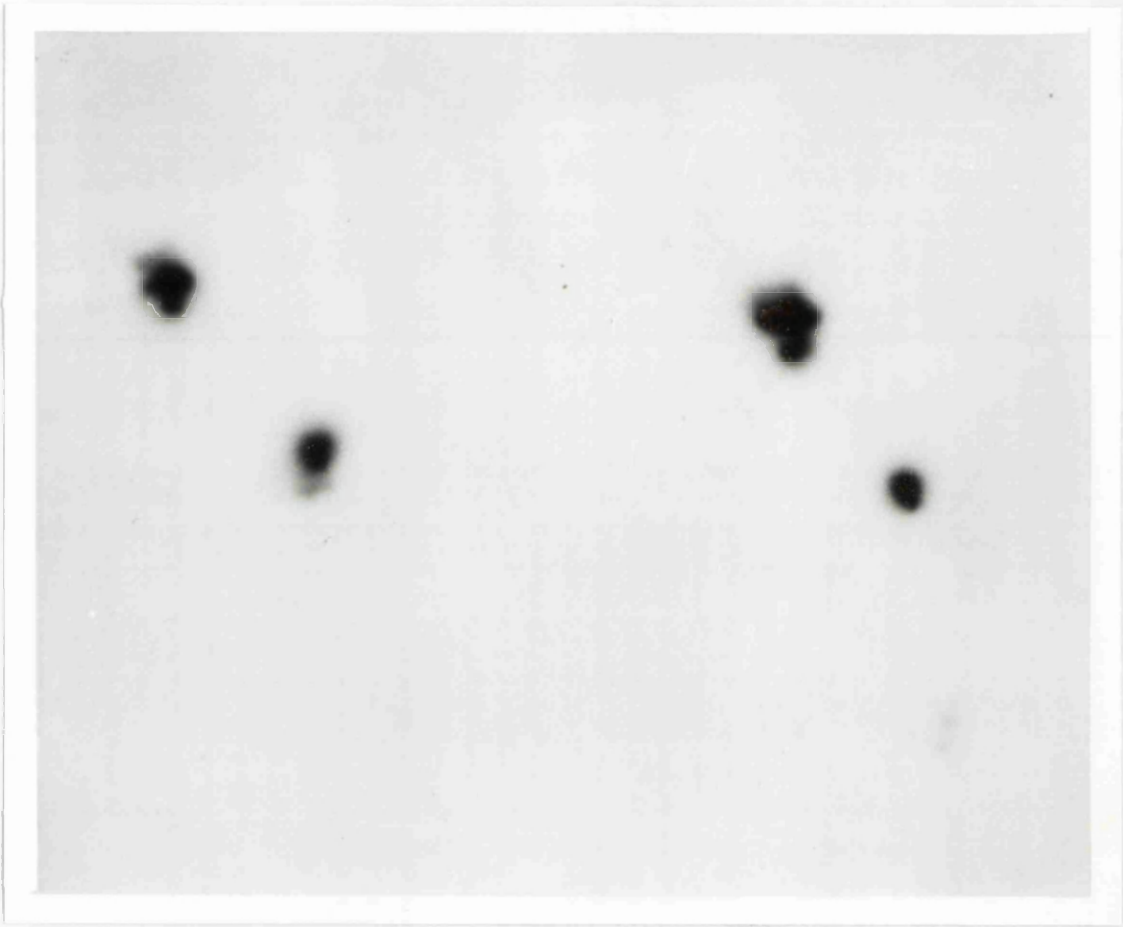


**Figure 5.6 Colony hybridisation of recombinant clones**

Recombinant pUC clones were screened with labelled oligonucleotide (figure 5.1) using colony hybridisation as described in section 2.18. Duplicate filters were made and screened, these are shown above.

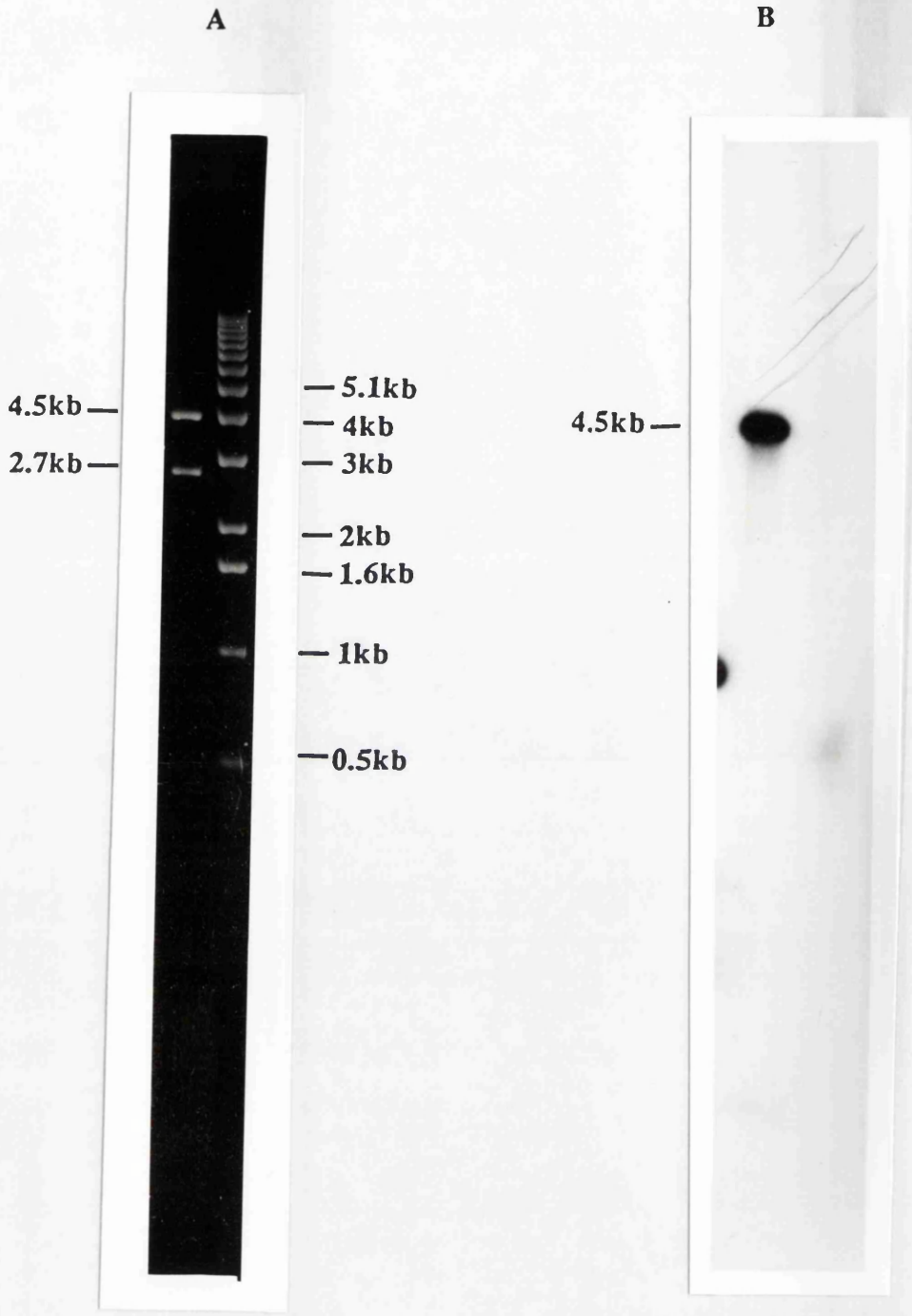



**Figure 5.7 Digestion of plasmid pCW1 with *Sal* I**

A 5µg of pCW1 were digested with *Sal* I as described in section 2.12.4. The restricton fragments obtained were resolved in a 0.8% agarose gel (section 2.12.5). Markers (1kb ladder ) were run alongside to estimate the size of the restriction fragments.

B The gel was Southern blotted (section 2.15), probed using the 35mer oligonucleotide (section 2.17) and autoradiographed. The probe hybridised strongly to the 4.5 kb insert fragment.

be 4.5kb, larger than the previous estimate of 4.2kb. Agarose gels were blotted and probed as in section 5.2.2. The probe was found to hybridise strongly to the 4.5 kb fragment figure (5.7B). The two recombinant plasmids were named pCW1 and pCW2.

### 5.2.6 Restriction mapping of pCW1

Plasmid DNA was prepared from the clone on a large scale by the method described in section 2.12.2. The plasmid obtained was either singly or doubly digested with a series of restriction enzymes, (section 2.12.4). Restriction fragments were analysed by gel electrophoresis and their sizes determined by comparison of their relative mobilities to those of molecular weight markers. Analysis of the fragments produced by single and double digests enabled the positions of several restriction sites within the plasmid pCW1 to be mapped (figure 5.8).

Southern blot analysis and probing of restriction digests indicated that the probe hybridised somewhere within the *Sal* I/*EcoR*I 1.1 kb fragment.

### 5.2.7 Confirmation that pCW1 contains the complete coding sequence for ICDH

A preliminary sequencing experiment confirmed that pCW1 contained the complete coding sequence for *B. caldotenax* ICDH. Double stranded template was prepared from pCW1 for sequencing by alkaline hydrolysis (Sambrook *et al.*, 1989) ( section 2.19.1 a). Nucleotide sequence was determined from the double stranded template using the chain termination sequencing method developed by Sanger (1977) as described in section 2.19.1. Two primers were used in these preliminary sequencing reactions;

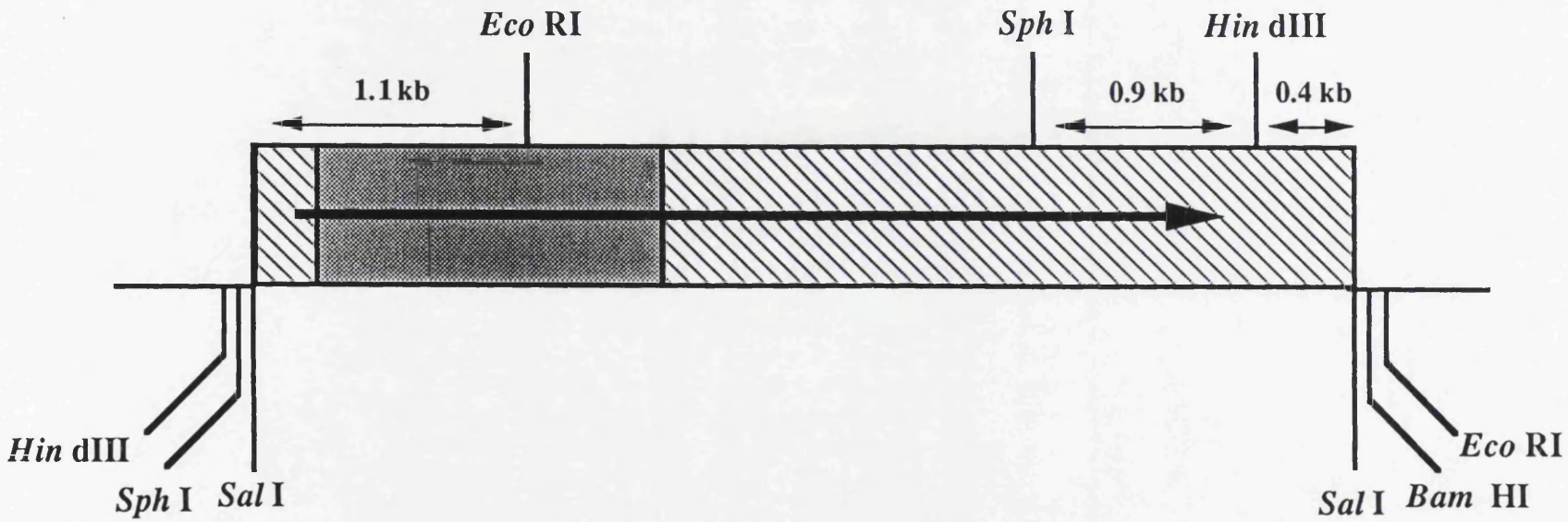



**Figure 5.8 Restriction map of pCW1**

A number of restriction sites were mapped onto pCW1 and their positions are indicated above Some of the polylinker restriction sites are also shown. The light shaded area represents the 4kb insert, the dark shaded area the *icd* gene. The arrow represents the direction of transcription.

a)The 35mer oligonucleotide probe designed from the ICDH amino acid sequence (figure 5.1).

The probe, originally designed to isolate the gene for ICDH, hybridises specifically to a region within it sufficiently well to be used as a primer for sequencing. Because the oligonucleotide probe is the reverse of the coding strand, sequence obtained using it as primer was also the reverse of the coding strand. It is likely (as explained in section 3.3) that the probe is homologous to the N-terminus of the ICDH coding sequence. If this is the case then sequence obtained would be just upstream to the gene for ICDH.

b) The -40 primer.

This is a universal primer homologous to a region of the pUC18 plasmid vector close to the polylinker. This primer was used to sequence into the plasmid insert.

The two sequences obtained were complementary over almost their entire lengths, i.e. sequencing in this manner gave the sequence of both strands of the same region of DNA. As explained previously this region of DNA is likely to be upstream to the *icd* gene. The size of the *icd* gene was calculated from the size of the protein to be approximately 1.4 kb, whereas the cloned insert is 4.5 kb in length. As a result of these sequencing experiments the 35mer oligonucleotide was found to hybridise approximately 300 base pairs from one of the *Sal* I sites with the *icd* gene lying in the direction leading into the insert. It was therefore concluded that the insert of pCW1 almost certainly contained the complete coding sequence for ICDH together with some DNA peripheral to the gene.

### 5.2.8 Determination of the complete nucleotide sequence of the *B. caldotenax* gene for ICDH

The gene for *B. caldotenax* ICDH was sequenced using the chain termination method of Sanger (1977), as described in section 2.19.1. Sequencing was carried out using double

stranded templates made by the method described in section 2.19.1 a) and 17mer oligonucleotides.

Nucleotide sequence obtained as described in section 5.2.9 was used to design an oligonucleotide primer. This was constructed in the correct orientation for sequencing into the *icd* gene. Successive oligonucleotide primers were designed as sequencing proceeded. Sequencing was facilitated by the construction of subclones from pCW1.

### 5.2.9 Construction of subclones from pCW1

Restriction mapping (figure 5.8) showed the presence of an *EcoR*I restriction site within the pCW1 insert, approximately 1.1 kb from one *Sal* I site. Knowing the size and location of the ICDH gene within the insert it could be concluded that this *EcoR* I site must fall inside the gene.

Plasmid pCW1 was digested as described in section 2.12.4 with both *Sal*I and *EcoR*I and the three fragments produced resolved in a 0.8% agarose gel (section 2.12.5 ). Two *Sal* I/*EcoR* I fragments were produced of 1.1kb and 3.4 kb in size. These two fragments were excised from the gel and the DNA was recovered by the method described in section 2.12.7. The two DNA fragments were subcloned into pBluescript KS$^+$ and transformed into XL1-blue (section 2.14 ). Plasmid isolated from the subclones and digested with *Sal*I and *EcoR*I confirmed the presence of either a 1.1kb ( pCW3) or a 3.4 kb insert (pCW4).

The cloning vector pBluescript KS$^+$ contains a universal priming site complementary to the commercially available primer T7. Sequence was obtained from both subclones using this primer. Further oligonucleotides were designed to the sequence obtained so that sequencing could proceed in both directions. The sequencing strategy used is summarised in figure 5.9.

**Figure 5.9 The strategy used to sequence *icd***

The complete region of 1555 nucleotides sequenced is outlined by the box. The arrows indicate the direction of second strand synthesis and length of readable sequence obtained. The clone or subclone used for sequencing is given together with the reference number of the oligonucleotide or commercial primer.

primers : T7 primer 5'-TAATACGACTCACTATAGGG-3'

-40 primer 5'-GTTTTCCCAGTCACGAC-3'

oligonucleotides:

01 5'-ACGCCGTTTTGGACCGTGATTTTTTCGCCTTGCAT- 3'

02 5'- CGCCCGCATGCACGGCT- 3'

03 5'- GAGGGGGATGGAACCGG -3'

04 5'- GACATCTTCCTGCAACA- 3'

05 5'- TTGCGCACGCCCATTTC -3'

06 5'- GACATCTTCCTGCAACA -3'

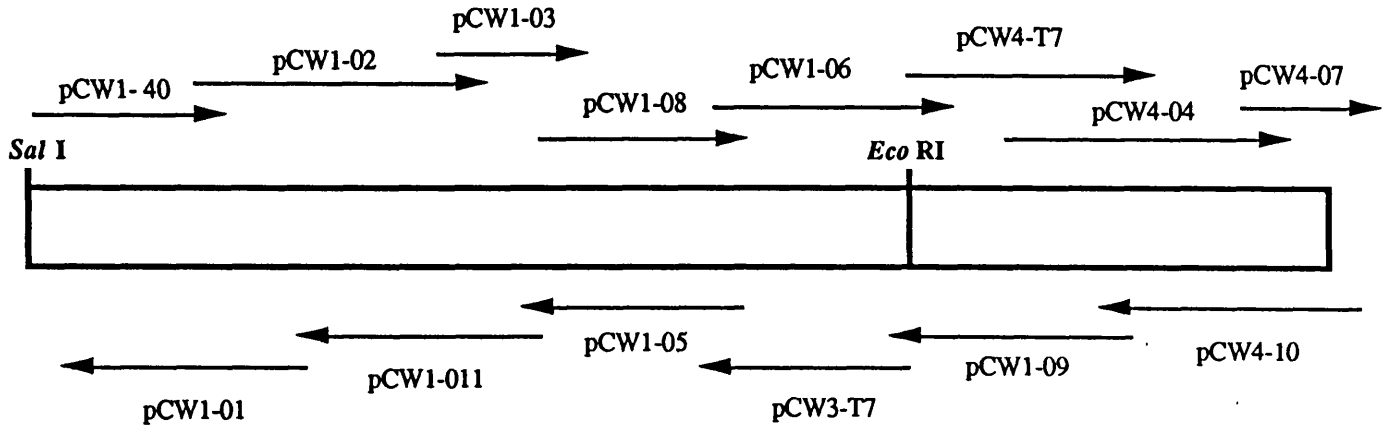
07 5'- GAAGCAGCGAAATTGAT -3'

08 5'- GCCATTAAAGGGCCGTT - 3'

09 5'- TGCCGTGCGTCGCTTCG -3'

10 5'- CGCCCCCGTGAATCCGG -3'

11 5'- GTTCAGCGAGCGGATGC -3'


pCW1-03
pCW1-02
pCW1-40
pCW1-06
pCW4-T7
pCW4-07
pCW1-08
pCW4-04
Sal I
Eco RI
pCW1-05
pCW4-10
pCW1-09
pCW1-011
pCW1-01
pCW3-T7

The complete coding sequence for ICDH was obtained on both strands of DNA together with some sequence of the flanking regions (figure 5.10). An open reading frame of 1272 nucleotides was evident, commencing with a GTG start codon and finishing with a TAA stop codon. The gene encodes a protein of 422 amino acids with a calculated molecular weight of 46,438 daltons.

### 5.2.10 Analysis of the nucleotide sequence

The nucleotide sequence of the region encoding *B. caldotenax* ICDH has been compared to that of *E. coli* ICDH using bestfit computer analysis (section 2.20). The two sequences aligned with 69.6% identity over their complete lengths. The total G+C content in the coding region of *B. caldotenax* ICDH was higher than in *E. coli*, 54.8% compared to 48.7%. A codon usage table was constructed for the *B. caldotenax icd* gene (table 5.2). Codon usage was quite different from that of *E. coli* as in *B. caldotenax* there was higher use of codons with either G or C as their third letter. For the *B. caldotenax icd* gene the G+C content in the third letter of the codon is 67.7% whereas in the *E. coli* gene it is only 52.5%.

The gene for ICDH from the extreme thermophile *Thermus thermophilus* has recently been cloned and sequenced (Miyazaki *et al.*, 1992). The nucleotide sequence of *B. caldotenax* ICDH showed 62% identity to that of *T. thermophilus*. The G+C content of the coding region of *T. thermophilus* ICDH is 65.6% and that in the third nucleotide of the codon is 90.3%. These values are higher than those obtained for the *B. caldotenax* ICDH coding region.

A testcode computer analysis (section 2.20) was performed on the nucleotide sequence obtained (figure 5.11). Testcode identifies a protein coding sequence by plotting a measure of the non-randomness of the composition at every third base. The plot is divided into

**Figure 5.10 Nucleotide sequence and derived amino acid sequence of the first 1555 nucleotides of the pCW1 insert**

The nucleotide sequence is shown with the derived amino acid sequence below. The putative ribosome binding site, -10 and -35 regions are indicated in bold print. The start and stop codons of ORFs are also shown in bold.

```
    NGTTTAGGAATCGATCATGATTTGTTTACGCCGATTTTTGCCGTCAGCCGCACCTCGGGA
1                                                                 60
     ?  L  G  I  D  H  D  L  F  T  P  I  F  A  V  S  R  T  S  G  -

    TGGTTGGCTCACATCTTGGAACAATACGACAACAACCGTCTCATCCGTCCGCGCGCTGAG
61                                                               120
     W  L  A  H  I  L  E  Q  Y  D  N  N  R  L  I  R  P  R  A  E  -

    TACACCGGCCCAGGCAAGCGGGCGTACGTGCCGATCGACGAGCGCGGCCAAAACAAGTGG
121                                                              180
     Y  T  G  P  G  K  R  A  Y  V  P  I  D  E  R  G  Q  N  K  W  -

    ACAAGGCGGTGAAGGCGCGCCTTCACGCCTTTTTATAAAAAAACTGCATTGGGGGTTTTG
181                                                              240
     T  R  R  *                                                  -

    CTCGTGACGCAAGGAGAAAAAATTACAGTCCAAAATGGTGTGCTCAACGTTCCGAACAAC
241                                                              300
           T  Q  G  E  K  I  T  V  Q  N  G  V  L  N  V  P  N  N  -

    CCGATCATTCCGTTCATCGAGGGGGATGGAACCGGCCCGGACATTTGGGCGGCCGCTTCG
301                                                              360
     P  I  I  P  F  I  E  G  D  G  T  G  P  D  I  W  A  A  A  S  -

    CGCGTGCTCGAAGCAGCGGTGGAAAAAGCGTACAAAGGTGAGAAAAAAATCGTCTGGAAG
361                                                              420
     R  V  L  E  A  A  V  E  K  A  Y  K  G  E  K  K  I  V  W  K  -

    GAAGTGCTCGCTGGTGAAAAAGCGTACAAGCTGACGGGCAGCTGGCTTCCGGATGAAACG
421                                                              480
     E  V  L  A  G  E  K  A  Y  K  L  T  G  S  W  L  P  D  E  T  -

    CTTGAGACGATCCGCGAATACATAATCGCCATTAAAGGGCCGTTGACGACGCCGGTCGGC
481                                                              540
     L  E  T  I  R  E  Y  I  I  A  I  K  G  P  L  T  T  P  V  G  -

    GGCGGCATCCGCTCGCTGAACGTAGCGCTCCGCCAAGAGCTCGACCTGTTTGTCTGCTTG
541                                                              600
     G  G  I  R  S  L  N  V  A  L  R  Q  E  L  D  L  F  V  C  L  -

    CGCCCGGTTCGCTACTTCCCAGGCGTTCCGTCGCCGGTGAAACGCCCGGAAGACACCGAT
601                                                              660
     R  P  V  R  Y  F  P  G  V  P  S  P  V  K  R  P  E  D  T  D  -
```

```
    ATGGTCATTTTCCGGGAAAACACGGAAGACATCTACGCTGGCATTGAATATGCCAAAGGC
661                                                             720
     M  V  I  F  R  E  N  T  E  D  I  Y  A  G  I  E  Y  A  K  G  -

    ACGCCGGAAGTGAAAAAAGTCATCGACTTTTTGCAAAACGAAATGGGCGTGCGCAAAATC
721                                                             780
     T  P  E  V  K  K  V  I  D  F  L  Q  N  E  M  G  V  R  K  I  -

    CGCTTCCCGGAAACGTCCGGCATCGGCATTAAACCGATTTCCGAACAAGGGACGAAACGG
781                                                             840
     R  F  P  E  T  S  G  I  G  I  K  P  I  S  E  Q  G  T  K  R  -

    CTTGTGCGCGCGGCGATCAACTACGCGATCGAACACGGCCGCAAGTCGGTGACGCTCGTT
841                                                             900
     L  V  R  A  A  I  N  Y  A  I  E  H  G  R  K  S  V  T  L  V  -

    CATAAAGGAAACATTATGAAATTCACCGAAGGCGCGTTTAAAAACTGGGGTTATGAATTG
901                                                             960
     H  K  G  N  I  M  K  F  T  E  G  A  F  K  N  W  G  Y  E  L  -

    GCGGAGGAAGAATTCGCCGACAAAGTGTTCACGTGGGCGCAATACGACCGAATCGTTGAA
961                                                             1020
     A  E  E  E  F  A  D  K  V  F  T  W  A  Q  Y  D  R  I  V  E  -

    ACGGAAGGCAAGGAAGCGGCGAACAAAGCGCTTGCTGATGCGGAAGCGTCCGGCAAAATC
1021                                                            1080
     T  E  G  K  E  A  A  N  K  A  L  A  D  A  E  A  S  G  K  I  -

    ATTATCAAAGATGTCATCGCCGACATCTTCCTGCAACAAATTTTGACGCGTCCGCGCGAA
1081                                                            1140
     I  I  K  D  V  I  A  D  I  F  L  Q  Q  I  L  T  R  P  R  E  -

    TTTGACGTCATCGCGACGATGAACTTAAACGGCGACTACATTTCCGACGCGCTGGCCGCT
1141                                                            1200
     F  D  V  I  A  T  M  N  L  N  G  D  Y  I  S  D  A  L  A  A  -

    CAAGTCGGCGGCATCGGCATCGCGCCGGGGGCCAACATCAACTACGAAACCGGCCACGCG
1201                                                            1260
   Q  V  G  G  I  G  I  A  P  G  A  N  I  N  Y  E  T  G  H  A  -
```

```
     ATTTTCGAAGCGACGCACGGCACGGCGCCGAAATACGCAGGGCTTGACAAAGTCAACCCG
1261                                                              1320
      I  F  E  A  T  H  G  T  A  P  K  Y  A  G  L  D  K  V  N  P  -

     TCGTCCGTCATTCTCTCGGGCGTCATGATGTTTGAGCATCTTGGTTGGAACGAAGCAGCG
1321                                                              1380
      S  S  V  I  L  S  G  V  M  M  F  E  H  L  G  W  N  E  A  A  -

     AAATTGATCATCAAAGCGATGGAGAAAACCATCGCCGCGAAAATCGTCACGTATGACTTC
1381                                                              1440
      K  L  I  I  K  A  M  E  K  T  I  A  A  K  I  V  T  Y  D  F  -

     GCCCGCCTGATGGAAGGGGCGACGGAAGTGAAATGCTCCGAATTTGCTGATGCGCTCATC
1441                                                              1500
      A  R  L  M  E  G  A  T  E  V  K  C  S  E  F  A  D  A  L  I  -

     CGCAATATGGACTAACCTTTGAAGGAAAGGGATGGCAAACGATGGCGATGAAACG
1501                                                        1555

      R  N  M  D  *                                             -
```

**Table 5.2 Codon usage in the *B.caldotenax* icd gene**

| | G | | A | | T | | C | | |
|---|---|---|---|---|---|---|---|---|---|
| G | Gly | 17 | Glu | 18 | Val | 39 | Ala | 60 | G |
| | Gly | 8 | Glu | 82 | Val | 3 | Ala | 7 | A |
| | Gly | 14 | Asp | 30 | Val | 16 | Ala | 13 | T |
| | Gly | 61 | Asp | 70 | Val | 42 | Ala | 20 | C |
| A | Arg | 0 | Lys | 13 | Met | 100 | Thr | 76 | G |
| | Arg | 0 | Lys | 88 | Ile | 2 | Thr | 4 | A |
| | Ser | 0 | Asn | 11 | Ile | 34 | Thr | 0 | T |
| | Ser | 8 | Asn | 89 | Ile | 63 | Thr | 20 | C |
| T | Trp | 100 | Stop | 0 | Leu | 22 | Ser | 46 | G |
| | Stop | 0 | Stop | 100 | Leu | 4 | Ser | 0 | A |
| | Cys | 0 | Tyr | 23 | Phe | 38 | Ser | 0 | T |
| | Cys | 100 | Tyr | 77 | Phe | 63 | Ser | 46 | C |
| C | Arg | 11 | Gln | 0 | Leu | 22 | Pro | 95 | G |
| | Arg | 6 | Gln | 100 | Leu | 0 | Pro | 5 | A |
| | Arg | 6 | His | 40 | Leu | 22 | Pro | 0 | T |
| | Arg | 78 | His | 60 | Leu | 30 | Pro | 0 | C |

Codon usage in the *B.caldotenax icd* gene was calculated using the WGCG program codon frequency (section 2.20) . The frequency with which each codon is used to code a particular amino acid is expressed as a percentage.

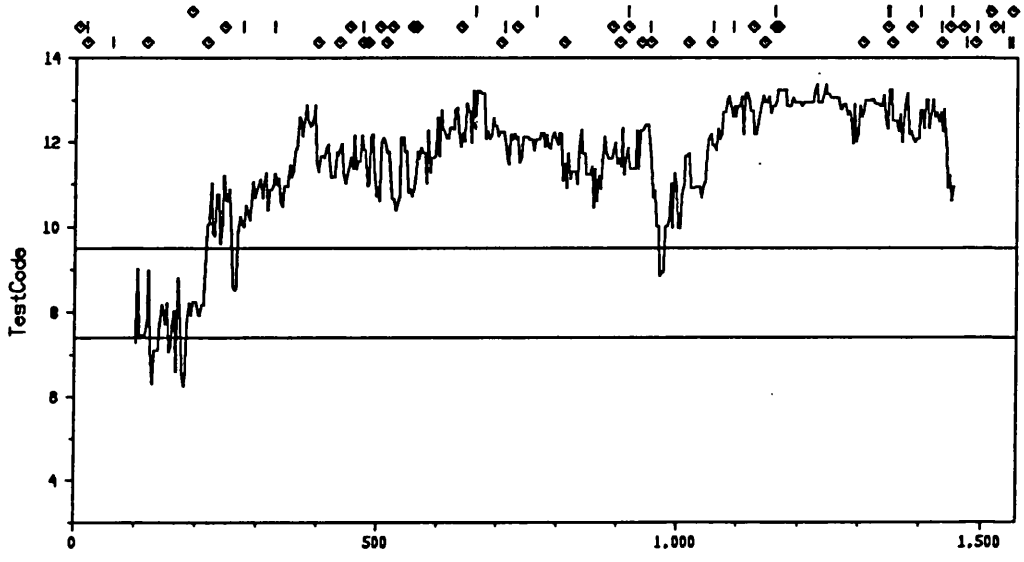



**Figure 5.11 TESTCODE plot of nucleotides 1-1555 of the pCW1 insert**

The top region of the plot predicts coding regions to a 95% level of confidence. The bottom region predicts non-coding regions to the same level of confidence. The middle region is the "window of vulnerability" for the method where the statistic can make no significant prediction. Above the curve, there are markings that identify the start and stop codons for each reading frame of the sequence. Starts are indicated by short vertical lines and stops by small diamonds.

three regions, the top region predicts coding regions to a 95% level of confidence and the bottom region predicts non-coding regions with the same level of confidence. The middle region is termed the "window of vulnerability" and makes no significant prediction. Testcode of the nucleotide sequence obtained predicted an open reading frame of approximately 1200 nucleotides with only one small "vulnerable" region. No conclusions could be drawn regarding testcode analysis of the region upstream of the *icd* gene. Testcode uses a window of 200 nucleotides to make predictions and there was not this much upstream sequence available.

### 5.2.11 Analysis of the predicted amino acid sequence of *B. caldotenax* ICDH

The nucleotide sequence of the *B. caldotenax icd* gene predicted a protein of 422 amino acid residues. This predicted sequence was compared to the sequence of 15 amino acid residues determined by Edman degradation (section 3.2.). This comparison confirmed, as previously suggested, that the sequence of 15 residues established by protein sequencing was in fact N-terminal. The two sequences were in complete agreement.

Bestfit computer analysis (section 2.20) was used to compare *B. caldotenax* and *E. coli* ICDH amino acid sequences (figure 5.12). The sequences aligned with 70.1% identity and 83.9% similarity. Comparison of the amino acid sequence of *B. caldotenax* ICDH with that of *T. thermophilus* showed 42% identity and 62% similarity (also shown in figure 5.12). Several stretches of amino acids present in *B. caldotenax* ICDH were absent from that of *T. thermophilus*. In addition *T. thermophilus* ICDH possessed a region of 140 amino acids at its C terminus which was not found in *B. caldotenax* ICDH. Miyazaki *et al.* (1992) compared the amino acid sequence of *T. thermophilus* ICDH to that of *E. coli*. The two sequences exhibited 33% identity and 37% similarity. *B. caldotenax* ICDH is much more closely related to ICDH of *E. coli* than that of *T. thermophilus*. Although

**Figure 5.12 Comparison of the *B. caldotenax* ICDH derived amino acid sequence with the known sequences of other ICDHs**

The derived amino acid sequence of *B. caldotenax* ICDH was compared to the derived amino acid sequences of *E. coli* (Thorsness and Koshland, 1987) and *T.thermophilus* (Miyazaki *et al.*, 1992). The sequences were aligned using the LINEUP program of the UWGCG package (section 2.20).

```
          1                                                   50
B.cald    ........TQ GEK....ITV QNG.VLNVPN NP..IIPFIE GDGTGPDIWA
E.coli    MESKVVVPAQ GKK....ITL QNG.KLNVPE NP..IIPYIE GDGIGVDVTP
T.ther    ..CKGGDSAP GGEPMPLITT ETGKKMHVLE DGRKLITVIP GDGIGPECVE

          51                                                  100
B.cald    AASRVLEAAV EKAYKGEKKI VWKEVLAGEK AYKLTG..SW LPDETLETIR
E.coli    AMLKVVDAAV EKAYKGERKI SWMEIYTGEK STQVYGQDVW LPAETLDLIR
T.ther    ATLKVLEAA. .......KAP LAYEVREAGA SVFRRGIASG VPQETIESIR

          101                                                 150
B.cald    EYIIAIKGPL TTPVGGGIRS LNVALRQELD LFVCLRPVRY FPGVPSPVKR
E.coli    EYRVAIKGPL TTPVGGGIRS LNVALRQELD LYICLRPVRY YQGTPSPVKH
T.ther    KTRVVLKGPL ETPVGYGEKS ANVTLRKLFE TYANVRPVRE FPNVPTPYAG

          151                                                 200
B.cald    PEDTDMVIFR ENTEDIYAGI EYAKGTPEVK KVIDFLQNEM GVRKIRFPET
E.coli    PELTDMVIFR ENSEDIYAGI EWKADSADAE KVIKFLREEM GVKKIRFPEH
T.ther    RGI.DLVVVR ENVEDLYAGI EHMQTPSVAQ .......... ..........

          201                                                 250
B.cald    SGIGIKPISE QGTKRLVRAA INYAIEHGRK SVTLVHKGNI MKFTEGAFKN
E.coli    CGIGIKPCSE EGTKRLVRAA IEYAIANDRD SVTLVHKGNI MKFTEGAFKD
T.ther    ...TLKLISW KGSEKIVRFA FELARAEGRK KVHCATKSNI MKLAEGP..K

          251                                                 300
B.cald    WGYELAEEEF ADKVFTWAQY DRIVETEGKE AANKALADAE ASGKIIIKDV
E.coli    WGYQLAREEF GGELIDGGPW LKV....... ......KNPN TGKEIVIKDV
T.ther    RAFEQVAQEY PD........ .......... .......... ....IEAVHI

          301                                                 350
B.cald    IADIFLQQIL TRPREFDVIA TMNLNGDYIS DALAAQVGGI GIAPGANINY
E.coli    IADAFLQQIL LRPAEYDVIA CMNLNGDYIS DALAAQVGGI GIAPGANIGD
T.ther    IVDNAAHQLV KRPEQFEVIV TTNMNGDILS DLTSGLIGGL GFAPSANIGN

          351                                                 400
B.cald    ETGHAIFEAT HGTAPKYAGL DKVNPSSVIL SGVMMFEHLG WNEAAKLIIK
E.coli    EC..ALFEAT HGTAPKYAGQ DKVNPGSIIL SAEMMLRHMG WTEAADLIVK
T.ther    EV..AIFEAV HGSAPKYAGK NVINPTAVLL SAVMMLRYLE EFATADLIEN

          401                                                 450
B.cald    AMEKTI.AAK IVTYDFARLM EGATEVKCSE FADALIRNMD ..........
E.coli    GMEGAI.NAK TVTYDFERLM DGAKLLKCSE FGDAIIENM. ..........
T.ther    ALLYTLEEGR VLTGDVVGYD RGA...KTTE YTEAIIQNLG KTPRKTQVRG

          451                                                 500
B.cald    .......... .......... .......... .......... ..........
E.coli    .......... .......... .......... .......... ..........
T.ther    YKPFRLPQVD GAIAPIVPRS RRVVGVDVFV ETNLLPEALG KALEDLAAGT

          501                                                 550
B.cald    .......... .......... .......... .......... ..........
E.coli    .......... .......... .......... .......... ..........
T.ther    PFRLKMISNR GTQVYPPTGG LTDLVDHYRC RFLYTGEGEA KDPEILDLVS

          551                          580
B.cald    .......... .......... ..........
E.coli    .......... .......... ..........
T.ther    RVASRFRWMH LEKLQEFDGE PGFTKAQGED
```

*B.caldotenax* and *E. coli* ICDHs show significant identity to the N-terminal region of that of *T.thermophilus* ICDH the C termini are considerably different. For this reason most comparisons were made between the *B. caldotenax* and *E. coli* enzymes.

The activity of ICDH in *E. coli* is controlled by phosphorylation of a serine residue. The position of the phosphorylated serine residue and the sequence of surrounding amino acids has been determined in *E. coli* (Borthwick *et al.*, 1984b; Malloy *et al.*, 1984) to be as follows;

104-Thr Thr Pro Val Gly Gly Gly Ile Arg Ser Leu Asn Val Ala-117

This sequence of amino acids is completely conserved in *B. caldotenax* ICDH between positions 94 and 107.

A consensus sequence for a nucleotide binding site in ICDH/IMDH type dehydrogenases has been defined in the computer analysis program MacPattern on the evidence of Hurley *et al.* (1991). This motif was identified in *B. caldotenax* ICDH between positions 341 and 351.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus ICDH/IMDH | E | [A/P] | X | H | G | [S/T] | A | P | X | X | [A/P] |
| *B.caldotenax* ICDH | E | A | T | H | G | T | A | P | K | Y | A |

The amino acid composition of *B. caldotenax* ICDH was calculated and is shown in table 5.3 together with that of *E. coli* ICDH for comparison. Generally there is little difference between the overall amino acid compositions of the two types of ICDH. However *B. caldotenax* ICDH does exhibit a slight increase in the overall number of polar residues and a decrease in cysteine residues. These differences may have implications in increasing the thermostability of the protein; the additional polar residues might be involved in stabilising hydrogen bonds.

**Table 5.3 The amino acid compositions of *B.caldotenax* and *E.coli* ICDH s**

| Amino acid | *B.caldotenax* ICDH | *E.coli* ICDH |
|---|---|---|
| Ala | 10.7 | 9.1 |
| Cys | 0.5 | 1.4 |
| Asp | 4.7 | 6.0 |
| Glu | 9.0 | 8.4 |
| Phe | 3.8 | 2.4 |
| Gly | 8.5 | 9.6 |
| His | 1.2 | 1.2 |
| Ile | 9.7 | 8.9 |
| Lys | 7.6 | 7.5 |
| Leu | 6.4 | 7.5 |
| Met | 2.1 | 3.1 |
| Asn | 4.3 | 3.6 |
| Pro | 4.5 | 4.8 |
| Gln | 2.1 | 2.6 |
| Arg | 4.3 | 4.1 |
| Ser | 3.1 | 3.1 |
| Thr | 5.9 | 4.3 |
| Trp | 1.4 | 1.4 |
| Tyr | 3.1 | 3.6 |
| Val | 7.1 | 7.2 |

The amino acid compositions of *B.caldotenax* and *E.coli* ICDH s were calculated from their amino acid sequence using the WGCG computer program Pepsort (section 2.20).

*B. caldotenax* ICDH lacks the first 8 amino acids at the N-terminus which are present in *E. coli* ICDH, these residues are also absent in *T. thermophilus* ICDH although the reason for this is unclear. In addition *B. caldotenax* ICDH contains an extra stretch of 13 amino acids between residues 257 and 270 which has no corresponding sequence in the *E.coli* enzyme. The region of nucleotides encoding these additional amino acids corresponded to the "vulnerable" coding region identified by testcode analysis (section 5.2.10). This region of unusual DNA was sequenced independently on the second ICDH clone, pCW2. The nucleotide sequence of this region was found to be identical in both clones, indicating that it is genuine and not an artefact resulting from a re-arrangement of the DNA during cloning. Examination of the structure of *E. coli* ICDH showed the position of this extra region. The region forms a loop on the surface of the smaller domain of the ICDH subunit. It is possible that this extra region of DNA has implications in enhancing the thermostability of the enzyme.

### 5.2.12 Analysis of DNA peripheral to the *icd* gene

#### a) Analysis of upstream DNA

In addition to determination of the complete nucleotide sequence of the *icd* gene the sequence of approximately 300 nucleotides upstream and 40 nucleotides downstream was also established. These flanking regions of DNA have been examined for evidence of ribosome binding sites and other upstream transcriptional elements, and searched for the presence of other open reading frames.

Moran *et al.* (1982) examined ribosome binding sites (RBS) of *B.subtilis* and showed the nucleotide sequence of these sites to be very similar to those for *E. coli* genes. Several genes from thermophilic bacilli have been cloned and potential RBSs identified for these

genes. In all cases the sequence of the RBS was similar to the consensus RBS defined for *E. coli* genes.

The RBS for the *B. caldotenax* ICDH gene would be expected to lie within 7-14 nucleotides of the GTG start codon (Moran *et al.*, 1982). This region of DNA was examined for a sequence of nucleotides similar to the consensus RBS sequence. The sequence most likely to be a RBS was found between positions -6 and-14 and is shown highlighted in figure 5.10. This potential RBS bears little similarity to the RBS consensus sequence. A putative transcriptional promoter has been identified, the -35 and -10 regions show considerable sequence homology to the *E. coli* -35 (TTGACA) and -10 (TATAAT) consensus sequences. The -35 and -10 regions for the putative *B. caldotenax icd* gene promoter are highlighted in figure 5.10.

Computer analysis of the upstream nucleotide sequence revealed the end of another open reading frame, just 50 nucleotides upstream to the *icd* gene and in the same reading frame. This ORF was translated and used to search the GenEmbl database for evidence of homology to other proteins of known sequence. The translated sequence was found to have 50% identity and 76 % similarity to the C-terminal end of the amino acid sequence of citrate synthase from the thermophile *Thermus acidophilum.* Similarly it showed 50% identity (74% similarity) to citrate synthase from the facultative thermophile *B. coagulans* and between 40% and 44 % identity with the C-terminus of citrate synthase from 6 other micro-organisms. An alignment of these sequences with the translated upstream ORF is shown in figure 5.13. It seems very likely from this alignment that the gene which encodes citrate synthase in *B. caldotenax* lies just upstream to the gene for ICDH.

**b) Analysis of downstream DNA**

The sequence of approximately 40 nucleotides after the TAA stop codon of the *icd* gene have been determined. This region of DNA contained a sequence of nucleotides which

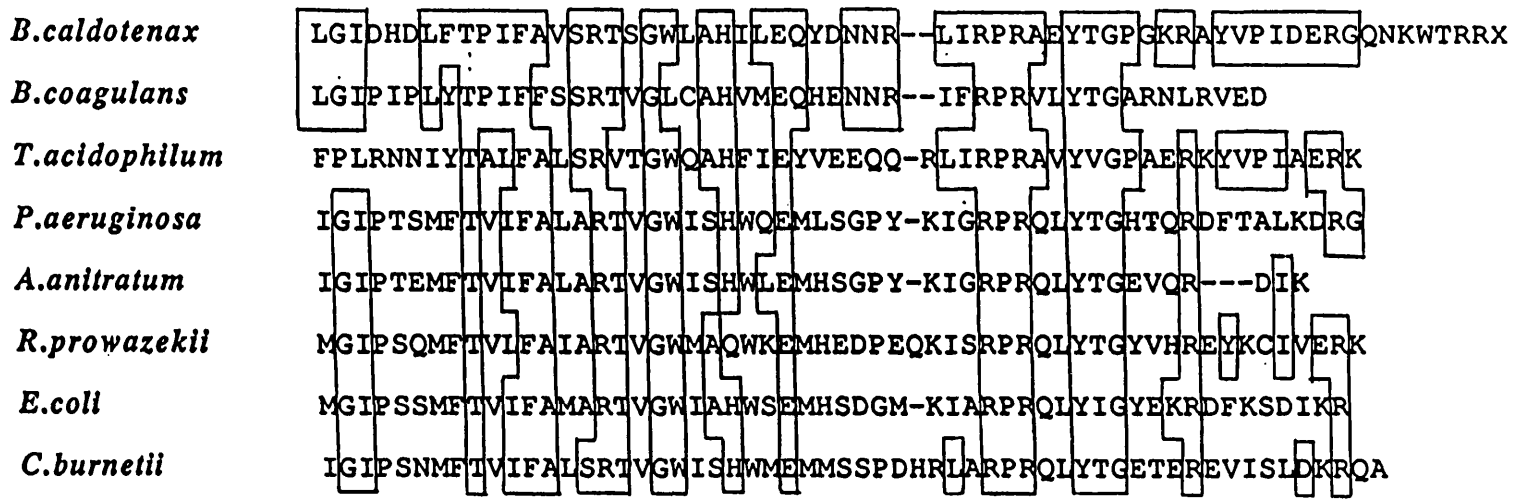



**Figure 5.13 Alignment of the amino acid sequences of several prokaryotic citrate synthases**

The translated open reading frame upstream to the *B.caldotenax* gene for ICDH is shown aligned to the C-terminal amino acid sequences of a number of bacterial citrate synthases. The *B.caldotenax* sequence aligns with between 40 and 50% identity to the other sequences. Conserved sequences are shown boxed.

TAACCTTT**GAAGGAAA**GGG**ATG**GCAAACG**ATG**GCGATGAAACG

1513 1555

M A N D G D E T

M A M K R

**Figure 5.14 Analysis of nucleotide sequence downstream to *icd***

The sequence of 40 nucleotides downstream to the gene for ICDH are shown. Translation of the sequence in all reading frames (section 2.20) showed the start of two possible ORFs. A potential ribosome binding site was identified for one of these ORFs and is indicated in bold. The derived amino acid sequences of these two ORFs are also shown.

resembled the consensus RBS sequence and was closely followed by two possible ATG start codons in two different open reading frames. The downstream region of DNA was translated in all 6 possible reading frames and the resulting peptide sequences (figure 5.14) used to search the GenEmbl database. None of the translated peptides showed significant similarity with any known protein sequences.

## 5.3 Discussion

A 35mer oligonucleotide probe was designed to *B. caldotenax* ICDH amino acid sequence and was found to hybridise to a 4.5kb *Sal* I fragment of *B. caldotenax* genomic DNA. The strength of the signal obtained from probing *B. caldotenax* genomic DNA was sufficient, at high stringency, to represent genuinely specific hybridisation. Strength and specificity of the signal depend on hybridisation and washing conditions. It is possible to calculate the Tm, melting temperature of a DNA duplex, (Suggs *et al.*, 1981; Anderson and Young, 1985; Albretsen *et al.*, 1988) and hybridisation temperatures of 5-10°C below Tm are recommended (Binnie, 1990). However in practice conditions are usually defined empirically.

A low stringency hybridisation wash is used initially and then the stringency increased progressively. The more stringent the hybridisation and washing conditions, the less likely the chance of mismatches between the oligonucleotide and target DNA. In this manner a set of conditions are determined which result in a single specific hybridisation signal being retained. In this case a single specific signal was obtained when filters were hybridised at 50°C in 6xSSC and washed with 1xSSC at 55°C.

The 35mer oligonucleotide probe was used to isolate a clone from a sub-library of *B.caldotenax* genomic DNA. The clone contains the complete coding sequence for the *B.caldotenax icd* gene. The gene consists of an open reading frame of 1266 base pairs

which encodes a protein of 422 amino acid residues with a molecular weight of 46,437 daltons. This value agrees well with that obtained for the subunit molecular weight determined by SDS PAGE (section 3.2.2).

A comparison was made of the inferred amino acid sequence with that obtained by protein sequence analysis (section 3.2.6). The two sequences were in complete agreement and confirmed the previous suggestion that the protein sequence was N-terminal.

It has been suggested that the ability of thermophiles to grow at elevated temperatures is a result of differences in the macromolecules which they contain, in comparison to mesophiles. Studies have shown DNA from thermophiles to possess greater thermal stability than that from mesophiles and investigations have been carried out to see how this increased thermostability is achieved (Stenesh *et al.*, 1968). Stenesh's group compared DNA from several strains of *B. stearothermophilus* with that from a number of mesophilic *Bacillus* species. They found thermophilic DNA contained an increased G+C content in comparison to mesophiles. Kagawa *et al.* (1984) studied the IMDH gene of *Thermus thermophilus* and showed thermophiles not only have an increased G+C content within their coding regions but more specifically to have high G+C in the third letter of codons. The results obtained for the G+C content within the *B. caldotenax* gene for ICDH support these observations. The G+C content in the coding region of this gene was 54.8% and in the third letter of codons to be 67.7%. These values were compared to those calculated from the ICDH coding regions of the extreme thermophile, *T. thermophilus* , and the mesophile, *E. coli* (table 5.4). A general trend was observed in which the more thermophilic the organism the higher the total G+C content in the coding region and in the third letter of the codon. This trend was also observed by Sekiguchi *et al.* (1986b) when comparing the G+C content of the IMDH coding regions from a variety of thermophilic and mesophilic organisms (table 5.4).Values for the G+C content in both *B. caldotenax* genes was found to be very similar.

**Table 5.4** **Comparison of the total G+C content and G+C content in the third letter of the codon in the coding region of ICDH and IMDH from different organisms**

| Micro-organism | Protein (%) | G+C content codon (%) | G+C content in 3rd letter | Study |
|---|---|---|---|---|
| *E.coli* | ICDH | 48.7 | 52.5 | Thorsness and Koshland (1987) |
| *S.cerevisiae* | IMDH | 43.5 | 39.6 | Andreadis *et al.* (1984) |
| *S.cerevisiae* | ICDH | 42 | 44.8 | Haselbeck and McAlister-Henn (1990) |
| *B.coagulans* | IMDH | 53.1 | 56.3 | Sekiguchi *et al.* (1986b) |
| *B.caldotenax* | IMDH | 56..7 | 65.2 | Sekiguchi *et al.* (1987) |
| *B.caldotenax* | ICDH | 54.8 | 67.7 | this study |
| *T.thermophilus* | IMDH | 70.1 | 89.4 | Kagawa *et al.* (1984) |

ICDH (isocitrate dehydrogenase)

IMDH (3-isopropylmalate dehydrogenase)

Any nucleic acid is stabilised by increasing its G+C content. Thermophiles utilise this property in order to gain increased thermostability of their DNA. In addition the increased G+C content in the third letter of the codon will help to stabilise dynamic structures formed during transcription and translation at high temperatures. A result of this is that the codon usage of thermophiles is quite different from that of mesophiles. Comparison of the nucleotide sequences of the *B. caldotenax icd* gene with that of *E. coli* showed 69% identity. Although significant, a high identity at the nucleotide level is less significant than at the amino acid level.

At the amino acid level the sequence of *B. caldotenax* had 70.1% identity and 83.9 % similarity to that of *E. coli* ICDH (figure 5.12). This is remarkable similarity considering that these are two quite different micro-organisms. Presumably this extreme level of conservation reflects the importance of the enzyme in central metabolism. In *E. coli* ICDH certain regions are known to be of functional importance to the enzyme, e.g. the sites for nucleotide binding and phosphorylation. The amino acid sequence of these regions are conserved in their entirety in *B. caldotenax* ICDH. The complete conservation of the phosphorylation site is further evidence that the activity of *B. caldotenax* ICDH may be controlled by phosphorylation as was originally suggested in chapter 4. *B. caldotenax* ICDH showed less identity (42%) with ICDH from *T. thermophilus*. *B. caldotenax* ICDH seems to be much more closely related to the *E. coli* enzyme than that of the extreme thermophile.

A number of workers in the past have tried to identify thermostabilising amino acid residues within thermophilic proteins by comparing them to a mesophilic form of the same protein. Several features of interest arose from comparisons of the amino acid composition and sequences of *E. coli* and *B. caldotenax* ICDHs. *B. caldotenax* ICDH contained only 2 cysteine residues whereas that of *E. coli* contained 6. It was suggested by Saiki *et al.* (1978) that non-essential cysteine residues might be replaced in thermophilic enzymes so as to prevent oxidative inactivation of the enzymes under aerobic conditions at high

temperatures. This may explain the reduced number of cysteine residues observed in *B.caldotenax* .

It is believed that an increase in the internal hydrophobicity of a protein will increase its thermostability (Yutani *et al.*, 1987). No significant differences in the number of hydrophobic residues present in *E. coli* and *B. caldotenax* ICDH were observed. Certain other amino acid replacements have been reported in thermophilic enzymes when compared to those of mesophiles. Zuber (1978) reported an increase in arginine residues in thermophilic enzymes at the expense of lysine residues. Zuber hypothesised that thermostabilty was gained by a difference in the arrangement of ionic bonds. Three such substitutions were apparent within *B. caldotenax* ICDH at residues 38, 177 and 255. Other similar thermostabilising substitutions have been suggested by Argos *et al.* (1979) who reported changes in thermophiles of Gly to Ala, Ser to Ala, Ser to Thr and Asp to Glu. At least one or more examples of all these substitutions can be identified in comparisons of the *E. coli* and *B. caldotenax* ICDH amino acid sequences. However it was suggested by Zuber (1978) that when carrying out such comparisons it was advisable to compare proteins from closely related bacteria with very similar metabolism, as thermophilic-mesophilic differences in amino acid sequence would be most visible when other differences were small. As a result of comparative studies on the amino acid sequences of mesophilic and thermophilic ferredoxins Zuber also advised that at least 2-3 thermophilic proteins must be compared with 2-3 mesophilic proteins in order to establish any critical thermostabilising residues. This being the case it is not possible to draw any firm conclusions, regarding thermostability, from similar comparisons between the amino acid sequences of *B. caldotenax* and *E. coli* ICDHs as phylogenetic differences will be large.

Examination of DNA upstream to the gene for ICDH has shown the presence of a second ORF in very close proximity to the *icd* gene. It seems very likely that this ORF codes for citrate synthase, another enzyme of the TCA cycle. The two ORFs are separated by only 51

nucleotides. The close proximity of the two genes might suggest that they form part of an operon. In addition it would seem logical for the genes of these two TCA cycle enzymes to be transcribed together. In contrast in *E. coli* none of the TCA cycle enzymes constitute an operon. Some of the TCA cycle genes are clustered together but this is not the case with the *icd* gene. It is thought that the genes do not form an operon possibly because of the cycle's dual role in anabolism and catabolism.

If the *B. caldotenax icd* gene does form part of an operon we would not expect to see distinct transcription initiation sites for this gene, however putative sites were identified. It is possible that *icd* is within an operon but under certain circumstances ICDH needs to be expressed separately from CS. Genes which are differentially expressed have been identified in *E. coli*. For example the genes for the component enzymes of the pyruvate dehydrogenase (PDH) complex (*aceE, aceF* and *lpd*) can be expressed as a single transcript from one promoter, or *lpd* can be transcribed singly from a different promoter (Spencer and Guest, 1985). The manner in which the genes are expressed is dependent on the growth conditions.

The hypothesis that the genes for CS and ICDH form an operon in *B. caldotenax* could be tested by the use of S1 nuclease transcriptional mapping. This approach was used by Spencer and Guest (1985) to analyse the organisation of the genes encoding components of the pyruvate and 2-oxoglutarate dehydrogenase complexes in *E. coli*. This method allows the size of transcripts to be established and it is possible to determine whether or not genes are transcribed singly or together. This approach and other methods of confirming the existence of a CS/ICDH operon are discussed further in the general discussion.

# Chapter 6

# General discussion

## General discussion

It has been firmly established in *E. coli* that the division of flux at the branch point between the TCA cycle and the glyoxylate bypass is controlled by the reversible phosphorylation and inactivation of ICDH. It is generally accepted that, during growth on acetate, phosphorylation of ICDH renders the enzyme rate limiting in the TCA cycle thus allowing the intracellular concentration of isocitrate, the common substrate of the competing pathways, to increase to a level that facilitates flux through the glyoxylate bypass.

The control of isocitrate dehydrogenase by phosphorylation in *E. coli* was the first example of prokaryotic phosphorylation to be well characterised, and both the physiological role and the molecular details of phosphorylation are well understood. During the elucidation of this control mechanism ICDH was intensively studied in many aspects. The gene for ICDH was cloned and sequenced and the 3-dimensional structure of the protein determined. No such comparable study has been carried out on ICDH from any other micro-organism.

The work of Bennett and Holms (1975) provided evidence that ICDH may be controlled in some other gram negative bacterial strains (*Serratia*, *Salmonella* and *Klebsiella*) in a similar manner to that of *E. coli*. However these workers provided no evidence for the existence of such a mechanism in any gram positive strains. It was the lack of knowledge in this area together with the desirability of learning more about central metabolism in thermophiles that prompted this study on ICDH from *B. caldotenax*. I have purified *B. caldotenax* ICDH, characterised some of its properties and investigated the possibility that the enzyme might be controlled by phosphorylation. In addition I have cloned and sequenced the gene for this enzyme. The results of these studies have allowed comparisons to be drawn between ICDH of *E. coli* and ICDH from a moderate thermophile *B. caldotenax*.

*B. caldotenax* ICDH was shown to be very similar in size and composition to *E. coli* ICDH. The similarity of the two enzymes led us to investigate the possibility that

*B.caldotenax* ICDH is controlled by phosphorylation. This led to the discovery that it was possible to phosphorylate and inactivate purified *B. caldotenax* ICDH using *E. coli* ICDH kinase/phosphatase *in vitro*. This was the first indication that phosphorylation might be used to control ICDH activity in a gram positive bacterium. However, initial attempts to look for evidence of ICDH phosphorylation *in vivo* proved unsuccessful.

Further support for the idea that *B. caldotenax* might be controlled by phosphorylation was provided when the complete amino acid sequence of the protein was determined. The sequence of the amino acids around the phosphorylation site in *E. coli* ICDH were established by Borthwick *et al.* (1984c) and Malloy *et al.* (1984). In this study, it was found that this sequence of amino acids is entirely conserved in *B. caldotenax* ICDH. Similarly the amino acid sequence of ICDH from the extreme thermophile *Thermus thermophilus* was recently determined (Miyazaki *et al.*, 1992). These workers reported the sequence of amino acids found at the *E. coli* phosphorylation site to be conserved within *T. thermophilus* ICDH. As a result of this finding, coupled with the knowledge that *T. thermophilus* is able to grow on acetate as sole carbon source, this group proposed that ICDH from this micro-organism may also be controlled by phosphorylation.

Many eukaryotic kinases have been shown to recognise and phosphorylate short linear sequences of amino acids. It might be thought therefore that the sequence conservation itself explains why *B. caldotenax* ICDH is a substrate for the *E. coli* kinase. However McKee *et al.* (1989) demonstrated that *E. coli* ICDH kinase/phosphatase was unable to phosphorylate a short synthetic peptide, the sequence of which corresponded to the ICDH phosphorylation site. In addition the kinase was unable to phosphorylate chymotryptic peptides of ICDH. *E.coli* ICDH kinase recognises the overall 3-dimensional structure of the protein and recognition cannot be achieved by the phosphorylation site alone. The *B. caldotenax* and *E.coli* ICDH amino acid sequences share 71% identity and the structures of these two enzymes are therefore likely to be similar. The 3-dimensional structure together with the presence of the serine residue at the correct site would allow phosphorylation of the enzyme.

The serine residue could have been conserved for two possible reasons, either to maintain the activity of the enzyme or for control by phosphorylation. The 3-dimensional structure of *E. coli* ICDH was determined by Hurley *et al.* (1990). The structure showed ser113 to form a hydrogen bond with the substrate isocitrate. These workers made mutants of ICDH in which ser113 was converted to other amino acids. The replacement of serine with similar sized amino acids, such as threonine or alanine resulted in enzymes which were still active, thus showing that ser113 is not essential for ICDH activity. The question arises as to why this serine residue is highly conserved even though it is not absolutely essential for activity. It is possible that it has been retained in order to maintain the ability of the enzyme to be controlled by phosphorylation.

It will only be possible to confirm the use of phosphorylation, as a regulatory mechanism, in the control of *B. caldotenax* ICDH by carrying out detailed *in vivo* phosphorylation studies, comparable to those performed by Borthwick *et al.* (1984b) in *E. coli*. These studies involved labelling a culture of *B. caldotenax*, grown on acetate, with $^{32}Pi$ and then looking for the incorporation of $^{32}P$ into ICDH together with a decrease in enzyme activity. This work was based on many years study of ICDH activity in *E. coli* under different growth conditions. An initial attempt to look for evidence of *B. caldotenax* ICDH phosphorylation *in vivo* produced negative results and, within the course of this project, there was insufficient time to repeat this work under different experimental conditions. Prior to any further attempts to look for ICDH phosphorylation *in vivo* it would be useful to carry out growth physiology studies on *B. caldotenax*. It would be necessary to monitor ICDH activity in *B. caldotenax* under different growth conditions in particular looking for changes in ICDH activity which correspond with changes in the growth conditions. In this way it should be possible to establish the most appropriate conditions in which to carry out future experiments to investigate the phosphorylation of *B. caldotenax* ICDH *in vivo*.

During the course of this study the gene encoding *B. caldotenax* ICDH (*icd*) was cloned and sequenced, together with the flanking regions of DNA. Determination of the nucleotide

sequence upstream to the gene for ICDH produced possibly the most interesting finding of this study. Analysis of the nucleotide sequence upstream to *icd* showed it to contain the 3' end of the gene encoding citrate synthase (CS). It seems likely from the extreme proximity of the two genes that they might constitute an operon.

Genes coding for functionally related proteins are sometimes found clustered together on the genome. For example it is generally accepted that the biosynthetic genes for each actinomycete antibiotic are clustered together.

One well characterised example of this concerns the genes encoding the enzymes involved in the biosynthesis of the antibiotic actinorhodin in streptomycetes. Malpartida and Hopwood (1986) determined the physical and transcriptional organisation of the set of genes involved in actinorhodin production. All the biosynthetic genes and at least one regulatory gene were shown to be clustered together in a region of approximately 26 kb. The genes were found to be organised in at least four transcriptional units, some of the genes being transcribed as polycistronic mRNA.

Similar gene clusters have been identified in *Bacillus* species. The genetics of *B. subtilis* have been well studied and the genes controlling development and sporulation extensively characterised. Some genes have been shown to be polycistronic as in the case of the spoIIA locus which encodes genes involved in sporulation (Piggot *et al.*, 1985). Other operons have also been identified in *B. subtilis*, Fujta *et al.* (1985) showed the genes for gluconate kinase and gluconate permease to form an operon. The enzymes coded for by these genes are necessary for the cell to utilise gluconate.

A few examples of gene clusters in thermophiles have been reported. The genes for the glycolytic enzymes phosphoglycerate kinase and GAPDH were found clustered together in *B. stearothermophilus* (Davies *et al.*, 1991) and *T. thermophilus* (Bowen *et al.*, 1988). This cluster is not unique to thermophiles but has also been identified in mesophiles

including *E. coli* (Alefounder and Perham, 1989). Mallinder *et al.* (1992) recently cloned a 4.2 kb *Eco*RI fragment from *B. stearothermophilus*. The fragment contains the gene encoding glycerol kinase, two other complete ORFs and two incomplete ORFs. The start and stop codons of two complete ORFs and one incomplete ORF are located appropriately so as to allow translational coupling and it was proposed that the genes could constitute a single operon. It is unclear what the genes encode.

In *E. coli* the genes of the TCA cycle enzymes do not constitute a single operon. This is probably a reflection on the cycle's dual role in anabolism and catabolism, and the complexity of its regulation. In addition it is thought possible that the cycle evolved from diverse segments of earlier metabolic pathways. If this is the case one would not expect the genes to constitute a single operon. However, some of the genes, including the gene for CS do form clusters. The genes for CS (*gltA*), succinate dehydrogenase (*sdhCDAB*) and the 2-oxoglutarate dehydrogenase complex (*sucCD*) form a large cluster at 17 min on the *E. coli* chromosome. The gene for ICDH (*icd*) does not form part of the cluster but is positioned on its own at 25 min.

CS catalyses the condensation of oxaloacetate with acetyl-CoA to form citrate at the start of the TCA cycle. The next enzyme of the cycle is aconitase which catalyses the interconversion of citrate to isocitrate, the substrate for ICDH. In this way CS and ICDH act in relatively close succession and it is likely that a requirement for the synthesis of both enzymes would occur simultaneously. It would therefore be logical for the genes of these two enzymes to form an operon. As the enzyme aconitase links the CS and ICDH reactions one might expect the gene for this enzyme present in the proposed operon. It is possible that the aconitase gene could lie upstream to the gene for CS, as this region of DNA has not yet been studied. Some nucleotide sequence of DNA downstream to the *icd* gene was obtained. The translated sequence showed no homology to any other known protein sequences including that of aconitase. It seems likely therefore that the aconitase gene does not lie

downstream to that of ICDH, though this possibility cannot entirely be ruled out as so little downstream sequence was available.

The arrangement of genes in *B. caldotenax* is quite different from that of *E. coli* and the question arises as to how this particular arrangement has developed. The most likely explanation seems to be that at some time during the evolution of this micro-organism a rearrangement of genetic material has occurred possibly a result of intrachromosomal recombination or a transposable element. The resulting order of the genes encoding CS and ICDH genes must have proved advantageous to the organism for it to be maintained within the species. No similar CS/ICDH operon has previously been identified in any other organism. The idea that CS and ICDH form an operon is proposed only on locational evidence and obviously further research is required in this area before any firm conclusions regarding such an operon can be made. However, it is possible to conclude that *B. caldotenax* exhibits clustering of the genes for CS and ICDH.

**Future work**

Further research on this project could concentrate on two main areas. Firstly, we need to determine conclusively if phosphorylation of *B. caldotenax* ICDH is genuinely of physiological significance. As mentioned previously further *in vivo* research is required. However an alternative approach to addressing this question would be to look for evidence of an ICDH kinase in *B. caldotenax*. In this study we showed that *E. coli* ICDH kinase/phosphatase was able to recognise and phosphorylate *B. caldotenax* ICDH as a substrate. It would be fairly reasonable to assume from this that any putative *B. caldotenax* ICDH kinase will be very similar to that of *E. coli*. It was also demonstrated that *B. caldotenax* ICDH cross-reacts with an antibody raised to *E. coli* ICDH, there apparently being little variation between the two species. A comparatively simple method of looking for

a *B. caldotenax* ICDH kinase would be to use an antibody raised to purified *E. coli* ICDH kinase/phosphatase. It is likely that such an antibody would then cross-react with the putative *B. caldotenax* ICDH kinase. The antibody could be used in Western blots of crude extracts and immunoprecipitation experiments. (We might expect to see a protein of approximately 66,000 daltons, the size of *E. coli* ICDH kinase, either 'lighting up' on a Western blot or being immunoprecipitated).

Attempts could also be made to look for a gene encoding an ICDH kinase/phosphatase within the *B. caldotenax* genome. The nucleotide sequences of *B. caldotenax* and *E. coli* ICDH showed appreciable similarity, (70% identity). The *E. coli* ICDH kinase gene and the putative *B. caldotenax* ICDH kinase genes might be expected to share similar identity. Genomic digests of *B. caldotenax* DNA could be screened using the complete gene for *E. coli* ICDH kinase, labelled with $^{32}P$, as a probe. A strong, specific hybridisation signal would not conclusively prove the presence of a *B.caldotenax* kinase gene but would strongly indicate this.

The second main area for future work would involve studies of the proposed CS/ICDH operon. The clone obtained in this study (pCW1) contained the complete gene for ICDH but only the 3' end of the gene for citrate synthase. Prior to any studies to determine the existence of a CS/ICDH operon it would be necessary to clone a stretch of DNA containing both genes in their entirety. This procedure will be facilitated by using pCW1 as a probe. It is possible that the proposed operon could consist of more than two genes. By sequencing further upstream to the gene for CS and also downstream from the gene for ICDH this could be clarified.

Several approaches could be used to establish the existence of a CS/ICDH operon. Firstly transposon insertional mutations could be constructed within one or other of the two genes. If the two genes do form an operon an insertional mutation in the gene for ICDH would only inhibit expression of *icd* having no effect on the synthesis of CS. However a similar

mutation in the gene for CS would prevent expression of both genes. If the genes do not constitute an operon any insertional mutation would only effect transcription of the mutated gene.

Insertional mutations could provide indirect evidence for the existence of a CS/ICDH operon. Direct evidence for the proposed operon could be provided by S1 nuclease transcriptional mapping. The method involves the purification of RNA followed by the production of DNA/RNA hybrids, using single stranded DNA of interest, in this case DNA encoding CS and ICDH. Treatment with S1 nuclease results in the digestion of any single stranded DNA, unprotected by bound RNA.In this manner the size of RNA transcripts can be deduced. From the size of the transcripts it should be possible to determine whether or not the genes for CS and ICDH are transcribed as a single unit, as in the case of an operon, or as individual genes.

Finally, it would be interesting to achieve expression of the gene for ICDH and also CS. Barstow *et al.* (1986) successfully expressed the *B.stearothermophilus* gene for lactate dehydrogenase in *E. coli*. The gene was cloned into an expression vector, expression proceeding from the vector's promoter. It should therefore be feasible to express the genes for *B. caldotenax* CS and ICDH in *E. coli*.

The main objectives set out at the start of this work have been achieved. ICDH has been purified from *B. caldotenax*, its regulatory properties have been studied and its gene has been cloned and sequenced. This represents a significant advance in our knowledge of the enzyme in thermophiles. In addition the work has led to a number of possibilities for further experiments, some of which are outlined above. It is hoped that at least some of these ideas will be followed up in the future.

# References

## References


Adler, H., von Euler, H., Gunther, G. and Plass, M. (1939) Biochem. J. **33**, 1028-1045

Albretson, C., Haukanes, B-I., Aasland, R. and Kleppe, K. (1988) Anal. Biochem. **170**, 193-202

Alefounder, P.R. and Perham, R.N. (1989) Molecular Microbiology **3**, 723-732

Antranikian, G., Herzberg, C.S. and Gottschalk, G. (1985a) FEMS Microbiol. Lett. **27**, 135-138

Andreadis, A., Hsu, Y-P, Hermodson, M., Kohihaw, G. and Schimmel, P. (1984) J. Biol. Chem. **259**, 8059-8062

Antranikian, G., Herzberg, C.S. and Gottschalk, G. (1985b) Eur. J. Biochem. **153**, 413-420

Argos, P., Rossmann, G., Grau, U.M., Zuber, H., Frank, G. and Tratschin, J-D. (1979) Biochemistry **18**, 5698-5703

Ashworth, J.M. and Kornberg, H.L. (1963) Biochim. Biophys. Acta. **773**, 519-522

Banerjee, P., Ahmad, M.F., Grove, J.R., Kozlosky, L., Price, D.J. and Avruch, J. (1990) Proc. Natl. Acad. Sci. USA **87**, 8550-8554

Barford, D. (1991) Biochim. Biophys. Acta. **1133**, 55-62

Barstow, D.A., Clarke, A.R., Chia, W.N., Wigley, D., Sharman, A.F., Holbrook, J.J, Atkinson, T. and Minton, N.P. (1986) Gene **46**, 47-55

Batteiger, B., Newhall, V.W.J. and Jones, R.B. (1982) J. Immun. Meth. **55**, 297-307

Bautista, J., Satrusteui, J. and Machado, A. (1979) FEBS Lett. **105**, 333-336

Bennett, P.M. and Holms, W.H. (1975) J. Gen. Microbiol. **87**, 37-51

Binnie, C. (1990) Adv. Gene. Tech. **1**, 135-154

Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. **7**, 1513-1523

Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984a) Eur. J. Biochem. **141**, 393-400

Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984b) Biochem. J. **222**, 797-804

Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984c) FEBS Lett. **174**, 112-115

Bowen, D., Littlechild, J.A., Fothergill, J.E., Watson, H.C. and Hall, L. (1988) Biochem. J. **254**, 509-517

Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254

Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) BioTechniques **5**, 376

Campbell, J.J.R., Smith, R.A. and Eagles, B.A. (1953) Biochim. Biophys. Acta. **11**, 594

Charles, A.M. (1970) Canad. J. Biochem. **48**, 95-103

Chung, A.E. and Franzen, J.S. (1969) Biochemistry **8**, 3175-3184

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. **252**, 1102-1106

Cohen, S.N., Chang, A.C.Y. and Hsu, L. (1972) Proc. Natl. Acad. Sci. USA **69**, 2110-2114

Cortay, J-C., Rieul, C., Duclos, B. and Cozzone, A.J. (1986) Eur. J. Biochem. **115**, 227-237

Cortay, J-C., Bleicher, F., Rieul, C., Reeves, H.C. and Cozzone, A.J. (1988) J. Bacteriol. **170**, 89-97

Cortay, J-C., Negre, D., Galinier, A., Duclos, B., Perriere, G. and Cozzone, A.J. (1991) E.M.B.O. **10**, 675-679

Davies, G.J., Littlechild, J.A., Watson, H.C. and Hall, L. (1991) Gene **109**, 39-45

Dean, A.M. and Koshland, D.E.Jr. (1990) Science **249**, 1044-1046

Dean, A.M., Lee, M.H.I. and Koshland, D.E.Jr. (1989) J. Biol. Chem. **264**, 20482-20486

Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. **12**, 387-395

Edlin, J.D. and Sundaram, T.K. (1989) Int. J. Biochem. **21**, 1203-1210

Eguchi, H., Wakagi, T., Oshima, T. (1989) Biochim. Biophys. Acta. **990**, 133-137

El-Mansi, E.M.T., Nimmo, H.G. and Holms, W.H. (1985) FEBS Lett. **183**, 251-255

El-Mansi, E.M.T., Nimmo, H.G. and Holms, W.H. (1986) J. Gen. Microbiol. **132**, 797-806

Epstein, I. and Grossowicz, N. (1969) J. Bacteriol. **99**, 414-417

Ernster, L. and Navazio, F. (1956) Experimental Cell Res. **11**, 483-486

Falmange, P., Vanderwinkel, E. and Wiame, J.M. (1965) Biochim. Biophys. Acta. **99**, 246-258

Ferro-Luzzi Ames, G. and Nikaido, K. (1981) Eur. J. Biochem. **115**, 525-531

Fujita, Y., Nihashi, J-I. and Fujita, T. (1985) in Molecular Biology of Microbial Differentiation (editors Hoch, J.A. and Setlow, P.) p203-208

Garland, D. and Nimmo, H.G. (1984) FEBS Lett. **165**, 259-264

Garnak, M. and Reeves, H.C. (1979a) J. Biol. Chem. **254**, 7915-7920

Garnak, M. and Reeves, H.C. (1979b) Science **203**, 1111-1112

Gilvarg, C. and Davies, B.D. (1956) J. Biol. Chem. **222**, 307-319

Hanahan, D. (1983) J. Mol. Biol. **166**, 557-580

Haselbeck, R.J. and McAlister-Henn, L. (1990) J. Biol. Chem. **266**, 2339-2345

Hawkins, C.F., Borges, A. and Perham, R.N. (1990) J. Biochem. **191**, 337-346

Holms, W.H. and Bennett, P.M. (1971) J. Gen. Microbiol. **65**, 57-68

Holms, W.H. and Nimmo, H.G. (1982) Biochem. Soc. Trans. **10**, 319-320

Howard, R.L. and Becker, R.R. (1970) J. Biol. Chem. **245**, 3186-3194

Hurley, J.M., Thorsness, P.E., Ramalingham, V., Helmers, N.H., Koshland, D.E.Jr. and Stroud, R.M. (1989) Proc. Natl. Acad. Sci. **86**, 8635-8639

Hurley, J.M., Dean, A.M., Thorsness P.E., Koshland, D.E.Jr. and Stroud, R.M. (1990) Science **249**, 1012-1016

Hurley, J.M., Dean, A.M., Sohl, J.L., Koshland, D.E.Jr. and Stroud, R.M. (1991) Biochemistry **30**, 8671-8678

Imada, K., Sato, M., Tanaka, N., Katsube, Y., Matsura, Y. and Oshima, T. (1991) J. Mol. Biol. **222**, 725-738

Ishii, A., Ochiai, T., Imagawa, S., Fukunaga, N., Sasaki, S., Minowa, O., Mizuno, Y. and Shiiokawa, H. (1987) J. Biochem. **102**, 1489-1498

Johanson, R.A. and Reeves, H.C. (1977) Biochim. Biophys. Acta. **483**, 24-34

Jones, M.D., Lowe, D.M., Borgford, T. and Fersht, A.R. (1986) Biochemistry **25**, 1887-1891

Kagawa, Y., Nojima, H., Nukiwa, N., Ishizuka, M., Nakajima, T., Yasuhara, T., Tanaka, T. and Oshima, T. (1984) J. Biol. Chem. **259**, 2956-2960

Klumpp, D.J., Plank, D.W., Bowdin, L.J., Stueland, C.S., Chung, T. and LaPorte, D.C. (1988) J. Bacteriol. **170**, 2763-2769

Kornberg, H.L. (1963) Biochim. Biophys. Acta. **73**, 517-522

Kornberg, H.L. (1966) Biochem. J. **99**, 1-11

Kornberg, A. and Pricer, W.E.Jr. (1951) J. Biol. Chem. **189**, 123-136

Kornberg, H.L. and Madsen, N.B. (1958) Biochem. J. **68**, 549-557

Kornberg, H.L. and Quayle, J.R. (1958) Biochem. J. **68**, 542-549

Kornberg, H.L., Phizackery, P.J.R. and Sadler, J.R. (1960) Biochem. J. **77**, 438-445

Krebs, H.A. and Johnson, W.A. (1937) Enzymologia **4**, 148-156

Kristjansson, J.K. (1989) Trends. Biotech. **7**, 349-353

Kuhn, F.H.J., Fischer, H.M. and Fiechter, A. (1980) Arch. Microbiol. **124**, 13-20

Laemmli, U.K. (1970) Nature (London) **227**, 680-685

LaPorte, D.C. and Koshland, D.E.Jr. (1982) Nature (London) **300**, 458-460

LaPorte, D.C. and Koshland, D.E.Jr. (1983) Nature (London) **305**, 286-290

LaPorte, D.C., Thorsness, P.E. and Koshland, D.E.Jr. (1985) J. Biol. Chem. **260**, 10563-10568

LaPorte, D.C. and Chung, T. (1985) J. Biol. Chem. **260**, 15291-15297

LaPorte, D.C., Stueland, C.S. and Ikeda, T.P. (1989) Biochimie **71**, 1051-1057

Leyland, M.L. and Kelly, D.J. (1991) FEBS Lett. **202**, 85-93

Lowry, O.H., Carter, J., Ward, J.B. and Glaser, L. (1971) J. Biol. Chem. **246**, 6511-6521

Mallinder, P.R., Prichard, A. and Moir, A. (1992) Gene **110**, 9-16

Malloy, S.R. and Nunn, W.D. (1982) J. Bacteriol. **149**, 173-180

Malloy, P.J., Reeves, H.C. and Spiess, J. (1984) Current Microbiology **11**, 37-42

Malpartida, F. and Hopwood, D.A. (1986) Mol. Gen. Genet. **205**, 66-73

Manai, M. and Cozzone, A.J. (1979) Biochem. Biophys. Res. Commun. **91**, 819-826

Marr, J.J. and Weber, M.M. (1968) J. Biol. Chem. **243**, 4973-4979

McKee, J.S. and Nimmo, H.G. (1989) Biochem. J. **261**, 301-304

McKee, J.S., Hlodan, R. and Nimmo, H.G. (1989) Biochimie **71**, 1059-1064

Messing, J. (1983) Meth. Enzymol. **101**, 20-24

Miyazaki, K., Eguchi, H., Yamagishi, A., Wakagi, T. and Oshima, T. (1992) Appl. Environ. Microbiol. **58**, 93-98

Moran, C.P.Jr., Lang, N., LeGrice, S.F.J., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J. and Losick, R. (1982) Mol. Gen. Genet. **186**, 339-346

Morikawa, M., Izui, K., Taguchi, M. and Katzuki, H. (1980) J. Biol. Chem. **243**, 5385-5395

Mousdale, D.M., Cambell, M.S. and Coggins, J.R. (1987) Phytochemistry **26**, 2665-2670

Moyle, J. (1956) Biochem. J. **63**, 552-558

Muro-Pastor, M.I. and Florencio, F.J. (1992) Eur. J. Biochem. **205**, 99-105

Nagaoka, T., Hachimari, A., Takeda, A. and Samejima, T. (1977) J. Biochem. **81**, 71-78

Negre, D., Cortay, J.C., Old, I.A., Garlinier, A., Richaud, C., Saint-Girons, I. and Cozzone, A.J. (1991) Gene **97**, 29-37

Nimmo, H.G. (1984) Trends. Biochem. Sci. **9**, 475-478

Nimmo, G.A. and Nimmo, H.G. (1984) Eur. J. Biochem. **141**, 409-414

Nimmo, H.G. (1986) Biochem. J. **234**, 317-323

Nimmo, H.G., Borthwick, A.C., El-Mansi, E.M.T., Holms, W.H., MacKintosh, C. and Nimmo, G.A. (1988) Biochem. Soc. Symp. **54**, 93-101

Ninfa, A.J. and Magasanik, B. (1986) Proc. Natl. Acad. Sci. USA **83**, 5905-5913

Norrander, J., Kempe, T. and Messing, J. (1983) Gene **26**, 101-106

Nygaard, A.P. and Hall, B.D. (1963) Biochem. Biophys. Res. Commun. **12**, 98-102

Olsen, J.A. (1954) Nature (London) **174**, 695-696

Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA **85**, 2444-2448

Piggot, P.J., Chapman, J.W. and Curtis, C.A.M. (1985) in Molecular Biology of Microbial Differentiation (editors Hoch, J.A. and Setlow, P.) p 15-21

Plaut, G.W.E. (1963) The Enzymes, 1st Edition, vol. III, 105-126. (eds. Boyer, P.D., Lardy, H.A., and Myrback, K.)

Ragland, T.E., Kawasaki, T. and Lowenstein, J.M. (1966) J. Bacteriol. **91**, 236-244

Rahmsdorf, H.J., Pai, S.H., Ponta, H., Herrlich, P., Roskoski, R., Schweiger, M. and Studier, F.W. (1974) Proc. Natl. Acad. Sci. USA. **71**, 586-589

Ramaley, R.F. and Hudock, M.O. (1973) Biochim. Biophys. Acta. **315**, 22-36

Reeves, H.C., Daumy, G.O., Lin, C.C. and Houston, M. (1972) Biochim. Biophys. Acta. **258**, 27-39

Reeves, H.C., Daumy, G.O., Lin, C.C. and Houston, R. (1981) Biochim. Biophys. Acta. **258**, 27-39

Reeves, H.C., O'Neil, S. and Weitzman, P.D.J. (1986) FEMS. Microbiol. Lett. **35**, 229-232

Reizer, J., Novotny, M.J., Panos, C. and Saier, M.H.Jr. (1983) J. Bacteriol. **156**, 354-361

Roberts, R.B., Abelson, P.H., Cowie, D.B., Bolton, E.T. and Britten, R.J. (1955) Publ.Carneg.Instn., no.607

Rossman, M.G., Moras, D. and Olsen, K.W. (1974) Nature (London) **250**, 194-199

Rubin, C.S. and Rosen, O.M. (1975) Biochemistry **44**, 831-887

Saiki, T., Ijima, S., Tohda, K., Beppu, T. and Arima, K. (1978) in Biochemistry of Thermophily (editor. S.M. Friedman) p 287-303

Saito, H. and Miura, K-I. (1963) Biochim. Biophys. Acta. **72**, 619-629

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, 2nd edition. Cold Spring Harbor Press

Sanger, F., Kiklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA. **74**, 5463-5467

Saz, H.J. (1954) Biochem. J. **58**, xx-xxi

Sekiguchi, T., Suda, M., Sekiguchi, T. and Nosoh, Y. (1986a) FEMS Lett. **36**, 41-45

Sekiguchi, T., Ortega-Cesena, J., Nosoh, Y., Ohashi, S., Suda, K. and Kanaya, S. (1986b) Biochim. Biophys. Acta. **867**, 36-44

Sekiguchi, T., Suda, M., Ishn, T., Nosoh, Y. and Tsuda, K. (1987) Nucleic Acids Res. **15**, 853-853

Self, C.H and Weitzman, P.D.J. (1972) Biochem. J. **130**, 211-219

Self, C.H, Parker, M.G. and Weitzman, P.D.J. (1973) Biochem. J. **132**, 215-221

Shiio, I. and Ozaki, H. (1968) J. Biochem. (Tokyo) **64**, 45-53

Smith, R.A. and Gunsalus, I.C. (1954) J. Amer. Chem. Soc. **76**, 5002-5003

Smith, T.F. and Waterman, M.S. (1981) Adv. Appl. Math. **2**, 482-489

Southern, E.M. (1975) J. Mol. Biol. **98**, 503-517

Spencer, M.E. and Guest, J.R. (1985) Mol. Gen. Genet. **200**, 145-154

Sprang, S.R., Acharya, K.R., Goldsmith, E.J., Stuart, D.I., Varvill, K., Fletterick, R.J., Madsen, N.B. and Johnson, L.N. (1988) Nature (London) **336**, 215-221

Stenesh, J., Roe, B.A. and Snyder, T.L. (1968) Biochim. Biophys. Acta. **161**, 442-454

Stetter, K.O. (1982) Nature (London) **300**, 258-260

Stock, J.B., Stock, A.M. and Mottonen, J.M. (1990) Nature **344**, 395-344

Stueland, C.S., Eck, K.R., Stieglbauer, K.T. and LaPorte, D.C. (1987) J. Biol. Chem. **262**, 16095-16099

Stueland, C.S., Ikeda, T.P. and LaPorte, D.C. (1989) J. Biol. Chem. **264**, 13775-13779

Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.H. and Itakura, K. (1981) Proc. Natl. Acad. Sci. USA **78**, 6613-6617

Sung, S.C. and Hsu, C.H. (1957) Journal of the Formosan Medical Association **56**, 103

Tabor, S. and Richardson, C.C. (1989) Proc. Natl. Acad. Sci. USA **86**, 4076-7080

Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA **76**, 4350-4354

Thorsness, P.E. and Koshland, D.E.Jr. (1987) J. Biol. Chem. **262**, 10422-10425

Varela, I. and Nimmo, H.G. (1988) FEBS Lett. **231**, 361-365

Vanderwinkel, E. and DeVliegher, M. (1968) Eur. J. Biochem. **5**, 81-90

Walsh, K. and Koshland, D.C.Jr. (1985) J. Biol. Chem. **260**, 8430-8437

Wang, J.Y.J. and Koshland, D.E.Jr. (1978) J. Biol. Chem. **253**, 7605-7608

Weber, K. and Osborne, M. (1969) J. Biol. Chem. **244**, 4406-4412

Weiss, B., Jacquemin-Sablon, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) J. Biol. Chem. **243**, 4543-4549

Wiegel, G.J. and Ljungdahl, L.G. (1986) Crit. Rev. Biotechnol. **3**, 39-109

Wierenga, R.K., Terpstra, P. and Hol, W.G.J. (1986) J. Mol. Biol. **187**, 101-107

Wong, D.T.O. and Ajl, S.J. (1956) J. Amer. Chem. Soc. **78**, 3230-3237

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. **118**, 197-203

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103-119

Yutani, K., Ogasahara, K., Tsujita, T. and Sugino, Y. (1987) Proc. Natl. Acad. Sci. USA **84**, 4441-4444

Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H.J., Ponta, H. and Hirsch-Kauffman, M. (1972) Proc. Natl. Acad. Sci. USA. **72**, 2506-2510

Zuber, H. (1978) in Biochemistry of Thermophily (editor S. M. Friedman) p 267-285

Zulli, F., Weber, H. and Zuber, H. (1987) Biol. Chem. **368**, 1167-1177