STRUCTURE AND FUNCTION OF ISOCITRATE DEHYDROGENASE FROM *ESCHERICHIA COLI* ML308

by

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For Mum and Dad

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ABBREVIATIONS

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

Bicine	N,N-bis(2-hydroxyethyl) glycine
BSA	Bovine serum albumin
cAMP	cyclic AMP
CD	Circular dichroism
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
FPLC	Fast Protein Liquid Chromatography
HPLC	High Pressure Liquid Chromatography
ICDH	Isocitrate dehydrogenase
ICL	Isocitrate lyase
MOPS	Morpholino propane sulphonic acid
NADP	Nicotinamide - adenine dinucleotide phosphate
ORD	Optical rotary dispersion
PAGE	Polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
PG	phenylglyoxal
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris (hydroxy methyl) aminomethane

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Summary

The level of activity of the NADP-dependent isocitrate dehydrogenase (ICDH) of E.coli ML308 is controlled by a reversible phosphorylation mechanism during growth on acetate as sole carbon source. Under such conditions the enzymes of the glyoxylate bypass, isocitrate lyase (ICL) and malate synthase are induced. This results in competition between ICDH and ICL for the available isocitrate. To allow efficient use of isocitrate via the glyoxylate bypass ICDH is phosphorylated on a single serine residue per subunit which completely inactivates it. Phosphorylation of ICDH is believed to occur at the $NADP^+$ binding site, and so the enzyme is inactive because it cannot bind its cofactor.

In this study chemical modification of ICDH has been used to study the active site of the enzyme. In particular we wished to identify any positively charged amino acid residues located at the $NADP^+$ binding site of ICDH.

Since arginine residues are often found at nucleotide binding sites we chose the arginine specific reagent phenylglyoxal. Phenylglyoxal was found to inactivate ICDH in a pseudo-first-order process. The inactivation was rapid and reproducible. Phenylglyoxal was found not to be an affinity label for ICDH. Protection against inactivation could be achieved either by $NADP^+$ binding to the enzyme or by phosphorylation. These results suggested the existence of an arginine residue at the cofactor binding site of ICDH. Analysis of the reaction using [7-^{14}C]-phenylglyoxal indicated that complete inactivation of the enzyme corresponded to incorporation of 1 mole of phenylglyoxal per mole of ICDH subunits.

Initial analysis of the arginine-[7-^{14}C]-phenylglyoxal adduct suggested the inactivation product was stable. However any

attempts to isolate a labelled peptide from peptic digests of ICDH revealed that the product was not stable under the conditions necessary to isolate a labelled peptide. However some indirect evidence was obtained that the same peptide in ICDH could be labelled by either phenylglyoxal or phosphorylation.

The analysis of substrate specificity of ICDH kinase/phosphatase using a 14-residue synthetic peptide corresponding to the known ICDH phosphorylation sequence indicated that the kinase activity did not recognise this primary sequence as a substrate. Furthermore attempts to phosphorylate chymotryptic digests of ICDH also failed. These results indicated that ICDH kinase appeared only to recognise intact ICDH as a phosphorylatable substrate. Possible reasons for this recognition mechanism will be discussed.

Finally, analysis of the conformation of ICDH on substrate binding or phosphorylation using circular dichroism in the near U.V. region was carried out. This indicated a change at 292nm for the active enzyme thought to occur as a result of $NADP^+$ binding to the enzyme. However no such change was detected for phosphorylated ICDH indicating an inability of the phospho-form to bind NADP. Comparison of the CD spectra for active and phosphorylated ICDH showed no detectable change in conformation occurred on phosphorylation. These results suggested that phosphorylated ICDH is inactive because it is unable to bind its cofactor.

CHAPTER ONE

Introduction

1.1 Isocitrate dehydrogenase from Escherichia coli ML308

1.1.1 Introduction

The oxidative decarboxylation of isocitrate to give 2-oxoglutarate and carbon dioxide is catalysed by the enzyme isocitrate dehydrogenase (Fig.1.1). The enzyme is found in most microorganisms, animals and plants (Plaut, 1963). The work reported in this thesis is concerned with the NADP-dependent isocitrate dehydrogenase from Escherichia coli. E.coli like most bacteria, contains only an NADP-dependent isocitrate dehydrogenase (Raglan et al., 1966). Eukaryotics, on the other hand, contain both an NAD and an NADP-dependent enzyme, distinct also from each other by their cellular localisation, mitochondrial and cytoplasmic respectively (Ernster & Navazio, 1956).

NADP-dependent isocitrate dehydrogenase (threo-D_s-isocitrate $NADP^+$ oxidoreductase (decarboxylating) EC 1.1.1.42), hereafter ICDH, requires Mn^{2+} for the oxidative decarboxylation of isocitrate (Adler et al., 1939). Further studies demonstrated that it was the decarboxylation reaction as opposed to the oxidation that required Mn^{2+} (Ochoa, 1948) and that Mg^{2+} could be substituted for the Mn^{2+} although it was less effective (Moyle, 1956). The natural coenzyme for this ICDH is $NADP^+$. NAD^+ is not active. ICDH is a TCA cycle enzyme; its function is to produce 2-oxoglutarate for further oxidation in the Krebs cycle and for biosynthesis.

1.1.2 Escherichia coli metabolism during growth on a single carbon source

In bacteria, as in animal tissues, catabolism is responsible for the breakdown of foodstuffs into a narrow range of small molecules which serve as the building blocks for the macromolecular constituents of the cell. The initial pathways of catabolism for most foodstuffs converge at the level of acetyl CoA. In most bacteria the Krebs cycle (Krebs and Johnston, 1937) serves as the final common pathway for the oxidation of these acetyl groups to CO_2 and H_2O with the concomitant

production of reduced pyridine nucleotides and flavins. These reduced coenzymes transfer their high potential-electrons to O_2 via the electron transport chain generating ATP as a result of oxidative phosphorylation.

The TCA cycle also plays a role in biosynthesis by providing the necessary intermediates. Cells continually draw off oxaloacetate, 2-oxoglutarate and succinyl CoA from the TCA cycle for biosynthetic reactions (Fig.1.2). When the cycle functions in its biosynthetic capacity, the removal of cyclic intermediates would prevent the regeneration of oxaloacetate and the cycle would cease to function. Thus there must exist pathways ancillary to the TCA cycle which function to replenish the pools of cyclic intermediates and lead to the regeneration of oxaloacetate. Kornberg (1966) reviewed the evidence that different ancillary or anaplerotic pathways are required depending on the nature of the carbon source on which the organism is growing.

(a) Growth on glucose

Analysis of the carbon flux of E.coli during growth on glucose suggests that flux through the Krebs cycle is relatively small and is almost exclusively for biosynthesis. Nevertheless an anaplerotic reaction is still required. Kornberg and his coworkers studied mutant strains of E.coli that were unable to grow on glucose unless the medium was supplemented with certain dicarboxylic acids. They found that these mutants lacked the enzyme phosphoenolpyruvate carboxylase (EC 4.1.1.31) (Canovas & Kornberg, 1965 and 1966; Ashworth & Kornberg, 1966). The reaction carried out by this enzyme (Fig.1.3c) thus replenished the supply of oxaloacetate in the Krebs cycle. Phosphoenolpyruvate carboxylase was found to be activated by acetyl CoA. The rationalisation of this effect is that if the pool of oxaloacetate is reduced the acetyl CoA levels build up in the cell which results in an activation of PEP carboxylase (Canovas & Kornberg, 1966). During growth

on glucose, or an equivalent carbon source, there is no evidence that ICDH plays a significant role in regulating the flux through the Krebs cycle. It is not under any allosteric control, however the enzyme is subjected to concerted inhibition by glyoxylate and oxaloacetate. This inhibition is partly caused by a condensation of glyoxylate and oxaloacetate to yield the unstable compound oxalomalate, which is an extremely potent competitive inhibitor of ICDH, and partly caused by the slow binding of glyoxylate and oxaloacetate to the isocitrate binding site of the enzyme. Neither mechanism, however, seems to be physiologically significant (Nimmo, 1986). Holms and Bennett (1971), however, found that growth of E.coli on glucose resulted in approximately 17% of the glucose carbon accumulating as acetate. When the glucose was exhausted the cells adapted to utilise the acetate. During oxidation of the acetate there was a drop in the activity of ICDH to approximately 25% of its original level. To understand the reasons for this decrease, we need to consider the metabolic pathways involved in growth on acetate.

(b) Growth on acetate

Growth of E.coli on acetate as sole carbon source requires the induction of the enzymes of the glyoxylate bypass, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) (Kornberg, 1966). Isocitrate lyase catalyses the formation of glyoxylate and succinate from the cleavage of isocitrate (Fig.1.3a). Malate synthase catalyses the condensation of acetyl CoA with glyoxylate to give malate (Fig.1.3b). The sequential operation of these enzymes was first demonstrated using extracts of Pseudomonas fluorescens (Kornberg & Madsen, 1957). Subsequent studies with E.coli showed that both enzymes of the glyoxylate bypass are synthesised adaptively when the organism is switched to acetate metabolism (Reeves & Ajl, 1960).

The induction of the enzymes of the glyoxylate bypass creates a branchpoint at isocitrate (Fig.1.2). This results in competition between ICDH and isocitrate lyase for the available common substrate isocitrate. If the isocitrate is used by ICDH then the net effect is loss of acetate carbon as CO_2. However, isocitrate lyase (ICL) bypasses the CO_2 releasing enzymatic reactions of the TCA cycle resulting in efficient use of acetate carbon for both cycle maintenance and supply of biosynthetic precursors. Table 1.1 shows data related to the competition arising between ICDH and ICL for isocitrate in E.coli. The K_m value of ICDH for isocitrate is much lower than ICL, and as a result the available isocitrate would be utilised by ICDH. Thus the acetate carbon would be lost as CO_2, there would be no available precursors for biosynthetic purposes, and cell growth would stop. Therefore in order to allow flux of carbon through the glyoxylate bypass to occur, there must be some means either of inhibiting ICDH or activating the ICL *in vivo*.

1.1.3 Reversible inactivation of isocitrate dehydrogenase

The observations made by Bennett and Holms during the comparison of the levels of three TCA cycle enzymes, ICDH, malate dehydrogenase and 2-oxoglutarate dehydrogenase, in E.coli ML308 grown on different carbon sources indicated that ICDH can be reversibly inactivated *in vivo* (Holms & Bennett, 1971; Bennett & Holms, 1975). These workers measured the specific activities of malate dehydrogenase, 2-oxoglutarate dehydrogenase and isocitrate dehydrogenase in cultures of E.coli ML308 over a four hour period after the cessation of growth on either limiting glycerol or limiting glucose. After cessation of growth on glycerol the three enzyme activities remained constant during the four hour period. However, in the four hour period after cessation of growth on glucose the activity of the ICDH fell and after two hours in stationary phase, 80% of the original activity had been lost. During the following two hours

ICDH activity rose again and four hours after growth ceased ICDH activity was restored to 75% of its maximum value. Malate dehydrogenase and 2-oxoglutarate dehydrogenase activities were unaffected during this four hour period (Holms & Bennett, 1971). This apparent inactivation/activation of ICDH after growth on glucose was attributed to the fact that E.coli excretes acetate during growth on glucose but not on glycerol (Britten, 1954; Holms & Bennett, 1971).

After growth on glucose the enzymes of the glyoxylate bypass were induced and the excreted acetate oxidised. During the period of adaption to and use of acetate, the activity of ICDH declined, and it recovered only after the exhaustion of acetate in the medium. The addition of chloramphenicol at the end of growth on limiting glucose prevented the increase in isocitrate lyase activity and the loss of ICDH activity (Holms & Bennett, 1971). Loss of ICDH activity after cessation of growth on limited glucose could be prevented by addition of further glucose, pyruvate or any TCA cycle intermediate. However, when growth ceased a second time loss of ICDH activity was observed (Holms & Bennett, 1971).

Since the variation in ICDH activity occurred on adaptation to acetate as sole carbon and energy source, Bennett and Holms (1975) examined the state of the enzyme in cells fully adapted to acetate. They found the apparent activity of ICDH during exponential growth on acetate was one third of the level found for growth on glucose. When pyruvate was added to acetate grown cultures there was an immediate and rapid 3- to 4-fold increase in ICDH activity accompanied by an increase in growth rate. Chloramphenicol added simultaneously with pyruvate barely affected the changes in activity indicating that protein synthesis was not involved (Bennett and Holms, 1975). Further experiments carried out by the same workers, including dialysis and mixing of the extracts showed that changes in the intracellular level of a reversible activator

were not involved in the observed changes in the level of ICDH activity (Bennett and Holms, 1975). E.coli ICDH is strongly inhibited by a mixture of glyoxylate and oxaloacetate (Shiio and Ozaki, 1968; Marr and Weber, 1968; Bennett and Holms, 1975). Shiio and Ozaki postulated that this inhibition in principle could be responsible for the low enzyme activities observed in cells with an operating glyoxylate bypass. However, Bennett and Holms (1975) showed that extracts exhibiting either maximum or minimum ICDH activity were inhibited to the same degree by the two metabolites. They also detected no difference in the K_m values of the ICDH for NADP or isocitrate or in its pH or temperature dependence.

The evidence of Bennett and Holms (1975) indicated that the observed changes in the activity of ICDH observed during shifts to and from media containing acetate as sole source of carbon and energy were independent of protein degradation and de novo synthesis and were unaffected by freely dissociable low molecular weight molecules which might affect enzyme activity.

Bennett and Holms (1975) concluded from their results that either reversible covalent modification of ICDH or some form of reversible association of ICDH with some other macromolecule was responsible for the modulation of ICDH activity of cells growing on acetate. They also proposed that the role of inactivation of ICDH was to allow isocitrate lyase to compete successfully for isocitrate under conditions in which the operation of the glyoxylate bypass was essential for growth (Bennett and Holms, 1975).

Phosphorylation of ICDH was first implicated by the work of Garnak and Reeves (1979a). They added $^{32}P_i$ to E.coli K12 grown to stationary phase on limiting glycerol in a low-phosphate medium. Addition of acetate to the culture caused both a partial reduction in ICDH activity and incorporation of ^{32}P-phosphate into the enzyme. At this stage, however, the relationship between the phosphorylation and the

activity of ICDH was not established. The system was, however, better characterised by the work of Nimmo and his coworkers (Borthwick et al., 1984a) and Koshland and coworkers (LaPorte and Koshland, 1983) (see Sections 1.2.3-1.2.5).

1.2 Phosphorylation of E.coli isocitrate dehydrogenase

1.2.1 Covalent modification

Recognition of the reversible covalent modification of proteins as a major regulatory process was a result of work on mammalian glycogen metabolism. Glycogen phosphorylase was shown to exist in two interconvertible forms, a relatively inactive form, phosphorylase b and an active form phosphorylase a. In resting muscle phosphorylase is in the b form. The regulation of phosphorylase b by AMP was the first example of the control of enzyme activity by an allosteric effector (Cori et al., 1938). Phosphorylase b is inactive in the absence of AMP, but is just as active as phosphorylase a in its presence. The regulation of phosphorylase b by AMP is however critically dependent on the substrate inorganic phosphate (P_i). As the concentration of P_i increases, the concentration of AMP required for half maximal activation decreases and conversely, the K_m for P_i decreases as AMP increases (Helmreich and Cori, 1964). On the other hand, activation by AMP is antagonised by both ATP and glucose-6-phosphate. Phosphorylase b is converted to phosphorylase a by an Mg-ATP requiring enzyme phosphorylase kinase. This phosphorylation alleviates the allosteric control of phosphorylase a by AMP.

Fischer and Krebs (1955) demonstrated the ATP dependent conversion of phosphorylase b to a in cell free extracts of rabbit muscle. At the same time Sutherland and Wosilait (1955) showed that phosphate is liberated from purified rabbit liver phosphorylase a as it is inactivated enzymatically. Together these early observations indicated that the ATP-dependent phosphorylation of phosphorylase b

converts the enzyme to the active form phosphorylase a and the subsequent release of this phosphate results in the regeneration of phosphorylase b. Since these initial observations of protein phosphorylation in glycogen metabolism a complex regulatory network has been uncovered. In 1983 more than six protein kinases, four phosphoprotein phosphatases and several thermostable regulatory proteins had been discovered (Cohen, 1983; review Cohen, 1988).

Little or no progress however was made in understanding the mechanism of activation of glycogenolysis in skeletal muscle until the 1950s. Earl Sutherland showed an enhancement of glycogenolysis and phosphorylase activity by incubating liver slices with adrenalin. However, the response to adrenalin was lost when the liver slices were homogenised. With the discovery of the chemical nature of the change in phosphorylase activity, phosphorylation, it became clear that ATP and Mg^{2+} would be necessary for inactivation. When added to the homogenised tissue they restored the sensitivity to adrenalin. These findings allowed the hormone response to be separated into two stages. The first was action of adrenalin on the membrane to produce a small heat stable factor and the second was replacement by this factor of the hormone signal to increase the proportion of phosphorylase a. The factor was identified as adenosine 3'5' cyclic monophosphate (cAMP) (Robison et al., 1971).

The discovery that cAMP promoted the conversion of phosphorylase b to a indicated that this molecule must activate phosphorylase kinase or inhibit protein phosphatase-1. The discovery by Krebs and his coworkers (Walsh et al., 1968) of the enzyme cyclic AMP dependent protein kinase resolved the problem of how cAMP affected certain enzyme activities. This enzyme phosphorylates phosphorylase kinase and activates it. This active form of phosphorylase kinase phosphorylates its substrate phosphorylase to give the a form.

It was soon realised, however, that cAMP-dependent protein kinase did not just phosphorylate phosphorylase kinase but a whole range of substrates (reviewed Cohen, 1983). Similarly cAMP itself is produced in a variety of tissues in response to the interaction of many different hormones with their receptors. The hypothesis, therefore, arose that the specificity of hormones which act through cAMP is determined by whether their receptors are present on the plasma membrane of a target cell and which physiological substrates for cAMP-dependent protein kinase are present within these cells (Kuo and Greengard, 1969; Krebs, 1972).

In 1983 about 40 enzymes were known to be controlled by phosphorylation (Cohen, 1983). It is now recognised as the major mechanism by which intracellular events are controlled by extracellular stimuli and also in control of intracellular processes from within the cell itself in eukaryotes.

Covalent modification, however, is not restricted to protein phosphorylation/dephosphorylation reactions. Modifications have been described including acetylation-deacetylation (Gottscholl et al., 1982), uridylylation-deuridylylation (Adler et al., 1973), adenylylation – deadenylylation (Holyer et al., 1967), methylation-demethylation (Springer and Koshland, 1977) and also limited proteolysis e.g. chymotrypsinogen activation (Stroud et al., 1977). For the scope of this thesis we will only discuss phosphorylation- dephosphorylation reactions. This modification is very widespread in biological systems.

1.2.2 Protein phosphorylation

Protein kinases which catalyse protein phosphorylation reactions can be distinguished by their specificity with respect to the particular amino acid residue which serves as a phosphate acceptor.

The major group of protein kinases phosphorylate serine or threonine residues, for example in glycogen metabolism (Cohen, 1983).

Protein kinases catalysing the transfer of phosphoryl groups to histidine or lysine residues have been reported by Smith et al. (1974). The most recently described group of kinases phosphorylate tyrosine residues. Examples include receptors for epidermal growth factor and insulin, and the src gene product. A common feature connecting many but not all of the eukaryotic protein kinases is their activation within the cell by a second messenger which is released in response to an extracellular stimulus such as hormone-receptor binding. Such second messengers include cAMP, cGMP, diacylglycerol and Ca^{2+}. The most studied example of a protein kinase is probably cAMP-dependent protein kinase. The formation of cAMP within the cell occurs in a variety of tissues in response to hormone-receptor interaction. This complex stimulates adenylate cyclase to produce cAMP within the cell. cAMP-dependent protein kinase appears to be the only protein in mammalian cells that binds cAMP with high affinity (apart from cAMP phosphodiesterase). The enzyme exists in isoenzymatic forms designated as type I and type II (Reimann et al., 1971), consisting of two catalytic subunits and two regulatory subunits, which bind cAMP. In the absence of cAMP the regulatory (R) and catalytic (C) subunits have a high affinity for each other, but on binding of the nucleotide this affinity is decreased by a factor of 10^4. The holoenzyme (R_2C_2) is inactive because a part of the regulatory subunit directly impedes the active site of the catalytic subunit. It has been proposed that cAMP binds to the R subunit of R_2C_2 forming an intermediate ternary complex $cAMP_4R_2C_2$. A conformational change then occurs in the R subunit and they dissociate from the C subunits ($R_2(cAMP)_4 + 2C$) (Hoppe, 1985).

Similarly, the only protein kinases whose substrate specificities are reasonably well understood are the cAMP and cGMP-dependent kinases and the casein kinases. It was originally

proposed for cAMP-dependent protein kinases that the tertiary structure of the protein substrate was important in substrate recognition (Langan, 1973). However Daile and Carnegie (1974) demonstrated that bovine cardiac and brain enzymes could phosphorylate a 17 amino acid residue peptide. Bylund and Krebs (1975) showed that the denaturation of lysozyme enhanced its susceptibility to phosphorylation and proposed that the kinase recognised some feature of the primary structure around the target residue. Further work proved this idea to be correct. On the basis of the amino acid sequence analysis and studies with synthetic peptides, reviewed by Krebs and Beavo (1979), three main patterns have emerged as target sites for cAMP-dependent protein kinases:

Lys-Arg-X-X-Ser(P)

Arg-Arg-X-Ser(P)

Arg-Arg-Ser(P)

Table 1.2 shows a range of recognition sequences for a variety of protein kinases, and examples of physiological substrates.

Protein phosphorylation has thus been recognised to be an important mechanism for controlling cellular processes in eukaryotic cells, and information concerning new phosphorylated proteins, substrate specificity, mechanism of action and metabolic significance is increasing every year. In contrast to eukaryotes, the occurrence of protein phosphorylation in prokaryotes was for a long time a matter of controversy. It is only over the last decade that the existence of protein kinases and phosphatases has been well established (reviewed by Cozzone, 1984).

Wang and Koshland (1978) labelled Salmonella typhimurium cells with $^{32}P_i$ and isolated at least four ribonuclease resistant and pronase sensitive ^{32}P-containing species using SDS polyacrylamide gel electrophoresis. Incubation of a cell free extract with [gamma-^{32}P]-ATP indicated that the same proteins could be labelled in

vitro and *in vivo*. The phosphorylation reaction required Mg^{2+}. Hydrolysis of the labelled proteins in 6N HCl at 110°C for 4 hours liberated labelled material which was found to co-migrate with phosphoserine and phosphothreonine standards. The finding of the two labelled phosphoamino acids in the labelled proteins was a strong argument that there was covalent modification (Wang and Koshland, 1978). If the phosphorylation reactions observed in prokaryotes are analogous to eukaryotic phosphorylation reactions it would be expected that prokaryotic phosphorylation would also serve to regulate some aspect of cellular function.

Such a function has been determined for the phosphorylation reaction of *Escherichia coli* isocitrate dehydrogenase. Only in this case of prokaryotic phosphorylation has the physiological role and the molecular details of the phosphorylation system been well studied on a level comparable to eukaryotic systems (reviewed by Nimmo, 1984).

1.2.3 Phosphorylation of E.coli isocitrate dehydrogenase

The ICDH of *E.coli* is a dimer of identical subunits of M_r 45000 (Borthwick *et al.*, 1984a). I have already discussed its location at the branchpoint between the TCA cycle and glyoxylate bypass and its inactivation/activation during growth on acetate as sole carbon source.

Results from Reeves' group first showed that during adaptation to acetate the ICDH of *E.coli* becomes phosphorylated (Garnak and Reeves, 1979a,b). When acetate was added to a culture of *E.coli* K12 grown to stationary phase and then incubated with $^{32}P_i$, this caused partial inactivation of ICDH. A phosphorylated form of ICDH containing ^{32}P as phosphoserine was isolated from the culture and shown to be immunologically identical to active ICDH from glucose grown cells. Borthwick *et al.* (1984a) isolated the active form of ICDH from glycerol grown cells and the inactive form from acetate grown cells. They showed that the two forms could be resolved by non-denaturing gel

electrophoresis and differed in charge but not molecular weight, amino acid composition or 2-dimensional peptide maps. The alkali-labile phosphate content of the two forms was determined. This showed that active ICDH contained no detectable phosphate whereas the inactive form contained somewhat less than one mole phosphate/45000g. Thus the data suggested a difference between the two forms of one phosphate group per subunit (Borthwick et al., 1984a).

The result that this reversible phosphorylation reaction only occurs during growth on acetate as sole carbon source, indicates a metabolically significant role for the inactivation reaction. Phosphorylation of ICDH during acetate growth, and hence inactivation of the ICDH, releases the competition between ICDH and ICL for the available isocitrate, thus allowing efficient use of acetate carbon via the glyoxylate bypass.

1.2.4 In vivo phosphorylation of ICDH

An important part of studying a phosphorylation system is to establish its occurrence in vivo. By using cells labelled with [^{32}P]-P_i, Borthwick et al. (1984b), showed that activation and inactivation of ICDH correlated with its dephosphorylation and rephosphorylation respectively. Their results indicated that the reversible changes in the activity of cells grown on acetate were solely due to phosphorylation/dephosphorylation. Isolation of the in vivo phosphorylated form followed by chymotryptic digestion indicated that the same phosphopeptide was present as compared to the in vitro phosphorylated ICDH (Borthwick et al., 1984b). Thus the serine residue labelled in vivo is the same as that phosphorylated by purified kinase in vitro.

1.2.5 A bifunctional ICDH kinase/phosphatase

Purification of ICDH kinase and ICDH phosphatase activities was first achieved by LaPorte and Koshland (1982). ICDH kinase was purified

using three steps: ammonium sulphate fractionation; DEAE-Sephacel chromatography and affinity chromatography using ICDH immobilised on Sepharose 4B. When they started to purify the phosphatase activity it was apparent that the same purification steps were needed, and that the ratio of the kinase and phosphatase activities remained constant throughout the purification procedure. They tried to separate the two activities using ion-exchange, Sephadex G150 and again on the ICDH affinity column but found that the two activities were always eluted superimposably. The purified enzyme, with both activities, gave only one band on SDS gels. This suggested either that the two activities were located on the same polypeptide chain or on two different subunits with the same electrophoretic mobility, and chromatographic behaviour. The former was later shown to be the case by cloning and deletion mutagenesis experiments (LaPorte and Chung, 1985), and maps the gene to the glyoxylate bypass operon.

An unusual feature of the ICDH phosphatase activity is its absolute requirement for ATP or ADP (LaPorte and Koshland, 1982). The radioactivity released from [^{32}P]-phospho-ICDH appears as inorganic phosphate rather than ATP, indicating that dephosphorylation had not simply resulted from the back reaction of the kinase (LaPorte and Koshland, 1982). During experiments intended to identify the role of the nucleotide in the phosphatase reaction, Stueland et al. (1987) discovered that ICDH kinase/phosphatase catalysed an ATPase reaction. Further characterisation of the ATPase activity indicated that the activity was associated with the bifunctional enzyme not a contaminating ATPase. Experiments carried out with mutant derivatives of ICDH kinase/phosphatase which were nearly devoid of ICDH phosphatase activity retain both ICDH kinase and ATPase activity, indicating that ATP hydrolysis does not result from the cyclic phosphorylation of ICDH. However, the ICDH kinase and ATPase activities of these mutant proteins

differ significantly from the wild type ICDH kinase/phosphatase expressed from the parent allele. This suggested that ICDH kinase and ICDH phosphatase do not reside on structurally independent domains. ATP hydrolysis was only partially inhibited by phospho- and dephospho-ICDH with saturating levels of phospho-ICDH decreasing the rate of ATP hydrolysis by a factor of approximately 5. During further analysis of the kinetic behaviour of the wild-type and mutant proteins, Stueland et al. (1987) found that the ICDH kinase, ATPase and residual ICDH phosphatase activities of the mutant proteins all exhibit reduction in their affinity for phospho-ICDH. They propose that the simultaneous loss of affinity for phospho-ICDH by all three activities might suggest that the ICDH kinase, ICDH phosphatase and ATPase reactions are catalysed by the same active site (Stueland et al., 1987). They propose that the kinase and phosphatase reactions occur at the same active site and that the phosphatase reaction results from the kinase back reaction tightly coupled to ATP hydrolysis and this model in part provides an explanation for the observation that the ICDH phosphatase reaction is absolutely dependent on either ATP or ADP (Stueland et al., 1987). The results of Varela and Nimmo (1988), using photoaffinity labelling confirm one prediction of this model, that the ICDH kinase/phosphatase contains only one ATP binding site.

Nimmo et al. (1984) went on to better characterise the two reactions, and their effect on ICDH activity using ICDH kinase/phosphatase purified over a thousand fold from E.coli ML308. Results showed that the kinase catalyses the transfer of the gamma-phosphate group of ATP to a serine residue of active ICDH with a stoichiometry of 1 per subunit. Phosphorylation essentially totally inactivated ICDH. ICDH phosphatase was capable of catalysing full reactivation of phosphorylated ICDH provided that the kinase was not active simultaneously. Nimmo et al. (1984) reported an absolute

requirement of a divalent metal ion for phosphatase activity and also confirmed the requirement for ADP or ATP. Analysis of the phosphatase reaction showed that the product was P_i and hence reactivation was not produced by reversal of the kinase (see above). Experiments carried out by Nimmo et al. (1984) showed that the kinase and phosphatase activities could be active simultaneously in vitro. In the presence of ATP alone, assaying for ICDH phosphatase activity, ^{32}P was released from the substrate ^{32}P-phospho-ICDH. However, in these conditions the activity of the ICDH remained at a very low level. This indicated that both ICDH kinase and ICDH phosphatase were active under these conditions: the $^{32}P_i$ removed by the phosphatase was replaced by unlabelled phosphate from the ATP present in the incubation. The results of Borthwick et al. (1984b) showed that both activities can be active simultaneously in vivo: addition of $^{32}P_i$ to cells growing on acetate in which the ICDH was about 75% phosphorylated resulted in more rapid ^{32}P-labelling of the ICDH than could be accounted for by de novo protein synthesis. Nimmo et al. (1984) proposed that the phosphorylation state of ICDH in intact cells, grown on acetate, represents a steady state balance between the activities of ICDH kinase and ICDH phosphatase.

Figure 1.4 shows the control of the two activities by a range of metabolites. Metabolites which inhibit the kinase stimulate the phosphatase therefore resulting in increased sensitivity of the system (Nimmo and Nimmo, 1984). These workers proposed that the phosphorylation of ICDH by the kinase renders it rate limiting in the TCA cycle, resulting in an increase in the level of isocitrate in the cell. This rise in isocitrate levels allows isocitrate lyase to operate at an increased level, and allows efficient use of acetate carbon via the glyoxylate bypass (Nimmo and Nimmo, 1984).

Isocitrate lyase has been purified from E.coli ML308 and its kinetic properties studied (MacKintosh and Nimmo, 1988). Product and

dead-end inhibition studies showed that the enzyme obeyed a random-order equilibrium mechanism. It is inhibited by several biosynthetic precursors, notably: phosphoenolpyruvate, 2-oxoglutarate and 3-phosphoglycerate. Kinetic analysis, however, showed that in all cases these metabolites merely act as substrate analogues and that allosteric effects are not involved. Table 1.1 shows a comparison of K_m values for ICDH and ICL, taking into consideration the known inhibitors of ICL (Nimmo et al., 1987). The inactivation of ICDH by phosphorylation would thus enable the isocitrate levels within the cells to increase to allow flux through isocitrate lyase. El-Mansi et al. (1985) measured the levels of isocitrate within the cell for acetate and glycerol growth and their results agree with the theory of a rise in intracellular isocitrate levels as a result of ICDH phosphorylation.

Nimmo and his coworkers therefore studied regulation of the enzyme activities of the components of the system to reach a conclusion about the metabolic significance of ICDH phosphorylation. At the same time LaPorte et al. (1984) reported the results of a theoretical analysis of the fluxes through the two limbs emerging from a branchpoint as a function of the kinetic parameters of the two competing enzymes. They showed that the partitioning of flux was extremely sensitive to changes in the activity of one enzyme if its K_m for the common substrate was much lower than that of the competing enzyme. They suggested that the conditions apply to ICDH (low K_m for isocitrate) and isocitrate lyase (high K_m for isocitrate) and that the 'branchpoint effect' may contribute to the control of flux between the TCA cycle and the glyoxylate bypass in intact cells. Walsh and Koshland (1984) developed techniques that allowed them to determine the net rates of carbon flux through the central metabolic pathways during growth on acetate. They then studied the transition caused by the addition of glucose to acetate grown cells (Walsh and Koshland, 1985). This perturbation caused

dramatic decreases in the flux through isocitrate lyase and the intracellular content of isocitrate. These changes apparently resulted from a 4-fold increase in ICDH activity, caused by dephosphorylation, and a 5-fold decrease in the flux through citrate synthase.

Two groups, working independently, and using different approaches, thus came to the same conclusions concerning the metabolic significance of ICDH phosphorylation, namely that it serves as a regulation mechanism to optimise the division of flux between the TCA cycle and the glyoxylate bypass.

1.3 Comparison of the active and inactive forms of ICDH

The phosphorylated and non-phosphorylated forms of ICDH can be separated by non-denaturing gel electrophoresis. They differ in charge but not in size (Borthwick et al., 1984a). The purification procedure for the two forms of ICDH employed by Borthwick and his coworkers involve a chromatographic step using Procion-red sepharose. It is thought that triazine dyes bind to the nucleotide-binding sites of many enzymes. They obtained specific elution of active ICDH from the matrix by $NADP^+$, which was consistent with such an effect. However, Borthwick et al. (1984a) found that the inactive, phosphorylated, form of ICDH was unable to bind to the triazine dye. Garland and Nimmo (1984) later proposed that enzyme phosphorylation occurred at the $NADP^+$ binding site and as a result was unable to bind its cofactor $NADP^+$.

Garland and Nimmo (1984) looked at the differences between phosphorylated and non-phosphorylated ICDH using limited proteolysis and fluorimetry. Digestion with chymotrypsin and other proteases revealed that the inactive form was resistant to proteolysis but the active form was not. $NADP^+$ could protect the active form but not the inactive form. In the case of eukaryotic enzymes regulated by phosphorylation/dephosphorylation there is evidence from proteolysis experiments that the phosphorylation site is not part of the active site,

and proteolytic removal of the phosphorylated residue can occur which mimics the effects of dephosphorylation (e.g. Huang and Cabib, 1974). Phosphorylated ICDH did not conform to this pattern. Garland and Nimmo (1984) were unable to reactivate ICDH by proteolysis.

Garland and Nimmo (1984) showed that addition of NADPH to active ICDH resulted in an enhancement of the fluorescence of the coenzyme with no wavelength shift in its emission and excitation spectra. The addition of NADPH to inactive ICDH had no effect on either fluorescence yield or the emission and excitation spectra of the coenzyme. The conclusion was reached that NADPH bound tightly to active ICDH but appeared unable to bind to inactive ICDH (Garland and Nimmo, 1984).

The hypothesis proposed by Nimmo (1984) to explain the differences between the active and inactive forms of ICDH proposes that binding of $NADP^+$ to the active form of ICDH promotes a conformational change, as shown by proteolysis protection, and that phosphorylation occurs close to or at the $NADP^+$ binding site producing a similar conformational change. The introduction of a negatively charged phosphate group near the coenzyme binding site could prevent binding of NADP by charge repulsion (Fig.1.5). This also explains why phosphorylated ICDH cannot be reactivated by proteolysis. Removal of the phosphorylated serine would disrupt the active site of the enzyme.

Thorsness and Koshland (1987) used site-directed mutagenesis to change the phosphorylatable serine residue to a negatively charged amino acid, aspartate. They found that this inactivated the enzyme. However, they did not isolate the mutant ICDH and check its conformation. It is therefore possible that the mutant protein is inactive due to an inability to fold correctly. Thus their data do not prove conclusively that inactivation was as a result of the introduction of the negative charge.

The amino acid sequence round the phosphorylation site has been determined for ICDH from E.coli ML308 (Borthwick et al., 1984c) and E.coli K12 (Malloy et al., 1984). The phosphoserine is located in a unique 22-residue chymotryptic peptide, the first 14 residues of which have been sequenced. This was the first phosphorylation sequence reported for a bacterial protein kinase. The sequence is unlike that of sequences at eukaryotic phosphorylation sites.

If the theory of how phosphorylation affects ICDH activity proposed by Nimmo (1984), see Figure 1.5, is correct then E.coli provides a phosphorylation system whose mechanism is quite different from those involved in most other phosphorylation systems known, and provides a good basic model for further structural analysis of the protein.

1.4 Aims of thesis

This introduction has described how in E.coli ICDH activity is controlled by a reversible phosphorylation mechanism catalysed by the bifunctional enzyme ICDH kinase/phosphatase. The components of the system have already been well studied and a hypothesis proposed as to how phosphorylation affects ICDH activity (Fig.1.5). It remains to look at the structural and functional aspects of how phosphorylation affects ICDH activity. When I started my research in October 1985 my objectives were divided into three main areas:-

i) Active site studies

With the use of chemical modification of amino acid residues I hoped to be able to identify residues located at the active site of ICDH. In particular I wanted to identify any positively charged residues involved in $NADP^+$ binding and to investigate whether these interacted with the phosphate group in phospho-ICDH.

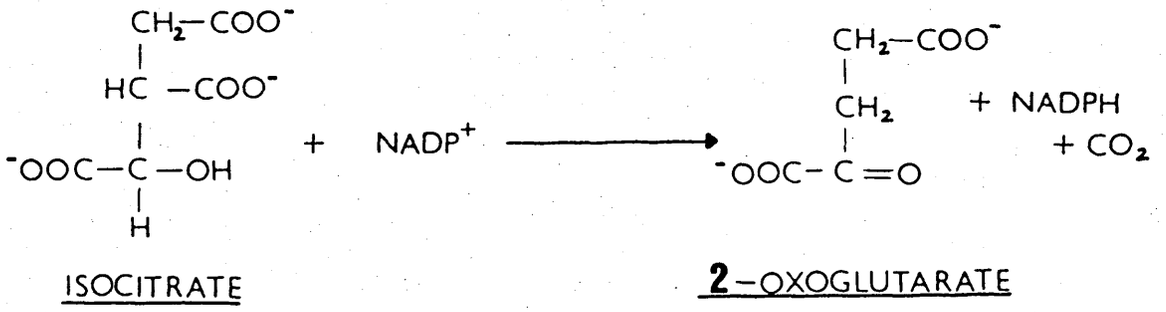
ii) Substrate recognition studies

What feature of ICDH does ICDH kinase recognise as a substrate and phosphorylate? In particular I intended to use synthetic peptides as trial substrates.

iii) Conformation studies

How is the conformation of ICDH affected by substrate binding and phosphorylation? I hoped to be able to detect and compare changes in the conformation of ICDH induced by binding of substrates or by phosphorylation.

Figure 1.1 Isocitrate dehydrogenase catalyses the oxidative decarboxylation of isocitrate to give 2-oxoglutarate



ISOCITRATE

2-OXOGLUTARATE

Figure 1.2 The TCA cycle and glyoxylate bypass in E.coli

1. Isocitrate lyase
2. Malate synthase A
3. Phosphoenolpyruvate carboxylase
4. Isocitrate dehydrogenase

→ represents routes to biosynthesis

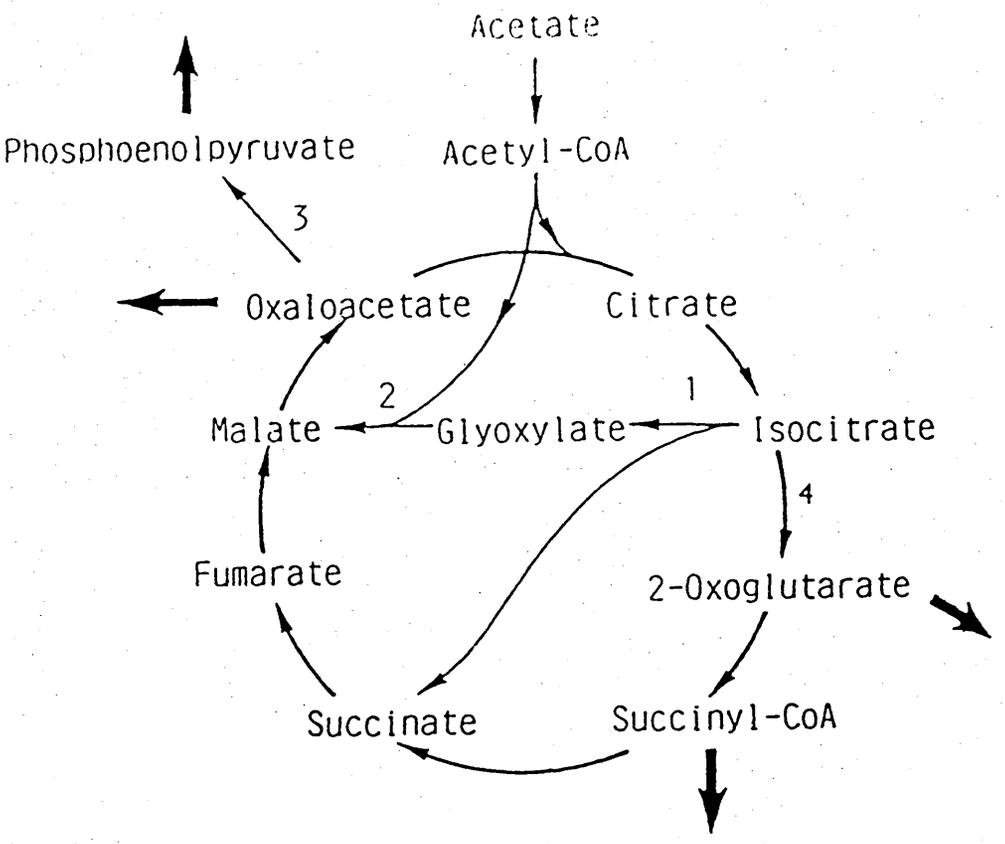

Acetate
Phosphoenolpyruvate
Acetyl-CoA
3
Oxaloacetate
Citrate
2
1
Malate
Glyoxylate
Isocitrate
4
Fumarate
2-Oxoglutarate
Succinate
Succinyl-CoA

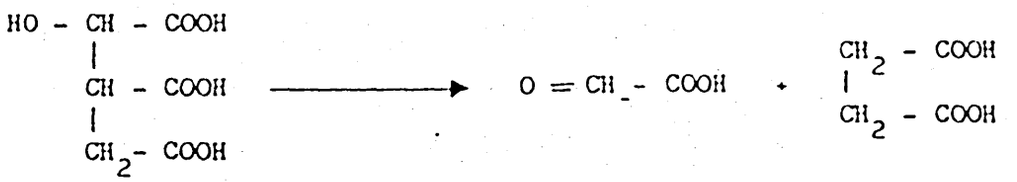
Figure 1.3

A ISOCITRATE LYASE catalyses the reversible aldol cleavage of isocitrate to form succinate and glyoxylate

B MALATE SYNTHASE catalyses the condensation of glyoxylate with acetyl CoA to form malate

C PHOSPHOENOLPYRUVATE CARBOXYLASE catalyses the carboxylation of phosphoenolpyruvate to form oxaloacetate

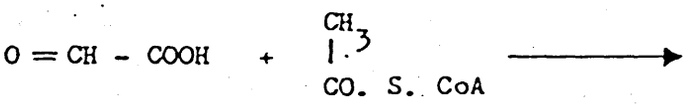
A



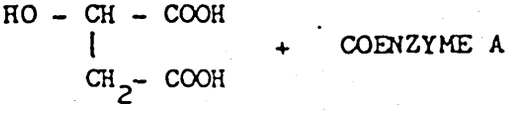
ISOCITRATE GLYOXYLATE SUCCINATE

ISOCITRATE LYASE

B



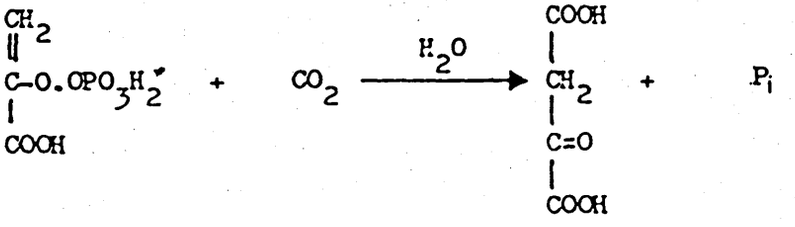
GLYOXYLATE ACETYL-COENZYME A



MALATE SYNTHASE

MALATE

C



phosphoenolpyruvate oxaloacetate

PHOSPHOENOLPYRUVATE CARBOXYLASE

Figure 1.4 Control of the phosphorylation state of isocitrate dehydrogenase

+ and − represent stimulatory and inhibitory effects respectively. Both ICDH kinase and ICDH phosphatase are associated with a single bifunctional protein. Figure from Nimmo (1984).

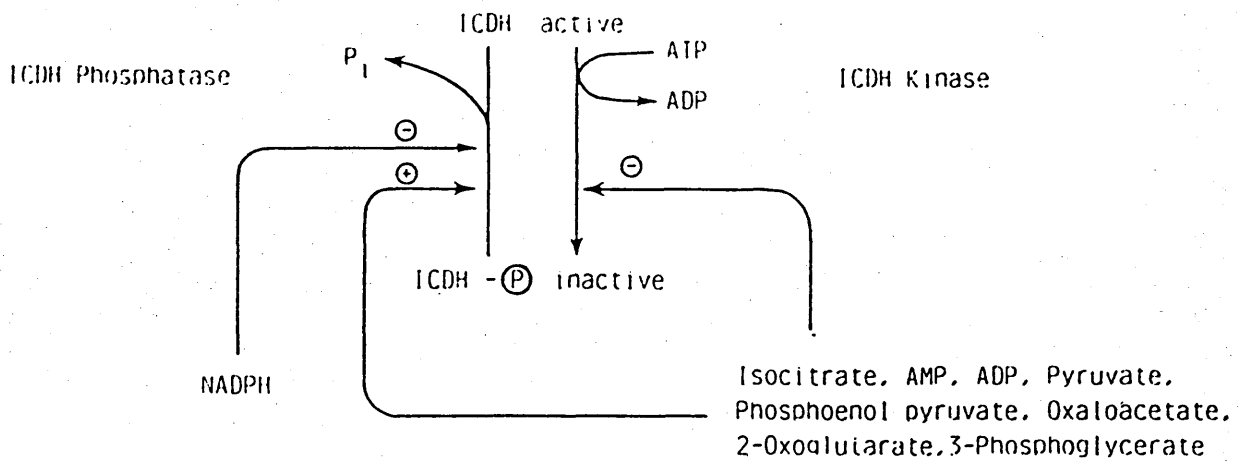

ICDH active
ICDH Phosphatase
P_i
ATP
ADP
ICDH Kinase
ICDH -Ⓟ inactive
NADPH
Isocitrate, AMP, ADP, Pyruvate,
Phosphoenol pyruvate, Oxaloacetate,
2-Oxoglutarate, 3-Phosphoglycerate

Figure 1.5 Hypothetical scheme for the inactivation of isocitrate dehydrogenase by phosphorylation

The hatched area represents the coenzyme binding site and the CH_2OH group is the side chain of the phosphorylated serine residue. The positive sign at the surface of the enzyme represents a positively charged group (or groups) that interact with the negatively charged phosphate groups of $NADP^+$. Taken from Nimmo (1984).

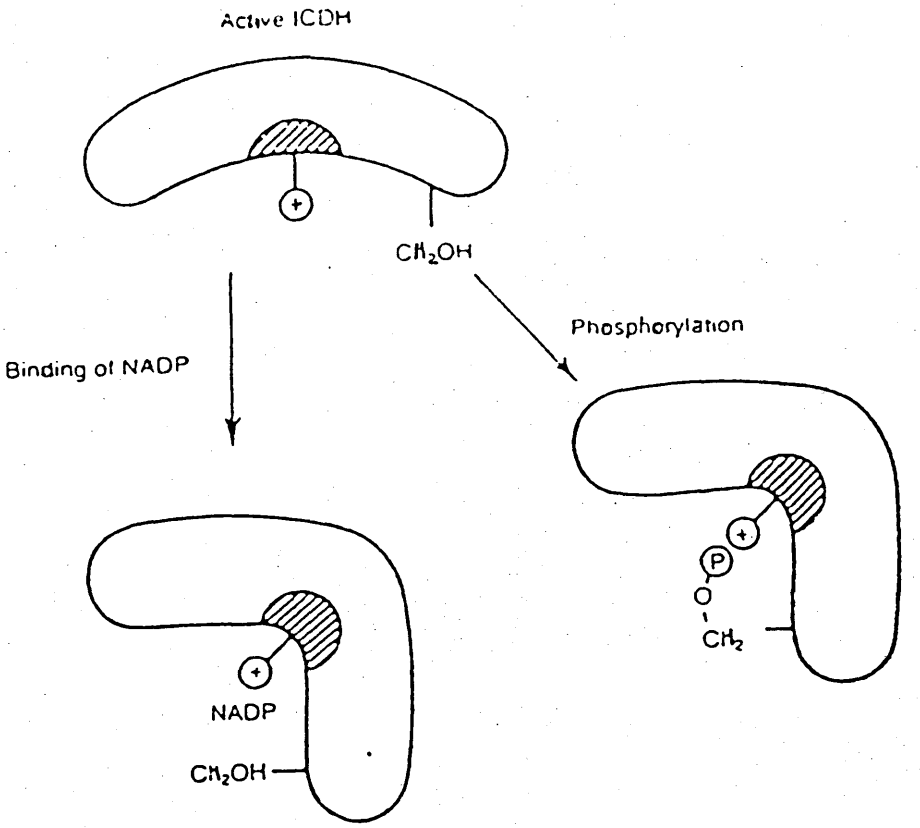

Active ICDH
CH_2OH
Binding of NADP
Phosphorylation
NADP
CH_2OH
P
O
CH_2

Table 1.1 Competition between isocitrate dehydrogenase and isocitrate lyase

Taken from Nimmo et al., 1987.

* at 40μM $NADP^+$ (Nimmo, 1986). The concentrations of isocitrate in E.coli ML308 growing on glycerol and acetate are $<$ 20μM and 0.57mM, respectively (El-Mansi et al., 1985).

ICDH - isocitrate dehydrogenase

ICL - isocitrate lyase

Table 1.1

	ICDH	ICL
Specific activity in acetate grown cells	0.9u/mg	0.8u/mg
K_m for D_s-isocitrate ignoring inhibitors	5μM*	63μM
Significant inhibitors	none	3-phosphoglycerate (K_i=0.8mM: intracellular conc. = 2.5mM) Sulphate (K_i=20mM: intracellular conc. unknown)
Estimated K_m for D_s - isocitrate in intact cells	5μM	260μM

Table 1.2 Phosphorylation sequences of selected eukaryotic protein kinases

(*Data from Pinna et al., 1986; ▲Data from Cohen, 1988)

Table 1.2

Kinase	Substrate	Phosphorylation sequence
cAMP dependent protein kinase▲	Glycogen synthase (1A)	Pro-Gln-Trp-Pro-Arg-Arg-Ala-Ser(P)-Cys-Thr-Ser
	Phosphorylase kinase (Beta)	Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser(P)-Val-Tyr-Glu
	Hormone Sensitive Lipase	Met-Arg-Arg-Ser(P)-Val-Ser-Glu
	Ribosomal protein S6	Ile-Ala-Lys-Arg-Arg-Arg-Leu-Ser(P)-Ser-Leu-Arg
Casein kinase 2▲	Troponin T	Ac -Ser(P),Asp-Glu-Glu-Val-Glu-His-Val-Glu-Glu-Glu
	Casein	Gln-Gln-Gln-Thr(P)-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His
Calmodulin dependent multiprotein kinase▲	Glycogen synthase (Rabbit muscle)	Pro-Leu-Ser-Arg-Thr-Leu-Ser(P)-Val-Ser-Ser-Leu
	Tyrosine hydroxylase (Pheochromacytoma)	Lys-Gly-Phe-Arg-Arg-Ala-Val-Ser(P)-Glu-Gln-Asp-Ala
Tyrosine protein kinase*	(Synthetic peptide)	Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr(P)-Thr-Ala-Arg
Protein kinase C*	(Synthetic peptide)	Gly-Ser(P)-Arg-Arg-Arg-Arg-Arg-Arg-Tyr
	(Synthetic peptide)	Arg-Arg-Leu-Ser(P)-Ser(P)-Leu-Arg-Ala

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Biochemicals

Ammonium sulphate (enzyme grade), glycine, hydrogen peroxide methionine, D,L-norleucine and urea were from BDH Chemicals, Poole, Dorset, U.K.

Ampicillin, Benzamidine, N,N-bis (2-hydroxyethyl) glycine (Bicine), Bromophenol blue, 2,3-butanedione, DL-isocitrate (trisodium salt), morpholino propane sulphonic acid (MOPS), naladixic acid, ninhydrin, 2-oxoglutarate, phenylglyoxal, phenylmethylsulphonyl fluoride (PMSF), Protamine sulphate (salmon, grade II), silver nitrate, sodium borohydride and tris-(hydroxymethyl) aminomethane (Tris) were from Sigma (London) Chemical Company, Poole, Dorset, U.K. Adenosine 5' diphosphate (ADP), adenosine 5' triphosphate (ATP), nicotinamide adenine dinucleotide phosphate ($NADP^+$), phenazine methosulphate, phosphoenol pyruvate (PEP) and tetranitro blue tetrazolium were from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K.

Dithiothreitol was from Koch Light Laboratories, Colnbrook, Berks, U.K.

1,2-cyclohexanedione was from Aldrich Chemical Company, Ltd., Gillingham, Dorset, U.K.

2.1.2 Enzymes

Protein kinase catalytic subunit (Bovine heart) was from Sigma (London) Chemical Company, Poole, Dorset, U.K. Alpha-chymotrypsin, pepsin and *Bacillus thermoproteolyticus* thermolysin were from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

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

Purified preparations of ICDH kinase/phosphatase (Nimmo *et al.*, 1984) were gifts from Dr. H.G. Nimmo.

2.1.3 Radiochemicals

All radiochemicals were from Amersham International, Bucks, U.K. and were as listed below:

Adenosine 5'-[gamma-^{32}P]-triphosphate (3000Ci/mmol)

[7-^{14}C]-phenylglyoxal (25mCi/mmol).

2.1.4 Chemicals for polyacrylamide gel electrophoresis

Acrylamide monomer, ammonium persulphate, 2-mercaptoethanol, N,N' methylenebisacrylamide, sodium dodecylsulphate (SDS), and N,N,N'N' tetramethyl ethylenediamine (TEMED) were 'analar' grade materials from BDH Chemicals, Poole, Dorset, U.K.

2.1.5 Chromatographic media and reagents

Silica gel thin layer chromatography plates were from Machercy-Nagel and Company, West Germany. Sephadex G50, Sephadex G25 and Mono Q ion exchange pre-packed column were from Pharmacia, Milton Keynes, Bucks, U.K.

Chromatography paper (3MM) and diethylaminoethyl (DEAE) cellulose (microgranular form, DE52) were from Whatman Biochemicals, Maidstone, Kent, U.K. Anion exchange resin AG1-X8 (100-200 mesh) was from Bio-Rad Laboratories, Richmond, California, U.S.A.

Procion red sepharose was prepared by Dr. H.G. Nimmo.

2.1.6 Miscellaneous Materials

Vitamin B_1 was from Fluka AG, Bucks S.G. Switzerland. Bovine serum albumin, coomassie brilliant blue G250, kemptide, low M_r standard proteins for SDS PAGE, lubrol PX and Tween 20 were from Sigma (London) Chemical Company, Poole, Dorset, U.K.

Chymostatin was from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K.

Nutrient broth (CM1) and Nutrient agar No.1 (CM3) were from Oxoid Ltd., Basingstoke, Hants, U.K.

Bactotryptone and yeast extract were from Difco Laboratories, Detroit, Michigan, U.S.A.

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

All other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of the highest grade obtainable.

2.2 General biochemical methods

2.2.1 pH and conductivity measurements

All pH measurements were made with a Radiometer pH probe calibrated at room temperature using standards of pH7 and pH4 prepared from tablets obtained from the manufacturer.

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

2.2.2 Glassware and plastics

Glassware was washed with 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 were used from a newly opened bag.

2.2.3 Micropipettes

For dispensing volumes of 5µl-1ml adjustable 'Finnpipettes' (Finnpipette Ky, Pulti 9, SF-00810, Helsinki 81, Finland) were used. Volumes of 0.5µl-5µl were dispensed using micro-syringes (Scientific Glass Engineering Pty Ltd., North Melbourne, Australia 3051).

2.2.4 Distilled water

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

2.2.5 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.

2.2.6 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 100ml of 85% (w/v) orthophosphoric acid. The reagent was made up to 1 litre with distilled water and filtered. For each set of assays, a standard curve in the range 0-25µg protein was obtained using a 1mg/ml BSA solution which was made up assuming a 1mg/ml BSA solution has an absorbance of 0.62 at 280nm. To each sample 2.5mls of Bradford Reagent was added, the samples vortexed and allowed to stand for 2 minutes. 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 elicited from columns were monitored at 280nm.

2.2.7 Concentration of protein samples

Samples of greater than 10ml were concentrated by vacuum dialysis. Smaller volumes were concentrated using Centricon 30 micro-concentrators (Amicon Ltd., Stonehouse, U.K.).

2.2.8 Preparation of chromatographic media

Sephadex G-25 and G-50 were swollen and poured according to the manufacturers instructions.

DEAE-cellulose (Whatman DE-52) was obtained pre-swollen. It was resuspended in starting buffer, and the pH adjusted to the correct value before equilibration in starting buffer. Procion-red Sepharose was suspended in starting buffer. After use it was washed with either 6M Guanidinium Chloride or 8M urea. Anion exchange resin AG1-X8 (Bio-Rad, Richmond, USA) was suspended in starting buffer and discarded after use.

All columns were poured at room temperature, stored in 0.02% (w/v) sodium azide and equilibrated with starting buffer before use.

2.2.9 Lyophilisation

Samples were frozen in a suitable vessel by dipping into a methanol/dry ice mixture. Tops were sealed with Nescofilm punctured with a needle. Samples were placed in a dessicator connected to a Flexi-dry (FTS Systems Inc., Stone Ridge, N.Y., USA) and vacuum maintained by a high vacuum pump (Javac Pty., Ltd., U.K.)

2.2.10 Sterilisation

Solutions for the preparation of growth media were autoclaved in a Manlove-Alliot Autoclave supplied with steam from a Speedlec-electrode boiler. Media were autoclaved 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 0.22μm pore-sized Millex G.V. filters (Millipore Ltd., U.K.) into sterile bottles: ampicillin, methionine, vitamin B_1 and naladixic acid.

2.3 Microbiological techniques

2.3.1 Bacterial strains

Escherichia coli ML308 (ATCC 15224) was originally obtained from the American Type Culture Collection (Rockville, Maryland, USA). It has been maintained in Glasgow for twenty years. It has the genetic configuration $i^-z^+y^+a^+$ for the lac operon. Because defective repressor is produced, synthesis of the products of the operon (beta-galactosidase, beta-galactosidase permease and thiogalactoside transferase) is constitutive. The identity of the organism can therefore be checked by assaying for beta-galactosidase which is present during growth on all media. In all other respects the strain is regarded as wild type.

Escherichia coli strains LE392 (a K12 derivative) and KAT-1 (an ace B^+ ace A^- mutant) derived from LE392 by transposon Tn10 insertion (Maloy and Nunn, 1982) were from the late Dr. W.D. Nunn (University of California, Irvine, USA). The plasmid bearing strain KAT-1/pEM9 was

constructed by Dr. E.M.T. El-Mansi (El-Mansi et al., 1987). Growth of this strain is described in Methods 2.3.4.

Escherichia coli EB106 was obtained from the E.coli genetic stock centre (Yale Univ., New Haven, USA). It has the genetic configuration icd$^-$, dad R1$^-$, trp A62, trp E61, tna S, lambda$^-$ (Apostolakus et al., 1982). Growth of this strain is described in Methods 2.3.4 (e).

Escherichia coli TG1/pCK505 a K12 derivative (lac pro, Sup E, thi, hsd D5/F^1, tra D36, pro A$^+$B$^+$, lac I^q, lac z, M15) contains a plasmid carrying the ICDH gene. It was constructed by Dr. K. Duncan. The original strain E.coli TG1 was from Anglian Biotechnology Ltd., Hawkins Road, Colchester, Essex and the plasmid pCK505 from D.E. Koshlands laboratory, University of California, Berkeley, California.

Growth of this strain is described in Methods 2.3.4 (f).

2.3.2 Storage of bacteria

Organisms were maintained in nutrient broth, stored at 4^{o}C. Subcultures were made at 6 to 12 monthly intervals. Samples were checked for homogeneity microscopically and in the case of E.coli ML308, by plating out on nutrient agar containing 10µg/ml 5-bromo-4-chloro-indoxy -beta-galactoside (BCIG agar). A cell constitutive for beta-galactosidase produces a blue colony on BCIG agar so a homogenous culture of lac-constitutive cells produces only blue clones.

2.3.3 Media

(a) Nutrient broth was prepared from Oxoid dehydrated granules dispensed in 10ml aliquots into Universals and sterilised by autoclaving at 15 p.s.i.

(b) Nutrient agar. 15g agar was dissolved in 1 litre nutrient broth, sterilised at 15 p.s.i., poured into petri dishes in a filtered air cabinet and allowed to solidify. Plates were stored at 4^{o}C.

(c) BCIG agar. BCIG (2mg/ml in dimethylformamide) was added to nutrient agar to a final concentration of 10µg/ml. Plates were stored at 4°C.

(d) L-broth. 10g bactotryptone, 5g yeast extract and 10g NaCl were dissolved in 1 litre of distilled water pH to 7.5. Solution was dispensed in the volumes necessary and sterilised by autoclaving at 15 p.s.i.

(e) Defined media for specifically pre-inducing inocula. This was prepared by mixing set volumes of the following three components.

i) PNS - 66.7mM potassium dihydrogen phosphate (9.06g/litre), 16.7mM ammonium sulphate (2.2g/litre) adjusted to pH7.0 with 5N sodium hydroxide. This was dispensed in 60ml volumes in 250ml conical flasks and autoclaved at 15 p.s.i.

ii) $FeSO_4$ - 0.8mM ferrous sulphate (0.22g/litre $FeSO_4.7H_2O$) adjusted to pH2 with HCl and autoclaved at 15 p.s.i.

iii) Carbon source - 2.5 times final concentration required plus 1.25mM magnesium sulphate (0.3g/litre $MgSO_4.7H_2O$).

Dispensed into 40ml volumes and autoclaved at 15 p.s.i. The complete media were prepared by adding 40ml carbon source to 1.25ml $FeSO_4$ and 60ml PNS.

(f) Defined media for large-scale growth

This was prepared by mixing set volumes of the following 4 components.

i) 3850ml of 67mM potassium dihydrogen phosphate (9.12g/litre KH_2PO_4) adjusted to pH7 with KOH, autoclaved at 15 p.s.i. in a 10 litre round bottom flask with a plugged 10ml pipette inserted through a cotton wool bung.

ii) MgNS contained 40mM magnesium sulphate (9.84g/litre $MgSO_4 7H_2O$ and 800mM ammonium sulphate (105.6g/litre $(NH_4)_2SO_4$), autoclaved in 50ml volumes at 15 p.s.i.

iii) $FeSO_4$ 0.8mM ferrous sulphate (0.22g/litre $FeSO_4.7H_2O$) adjusted to pH2 with HCl, autoclaved in 50ml volumes at 15 p.s.i.

iv) Carbon source 3.2M sodium acetate or 1.6M glycerol, autoclaved in 50ml volumes at 5 p.s.i. The complete media were prepared by adding 50ml of each MgNS, $FeSO_4$ and Carbon source to potassium dihydrogen phosphate, to give 4000ml.

(g) Specific supplements to defined media

E.coli strain KAT-1/pEM9 required addition of Vitamin B1 (to 1µg/ml from a 0.5mg/ml stock solution), methionine (to 50µg/ml from a 25mg/ml stock solution) and ampicillin (to 100µg/ml from a 10mg/ml, pH7 stock solution).

2.3.4 Growth of bacteria

(a) Preparation of specifically pre-induced inocula

100ml of complete defined medium (Methods 2.3.3 (e) and 2.3.3.(g)) was inoculated with 0.5ml of a nutrient broth culture and grown on an orbital shaker at 37°C. After overnight growth 1ml of this culture was inoculated into another 100ml of identical medium (2nd passage). A third passage was also grown in this way and could be stored for upto 24 hours at 4°C before use. Growth times depended on the carbon source but were generally overnight and growth was stopped in mid log phase.

(b) Large-scale batch culture

Four 4000ml flasks of defined media (Methods 2.3.3 (f) and 2.3.3.(g)), at 37°C were inoculated with third passage cultures to the required cell density. Cultures were grown at 37°C on a 4-place stirrer (Harvey et al., 1968). Stirring magnets were turned, by horse-shoe magnets under the flasks, fast enough to break the vortex in the flask causing bubbles to form. Compressed air was passed through a cotton-wool plug at a rate of 400ml/min.

(c) Measurement of growth

Bacterial cell density was measured as an apparent absorption at 420nm. The measurements were carried out in 1cm 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 A_{420} of less than 0.5.

(d) Harvesting of cells

Cells were harvested by centrifugation at 6000g for 15 min at 4°C (MSE Mistral 6L with a 6x750ml rotor). To harvest 16 litres of culture, the same six centrifuge pots were used for 5 runs. The accumulated pellets were resuspended in chilled Buffer A (Methods 2.5.1 (a)) and then centrifuged at 30,000g for 10 min at 4°C (MSE18 with an 8x50ml rotor). The wet weight of the pellets was recorded and the cells stored at -20°C.

(e) Growth of E.coli EB106

Eight 2500ml flasks of L-broth at 30°C containing 1% glucose and 10µg/ml naladixic acid in a final volume of 500ml were inoculated with 10ml of an overnight L-broth culture of E.coli EB106. Cultures were grown at 30°C on an orbital shaker. Growth was monitored and cells harvested as before.

(f) Growth of E.coli TG1/pCK505

Eight 2500ml flasks of L-broth at 30°C containing 0.1mg/ml ampicillin in a final volume of 500ml were inoculated with 5ml of an overnight L-broth culture of E.coli TG1/pCK505. Cultures were grown at 30°C on an orbital shaker. Growth was monitored and cells harvested as before.

2.3.5 Disruption of bacteria

The cell pellets (Methods 2.4.4 (d)) were resuspended in two volumes of Buffer A (Methods 2.5.1 (a)) at 4°C and disrupted by two passages through the French pressure cell (Cat. No.4-3398A, American Instruments Company, Maryland, USA) at a pressure of 98 MPa (14,300 lb/in^2. The pressure cell, capacity 40ml, was cooled in ice before use.

2.4 Enzyme Assays

2.4.1 Instrumentation

All spectrophotometric assays were carried out at 37°C using a thermostatted Unicam SP500 spectrophotometer (Pye Unicam Instruments Ltd., U.K.) equipped with a Gilford photoelectric detector and chart recorder. Quartz cuvettes, 1ml, with a path length of 1cm were used. Assay mixes were allowed to equilibrate then the component to initiate the reaction was added and mixed in.

One unit of enzyme activity is defined as the amount of enzyme required to catalyse the disappearance of one micromole of substrate, or the appearance of one micromole of product per minute.

2.4.2 Assays

(a) Isocitrate dehydrogenase

ICDH was assayed by monitoring the reduction of $NADP^+$ at 340nm (Borthwick et al., 1984a). Each cuvette contained in a final volume of 1ml : 0.15M Tris/HCl pH7.5, 0.5mM $MnCl_2$, 0.4mM $NADP^+$, 2.5mM D.L-isocitrate and enzyme. The reaction was initiated by the addition of enzyme.

(b) Isocitrate dehydrogenase kinase

ICDH kinase activity was assayed by measuring the rate of inactivation of ICDH (Nimmo et al., 1984). 15µl of assay 'cocktail' (25mM Bicine-NaOH pH9.0, 10mM ATP, 20mM $MgCl_2$, 1mM DTT, 6.0µM active ICDH, 2% v/v glycerol) was incubated at 37°C with 15µl ICDH kinase

diluted in 50mM MOPS-NaOH pH7.3, 1mM EDTA, 1mM DTT, 1µl samples of the incubation were withdrawn at intervals and assayed for ICDH activity.

The amount of kinase was adjusted so that the inactivation of ICDH was linear with time for at least 10 min. 1mU kinase is the amount of enzyme required to inactivate 1nmol ICDH subunits/min.

(c) Isocitrate dehydrogenase phosphatase

ICDH phosphatase was assayed by monitoring the release of ^{32}P from ICDH (Nimmo et al., 1984) that had been phosphorylated in vitro using [gamma-^{32}P] ATP (see later). The following components were incubated in microfuge tubes at 37°C in a final volume of 40µl: 50mM MOPS-NaOH pH6.7, 12.5mM $MgCl_2$, 0.5mM ATP, 0.5mM phosphoenolpyruvate, 5% (v/v) glycerol and ICDH phosphatase. The reactions were started by the addition of ^{32}P-ICDH. After a set time (5-30 min) the reactions were terminated by the addition of 10µl 100mg/ml BSA and 0.2ml of 5% (w/v) TCA. After 10 min at 0°C the tubes were centrifuged at 12,000g for 2 min and 0.2ml portions of the supernatant were counted in 2ml portions of Ecoscint (National Diagnostics, Somerville, New Jersey, USA) in a Beckman LS8100 scintillation counter. Dephosphorylation of ICDH was linear with time provided less than 30% of the ^{32}P was released. 1mU ICDH phosphatase catalyses the release of 1nmol of phosphate from phosphorylated ICDH/min.

ICDH phosphatase was also assayed in some experiments by measuring reactivation of phosphorylated ICDH. The incubation was as above and 1µl samples were withdrawn and assayed for ICDH activity.

^{32}P-ICDH was prepared as described by Nimmo et al. (1984). The following were incubated at 37°C: 32mM Bicine-NaOH pH9.0, 0.64mM DTT, 15.5µM active ICDH, 10mM $MgCl_2$, 0.5mM [gamma-^{32}P] ATP (50-100Ci/mol) and 0.25-0.50mU/ml ICDH kinase. The reaction was started by the addition of kinase and continued until the ICDH activity had fallen to less than 0.5% of the original value. The reaction was stopped by the addition of EDTA to a final concentration of 10mM. The

^{32}P-ICDH was separated from ATP by gel filtration at room temperature through a column of Sephadex G-50 (3cmx15cm) equilibrated in MOPS-NaOH pH7.3, 1mM EDTA, 1mM DTT. Protein was dialysed overnight against 50mM MOPS-NaOH pH7.3, 1mM EDTA, 1mM DTT, 40% (v/v) glycerol and stored at -20°C.

2.5 Methods of protein purification

2.5.1 Purification buffers

(a) ICDH, ICDH from E.coli EB106

Buffer A: 10mM potassium phosphate, 0.5M potassium chloride 2mM magnesium chloride pH6.5

Buffer B_1: 40mM potassium phosphate, 5mM potassium citrate, 2mM magnesium chloride pH6.5

Buffer B_2: 100mM potassium phosphate 5mM potassium citrate, 2mM magnesium chloride pH6.5

Buffer C: 10mM potassium phosphate, 5mM potassium citrate, 2mM magnesium chloride pH6.5 containing 10% (v/v) glycerol)

Buffer D: 10mM potassium phosphate, 5mM potassium citrate, 2mM magnesium chloride pH6.5 containing 40% (v/v) glycerol

(b) ICDH kinase/phosphatase

Buffer E: 10mM potassium phosphate, 0.5M potassium chloride pH6.5, 2mM magnesium chloride, 1mM benzamidine, 1mM DTT, 1.2mM PMSF

Buffer F: 50mM MOPS-NaOH pH7.3, 1mM EDTA, 1mM benzamidine, 1mM DTT, 1.2mM PMSF

Benzamidine and DTT were stored as 1M solutions at -20°C and added just before use. PMSF was dissolved in ethanol (20mg/ml) and added just before use.

2.5.2 Purification of isocitrate dehydrogenase from E.coli ML308

Active ICDH was purified from glycerol-grown cells by the method of Borthwick et al. (1984a). Steps a-c were carried out at 0-4°C, the rest at room temperature.

(a) Preparation of crude extract

20-30g E.coli ML308 grown to the end of log phase and harvested as described previously (Methods 2.3.4 (b) and 2,3,4 (d)) were passed through the French pressure cell twice (Methods 2.3.5) in two volumes Buffer A. The resulting material was centrifuged at 40,000g for 20 min at 4°C (MSE 18 with an 8x50ml rotor) and the supernatant was decanted through glasswool to give the crude extract.

(b) Protamine sulphate treatment

Protamine sulphate (100mg/ml in Buffer A) was added dropwise with stirring to give a ratio of 0.3mg protamine sulphate per mg protein. The suspension was stirred for 15 min and centrifuged at 40,000g for 10 min. The supernatant was retained.

(c) Ammonium sulphate fractionation

Powdered $(NH_4)_2SO_4$ was gradually added to the supernatant to give 50% saturation (312g/litre) while the pH of the solution was maintained in the range pH6.3-6.5 by the addition of 5M NH_3. The solution was stirred for 30 min and centrifuged at 40,000g for 10 min, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation (65g/litre) and the solution was stirred and centrifuged as before; the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to give 75% saturation (103g/litre) and the solution was stirred and centrifuged as before. The pellet was retained and dissolved in a small volume of Buffer B_1.

(d) Ion-exchange chromatography

The dissolved pellet was desalted into Buffer B_1 on a column of Sephadex G-25. The material was then loaded onto a column of DEAE-cellulose (1x10cm) equilibrated in Buffer B_1. The column was washed with this buffer at a flow rate of 100ml/hour until the A_{280} readings of the effluent were lower than 0.1. The column was then washed with Buffer B_2. ICDH activity was eluted in a sharp peak and

fractions with an activity greater than 10 units/ml were pooled and dialysed overnight against Buffer C.

(e) Procion-red Sepharose chromatography

The dialysed pool was loaded onto a column of Procion-red Sepharose (1x10cm) equilibrated in Buffer C at a flow rate of 48ml/hour. The column was washed exhaustively with Buffer C until the absorbance at 280nm of the effluent was below 0.05. The column was then washed with Buffer C containing 0.5mM $NADP^+$. The ICDH activity was eluted as a sharp peak with the $NADP^+$ front; fractions containing an activity of more than 5 units/ml were pooled, concentrated by vacuum dialysis and dialysed against Buffer D.

The whole procedure was completed in 48 hours. The enzyme was stored in Buffer D at -20^oC.

2.5.3 Purification of ICDH kinase/phosphatase from E.coli KAT-1/pEM9

The purification scheme was developed by Dr. H.G. Nimmo and I. Varela and was based on that devised for purification of ICDH kinase/phosphatase from E.coli ML308 (Nimmo et al., 1984, and Varela, I. & Nimmo H.G. 1988).

(a) Preparation of crude extract

20-30g of E.coli KAT-1/pEM9 grown on acetate to the end of log phase were passed through the French Pressure cell twice (Methods 2.3.5), in two volumes Buffer E. The resulting material was centrifuged at 40,000g for 20 min at 4^oC (MSE 18 with an 8x50ml rotor) and the supernatant was decanted through glass wool to give the crude extract.

(b) Protamine sulphate treatment

This was as described in 2.5.2 (b).

(c) Ammonium sulphate fractionation

The fraction precipitating between 30 and 45% was collected as described in 2.5.2 (c). The precipitate was redissolved in a small volume of Buffer F containing 150mM NaCl. It was desalted into this buffer by gel filtration on a column of Sephadex G-25 (2.2x50cm).

(d) Ion exchange chromatography at pH7.3

The desalted material was loaded onto a column of DEAE-cellulose (3.3x6cm) equilibrated in the same buffer. The column was washed with this buffer until the A_{280} of the eluate was zero. The enzyme was then eluted with Buffer F containing 250mM NaCl. The flow rate was 200ml/hr and fractions of 10ml were collected and assayed for ICDH kinase activity. The active fractions were pooled and dialysed overnight into Buffer F containing 100mM NaCl.

(e) Chromatography on blue dextran Sepharose

The dialysed material was loaded onto a column of blue dextran Sepharose (2.2x12cm) equilibrated in Buffer F containing 100mM NaCl. The column was washed with this buffer until the A_{280} of the eluate was zero and was then washed in the same buffer containing 1mM NAD^+. The enzyme was then eluted with Buffer F containing 400mM NaCl. The flow rate was 60ml/hr and 5ml fractions were collected. A solution of 10% (w/v) Lubrol PX was added to the fractions to give a final concentration of 0.05% (w/v). The fractions were assayed for ICDH kinase, the active fractions were pooled and concentrated by vacuum dialysis to approximately 1ml.

(f) Ion-exchange chromatography at pH7.3 on Mono Q

The concentrated material was diluted with 50mM MOPS-NaOH 1mM EDTA containing 1mM benzamidine, 1mM DTT, 1.2mM PMSF and 0.05% (w/v) lubrol to reduce the NaCl concentration to 100mM and loaded onto an FPLC Mono Q column in Buffer F containing 0.05% (w/v) Lubrol PX. The flow rate was 1ml/min and 0.5ml fractions were collected. The column was developed with increasing concentrations of NaCl in Buffer F: 0-0.23M over 4 min, then 0.23-0.39M over the next 20 min. ICDH kinase/phosphatase was eluted at a NaCl concentration of approx 0.27M. The most active fractions were pooled.

(g) Ion-exchange chromatography at pH9.0 on Mono Q

The pool fractions were diluted 1 in 4 in 50mM Bicine-NaOH pH9.0, 0.05% (w/v) lubrol PX and were loaded on to a Mono Q column equilibrated in this buffer containing 100mM NaCl. The column was washed in this buffer and then developed with a linear gradient of 100-400mM NaCl over 30 min. The flow rate was 1ml/min and 0.5ml fractions were collected. The pH of the most active fractions was reduced to pH7.3 by the addition of 200µl of 0.5M MOPS-NaOH pH7.3. These fractions were pooled and concentrated to 200µl.

(h) Gel filtration on an FPLC Superose 12 column

The concentrated material was loaded onto a Superose 12 column equilibrated in Buffer F containing 0.05% (w/v) lubrol PX. The flow rate was 0.3ml/min and 1 min fractions were collected. Active fractions were pooled and dialysed into 50mM MOPS-NaOH pH7.3, 1mM EDTA, 1mM DTT, 1mM benzamidine hydrochloride 0.05% (w/v) lubrol PX 40% glycerol and stored at -20°C.

2.5.4 Purification of ICDH from E.coli TG1/pCK505

ICDH was purified from E.coli TG1/pCK505 grown on L-broth. The method used was adapted from the purification of ICDH from E.coli ML308 (Method 2.5.2). Steps a-c were carried out at 0-4°C, step d at room temperature.

(a) Preparation of crude extract

A crude extract of E.coli TG1/pCK505 was prepared as in 2.5.2(a).

(b) Protamine sulphate treatment

This was as described in 2.5.2(b).

(c) Ammonium sulphate fractionation

This was as described in 2.5.2(c).

(d) Ion exchange chromatography at pH6.5 on Mono Q

The dissolved pellet was desalted into Buffer B_1 on a column of Sephadex G-25. The material was then dialysed overnight against 20mM potassium phosphate, 5mM potassium citrate, 2mM $MgCl_2$ pH6.5. The dialysed material was loaded onto an FPLC Mono Q column (8ml) equilibrated in 20mM potassium phosphate, 5mM potassium citrate, 2mM $MgCl_2$ pH6.5. The flow rate was 4ml/min and 4ml fractions were collected. The column was washed in this buffer and then developed with a linear gradient of 0-100% 200mM potassium phosphate, 5mM potassium citrate, 2mM $MgCl_2$ pH6.5 over 25 min. ICDH was eluted at a phosphate concentration of approximately 120mM. The most active fractions were pooled and dialysed into Buffer D and stored at -20°C.

2.6 Polyacrylamide gel electrophoresis techniques

2.6.1 Non-denaturing polyacrylamide gel electrophoresis

This was carried out on 7% (w/v) polyacrylamide gels at pH8.0 (Davis, 1964).

The following stock solutions were stored at 4°C:

A 36.3g Tris
0.23ml TEMED
48ml 1M HCl
H_2O to 100ml

B 28g acrylamide
0.74g N,N'-methylenebisacrylamide (bis)
H_2O to 100ml

C 30g Tris
144g glycine
H_2O to 1000ml

'Gel monomer' was prepared by mixing 1 volume A, 2 volumes B and 1 volume H_2O. An equal volume of freshly prepared ammonium

persulphate (1.4mg/ml) was prepared. Solutions were degassed separately and then mixed and poured into gel tubes (dimensions 0.5x10cm). 9cm gels were poured, carefully overlayed with H_2O and allowed to set. The tank buffer was solution C which had been diluted 25-fold with H_2O at 4°C containing 0.1% (v/v) 2-mercaptoethanol. The gels were pre-electrophoresed for 30 min at 3mA per tube at 4°C. 5µl of tracking dye (0.01% (w/v) bromophenol blue in 20% (v/v) glycerol) was layered onto the top of each gel and electrophoresis was carried out until the dye had penetrated the gel. The samples (less than 50µl in 20% v/v glycerol) were loaded onto the gels and electrophoresis was performed at 3mA per tube at 4°C until the tracking dye approached the bottom of the gel. The dye front on each gel was marked with a piece of wire inserted through the gel.

2.6.2 Staining for ICDH activity in non-denaturing gels

Gels to be stained for ICDH activity were soaked in 0.15M Tris-HCl pH7.5, 0.5mM $MnCl_2$ for 30 min to remove 2-mercaptoethanol. The gels were then incubated in a reaction mixture containing 0.15M Tris-HCl pH7.5, 2.5mM D,L-isocitrate, 0.4mM $NADP^+$, 0.5mM $MnCl_2$, 0.24mM tetranitro blue tetrazolium and 78µM phenazine methosulphate. ICDH activity was visualised by the production of a blue precipitate of Formazan. The reaction was stopped by storing the gel in the destaining solution described in Methods 2.6.4 (a).

2.6.3 SDS Polyacrylamide gel electrophoresis (discontinuous system)

Protein samples were analysed by discontinuous slab gel electrophoresis in the presence of SDS according to Laemmli (1970). Glass plates were separated by 0.8mm or 1.5mm spacers and sealed with 1% (w/v) agarose.

Gels were prepared by mixing the appropriate volumes shown by Table 2.1. The acrylamide Tris and H_2O were mixed and degassed. SDS, ammonium persulphate and TEMED were then added and the mixture was

poured into the cast immediately. Isopropanol was layered onto the separating gel and rinsed off after polymerisation. Then a stacking gel was made up and poured around a well-forming template. The wells were rinsed after polymerisation. Electrophoresis buffer consisted of 3g Tris, 14.4g glycine and 0.1% (w/v) SDS per litre. Samples were denatured by addition of at least equal volumes of 'sample buffer' which comprised 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, and boiled for 2 minutes. The samples were then loaded into the wells and electrophoresis was carried out at a constant current of 50mA until the tracking dye reached the bottom of the gel.

2.6.4 Staining gels for protein

(a) Coomassie blue

Routinely, gels were stained in 0.1% (w/v) Coomassie Brilliant Blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid for 1 hour at 45^{o}C and destained in several changes of 10% (v/v) methanol, 10% (v/v) acetic acid at 45^{o}C.

(b) Silver staining

A more sensitive method used to locate protein on gels was silver staining, based on the method of Wray et al. (1981). Gels were soaked for at least 24 hours in 3 changes of 50% (v/v) methanol. Staining solution was prepared by adding Solution A (0.8g $AgNO_3$ in 4 ml H_2O) dropwise to Solution B (1.4ml 14.8M NH_4OH plus 21ml 0.36% (w/v) NaOH) with vigorous shaking. Distilled water was added to give a final volume of 100ml. The gel was gently agitated in staining solution for 8 minutes and then rinsed for 1 hour with 6 changes of distilled H_2O. The stain was developed by immersing the gel in a solution comprising 0.12ml 1M citric acid and 0.25ml 38% (v/v) formaldehyde in 500ml distilled water. Staining was stopped by washing the gel immediately in distilled H_2O.

2.6.5 Drying and autoradiography of polyacrylamide gels

Slab gels were dried for easy storage or autoradiography. A destained slab gel was placed on Whatman 3mm chromatography paper and dried using a Biorad Laboratories Slab Gel Dryer model 1125 connected to an Aquavac junior (Uniscience Ltd., London, U.K.). Dried down slab gels were autoradiographed using Kodak X-OMAT R X-ray film with an intensifying screen. The period of exposure was usually from 6 hours to 14 days.

2.7 Modification of ICDH with phenylglyoxal

2.7.1 Incubations with phenylglyoxal

ICDH was diluted in 50mM MOPS-NaOH, 1mM EDTA pH7.3 at $4^{o}C$ to give a final concentration of 0.2mg/ml. Other additions were as given in the text. A sample of the mixture was assayed to establish the initial activity of ICDH. Then at zero time a solution of 100mM phenylglyoxal was added to give a final concentration of 10 or 20mM. Samples (1-5µl) were assayed for ICDH activity at various times thereafter. Results were plotted as log % activity remaining against time.

Some incubations were carried out at increased temperatures as stated in the Results Chapter 3. Others contained a variety of phenylglyoxal concentrations in the range 1-30mM, the results were plotted as above and $t_{1/2}$ for ICDH inactivation determined and replotted as $t_{1/2}$ vs phenylglyoxal concentration.

2.7.2 Incorporation of [7-^{14}C]-phenylglyoxal

Conditions were as Methods 2.7.1 except [7-^{14}C]-phenylglyoxal (5µCi/umol) was used. To separate protein bound phenylglyoxal from unreacted reagent 10µl samples were spotted onto 2.5cm discs of Whatman 3mm filter papers. The discs were then placed in 10% TCA for 1 hour (approx. 10ml TCA/disc) and then washed a further three times in 10% TCA, with each wash lasting 1 hour. The discs were then placed in 4ml

Ecoscint and the counts measured in a Beckman Liquid Scintillation Counter model 8100.

2.7.3 Large scale labelling of ICDH for HPLC

(a) Labelling

ICDH was incubated with 10mM [7-^{14}C]-phenylglyoxal (5µCi/umol) for two and half hours either in the presence or absence of enzyme substrates. The activity of the sample was assayed at zero time and again at the end of the incubation, as was incorporation of label. A Sephadex G-50 column (1.5x25cm) equilibrated in 10% formic acid was then used to separate the protein bound phenylglyoxal from the free. The fractions obtained were counted for radioactivity and the protein containing fractions pooled and freeze-dried (Methods 2.2.9).

(b) Digestion with pepsin

After freeze drying the samples were dissolved in 1ml 10% formic acid and 20µl of a 1mg/ml pepsin solution added. The digestion mixes were left overnight at 37^{o}C on a shaker. Samples were then boiled for 2 mins, freeze-dried and subjected to HPLC analysis.

(c) HPLC reverse phase chromatography

Peptides were applied to a Waters µbondapak C-18 column (30cmx3.9mm) equilibrated in 0.1% trifluoroacetic acid and eluted at 1ml/min with gradients of increasing acetonitrile concentrations as described in the text. Peptides were detected at 215nm, 1ml fractions were collected.

2.7.4 Reaction with $NaBH_4$

ICDH was inactivated with 10mM phenylglyoxal (5µCi/umol). 1M $NaBH_4$ in 50mM NaOH was added in 10µl aliquots to give a final concentration 10-fold in excess of the phenylglyoxal concentration. The incubation was then split. 10µl samples were spotted onto filter discs and washed in 10% TCA overnight. The remaining incubation was dialysed overnight against 50mM MOPS-NaOH, 1mM EDTA pH7.3. Disc samples and

dialysate were counted in the liquid scintillation counter to determine the amount of label still associated with the protein.

2.7.5 Inactivation of dephosphorylated ICDH-P with phenylglyoxal

ICDH was phosphorylated with unlabelled ATP as in Methods 2.4.2 (c). It was incubated at 37°C in the presence of 0.5mM ADP, 10mM $MgCl_2$, 1mM PEP, 0.25mU/ml ICDH kinase in 50mM MOPS-NaOH, 1mM EDTA pH7.3 at set times 1µl samples were assayed for ICDH reactivation. After complete reactivation 1M EDTA pH7.0 was added to a final concentration of 10mM and the sample was dialysed overnight against 50mM MOPS-NaOH, 1mM EDTA pH7.3. After dialysis 100mM phenylglyoxal was added to give a final concentration of 10mM and 1µl samples assayed for loss of ICDH activity.

2.7.6 Digestion of [^{32}P]-ICDH and [7-^{14}C] phenylglyoxal-ICDH with thermolysin

[^{32}P]-ICDH was prepared as in Methods 2.4.2 (c). ICDH was inactivated with [7-^{14}C]-phenylglyoxal (5µCi/umol) immediately before the addition of the protease. The two labelled samples were digested for two hours at 37°C with thermolysin (ICDH-P:thermolysin 4:1, ICDH-PG:thermolysin 850:1), denatured by boiling in SDS 'sample buffer' and loaded onto a 15% SDS gel (Methods 2.6.3). Duplicate tracks of each sample were run on opposite sides of the gel, and after completion of the run the gel was halved. One portion was soaked in 50% methanol prior to silver staining, and the remaining half was immediately dried down, without any staining, and autoradiographed (Methods 2.6.5).

2.8 Digestion of ICDH and ICDH-P with chymotrypsin

ICDH, phosphorylated ICDH and ICDH inactivated with phenylglyoxal (Methods 2.7.1) were digested with chymotrypsin (chymotrypsin:ICDH 1:72) in 50mM MOPS-NaOH, 1mM EDTA pH7.3 at 37°C. At set time intervals between 0 and 24 hours, samples each containing 10µg protein, were removed, denatured and run on a 15% SDS gel.

Similarly, ICDH was digested in this manner in the presence of either 200μM $NADP^+$ or 100μM isocitrate. A sample of phosphorylated ICDH which had been incubated with 10mM phenylglyoxal was digested under the same conditions. The unreacted phenylglyoxal was not removed from any of the incubations to which it was added. All gels from these experiments were soaked in 50% methanol and stained with silver.

2.9 Synthetic peptide studies

2.9.1 Preparation of peptide

The 14-mer synthetic peptide N-Thr-Thr-Pro-Val-Gly-Gly-Gly-Ile-Arg-Ser-Leu-Asn-Val-Ala-C was synthesised by solid phase techniques essentially as described by Gutte and Merrifield (1971) on a Biosearch 9500 (San Rafael, California, USA) peptide synthesiser using t-Boc (tert-butyloxycarbonal) amino acid substrates. The completed peptide was deprotected (side chain groups) and cleaved from the resin with hydrogen fluoride treatment (Fig.2.1). The purity of the deprotected synthetic peptide was checked on an HPLC reverse phase column (C18 Waters μ bondpak 30cmx3.9mm) equilibrated in 0.1% TFA and eluted with a gradient of increasing acetonitrile concentration.

2.9.2 Analysis of peptide HN2

(a) Amino acid analysis

The peptide sample was hydrolysed for 35 min in 6N HCl at 165°C under argon. The hydrolysed sample was then dried, reconstituted in 20μl of EDTA solution (250μg/ml). Analysis was carried out by Mr. B. Dunbar at the University of Aberdeen, on an Applied Biosystems 420A Derivatizer/130A Separation System.

(b) Liquid phase sequencing

This was carried out in collaboration with Professor J.E. Fothergill and Mr. B. Dunbar at the SERC-funded protein sequencing facility, Aberdeen University. Automated Edman degradation of the peptide sample was performed using an Applied Biosystems Gas Phase

sequencer operated by Mr. B. Dunbar. The anilinothiazoline products were converted to phenylthiohydatoins by treatment with a 1M HCl solution containing 1% (v/v) ethanethiol according to the general procedure of Beckman Instruments. The phenylthiohydantoins were identified and quantitated by HPLC on a Waters ABI PTH C_{18} reverse phase column with a pH5 sodium acetate-acetonitrile buffer system.

2.9.3. Phosphorylation of synthetic peptides

5-10mg/ml 14-mer synthetic peptide was incubated with 0.5mM ATP (10μCi/umol), 10mM $MgCl_2$, 0.25mu/ml ICDH kinase/phosphatase in 50mM MOPS-NaOH, 1mM EDTA pH7.3 at 37°C. At set times samples were removed and the reaction was stopped in one of 3 ways, according to the method of detecting phosphorylation used.

The synthetic peptide Kemptide (Kemp et al., 1976) and cAMP dependent protein kinase catalytic subunit were used in control phosphorylation mixes. 1mg/ml Kemptide was incubated with 0.5mM ATP (10μCi/umol), 10mM $MgCl_2$, 10 units cAMP dependent protein kinase catalytic subunit per 100μl in 50mM MOPS-NaOH, 1mM EDTA pH7.3. The mix was incubated at 37°C and samples were removed and analysed as above.

2.9.4 Detection of phosphorylated peptides

(a) Thin layer electrophoresis

After set incubation times kinase incubations were immediately put on ice. Samples (5-10μl) were rapidly spotted onto a TLC sheet (Machercy and Nagel, W. Germany) 10cmx10cm along a central line (Fig.2.2a) The sheet was carefully saturated with pH6.5 buffer (10% pyridine (v/v), 0.5% acetic acid (v/v). Electrophoresis (400 V for 20 min) was carried out in a Shandon tank and the plates were dried, stained with ninhydrin and autoradiographed.

(b) High voltage electrophoresis

A sample of the incubation mixture (15-20μl) was rapidly spotted onto a sheet of Whatman 3MM paper (57x25cm) along a central line

(Fig.2.2b). The paper was carefully saturated with pH6.5 buffer (10% pyridine (v/v), 0.5% acetic acid (v/v), electrophoresis (40V/cm for 90 min) was carried out in a 10kV electrophoresis unit (Miles Hivolt Ltd., Shoreham, Sussex, U.K.) water cooled system. The paper was dried, stained with ninhydrin and autoradiographed.

(c) Anion exchange resin analysis

Reactions were terminated in individual samples by the addition of acetic acid to a final concentration of 30%. The terminated reaction mixture was applied to a small column containing 2ml AG1-X8 anion exchange resin equilibrated with 30% acetic acid. The reaction tube was rinsed with 1ml of 30% acetic acid, which was allowed to completely run into the column. The [^{32}P]-phosphopeptide (as well as the dephosphopeptide) was then quantitatively eluted with an additional 3ml of 30% acetic acid. The eluant was collected directly into scintillation vials in 1ml aliquots. The fractions were counted for radioactivity by counting Cerenkov radiation (Ross, H.H. (1969)). The unreacted [^{32}P]-ATP remains bound to the resin.

2.10 Phosphorylation of chymotrypsin digests of ICDH

ICDH was digested overnight at 37^{o}C with chymotrypsin (chymotrypsin:ICDH, 1:100) in 50mM MOPS-1mM EDTA pH7.3. After digestion a 0.5mg/ml solution of chymostatin (15% v/v acetic acid, 50mM MOPS-NaOH, 1mM EDTA pH7.3) was added to give a final 10-fold excess with respect to the chymotrypsin present. The mix was left at 37^{o}C for 30 min. ICDH kinase assays were then carried out on the mix in the presence of 0.5mM ATP (10μCi/umol), 10mM $MgCl_2$ and 5μl partially purified ICDH kinase. The experiment was run as a time course. Samples were removed at set times, denatured and run on a 15% SDS gel, with suitable controls. The gel was stained with Coomassie Blue dried and autoradiographed.

2.11 Immunoblotting techniques

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

2.11.1 Stock solutions

Buffer A (Transfer buffer)

20mM Tris-HCl pH7.2

150mM NaCl

0.5% w/v Tween 20

Buffer B (Blocking buffer)

20mM Tris-HCl pH7.2

150mM NaCl

Buffer C (Chloronaphthol stain)

50ml 10mM Tris-HCl pH7.4

30mg chloronaphthol in 10ml methanol

150µl 4% v/v H_2O_2

H_2O_2 was mixed immediately before use.

2.11.2 Conditions

SDS-PAGE was carried out (Method 2.6.3). When electrophoresis was complete, the proteins were transferred to 0.45µ nitrocellulose paper (Adderman and Co., Kingston-upon-Thames, Surrey KT2 6NH) using a Bio-Rad trans-blot cell containing Buffer A containing 16.6% methanol. The transfer was run at 350mA for 4 hours, after which the nitrocellulose paper was placed in Buffer B containing 5% normal goat 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 goat serum and 0.5% (v/v) antisera at 25°C for 90 min, after

which the nitrocellulose paper was washed 4x12 min in Buffer A and then 1x12 min in buffer B. The nitrocellulose paper was then soaked in Buffer B containing 5% (v/v) normal goat serum and 0.05% peroxidase conjugate goat anti rabbit IgG at 23°C for 90 min, washed 5x12 min in Buffer B and developed by soaking in Buffer C for 5 min at 23°C. The final product was then washed in distilled H_2O, dried and stored.

2.12 Circular dichroism

Circular dichroism analysis of ICDH and ICDH-P in the presence and absence of cofactors $NADP^+$ and NADPH was carried out in 50mM MOPS, 1mM EDTA pH7.3. The analysis was carried out at the Department of Biochemistry, University of Newcastle by Roman Hlodon on a Jobin-Yvon dichrograph III equipped with a temperature controlled cell holder. Ellipticity values (mean residue molar ellipicity in deg.cm^2 $decimole^{-1}$) were calculated using the following equation:-

$$= \frac{\text{m.r.w. x sensitivity x displacement x 3300}}{\text{concentration x pathlength}}$$

where m.r.w. -= mean residue weight

displacement = difference between sample and reference at any given time

concentration = 1mg/ml

pathlength = 1cm

Figure 2.1 Basic scheme of solid phase peptide synthesis (Stewart & Young, 1984)

L	Labile group protecting amino group
S	Stable blocking group protecting functional amino acid group
X	Polymer reactive group
R^1, R^2	Amino acid side chain
DCM	Dichloromethane
DIPC	N,N-diisopropylcarbodiimide

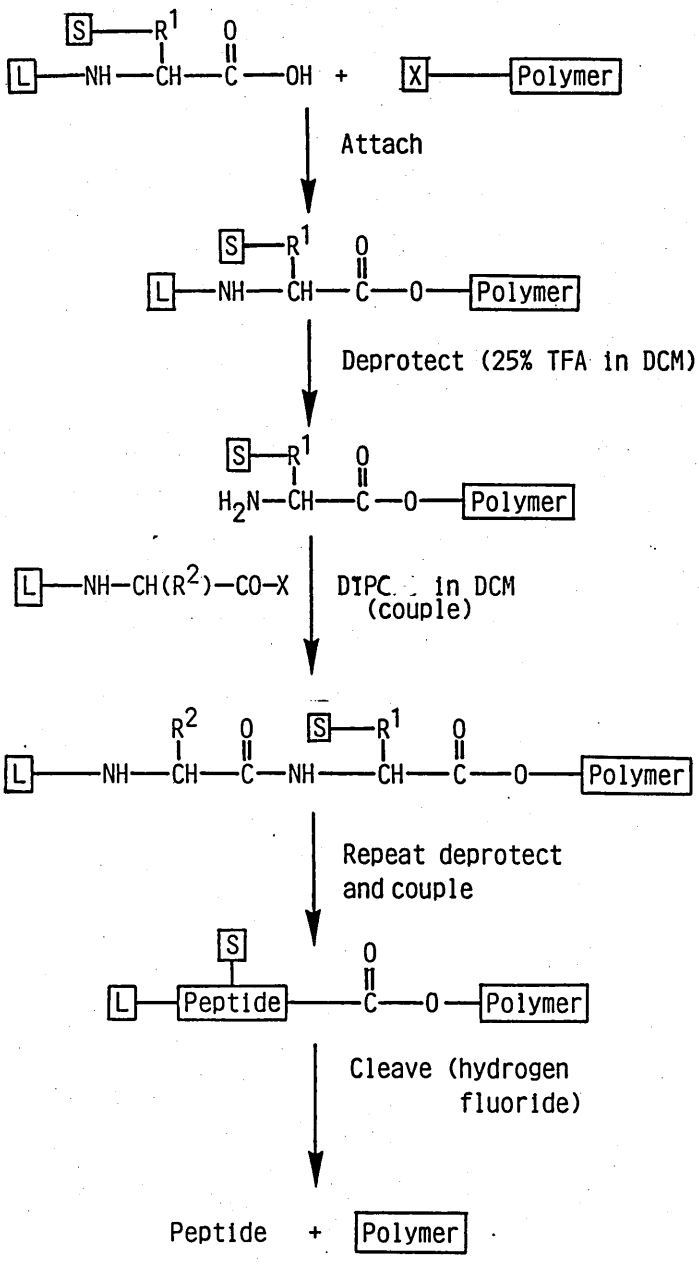

Attach
Deprotect (25% TFA in DCM)
DIPC in DCM
(couple)
Repeat deprotect
and couple
Cleave (hydrogen
fluoride)
Peptide + Polymer

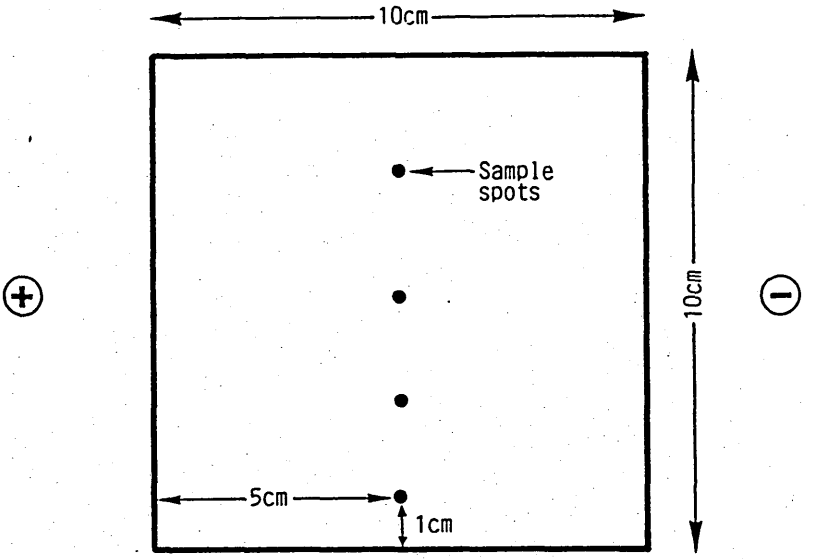
Figure 2.2 Electrophoretic analysis of peptides

Diagram of sample loading

(a) Thin layer chromatography, pH6.5

(b) High voltage paper electrophoresis, pH6.5

a) Thin layer electrophoresis pH6.5



b) High voltage paper electrophoresis pH6.5

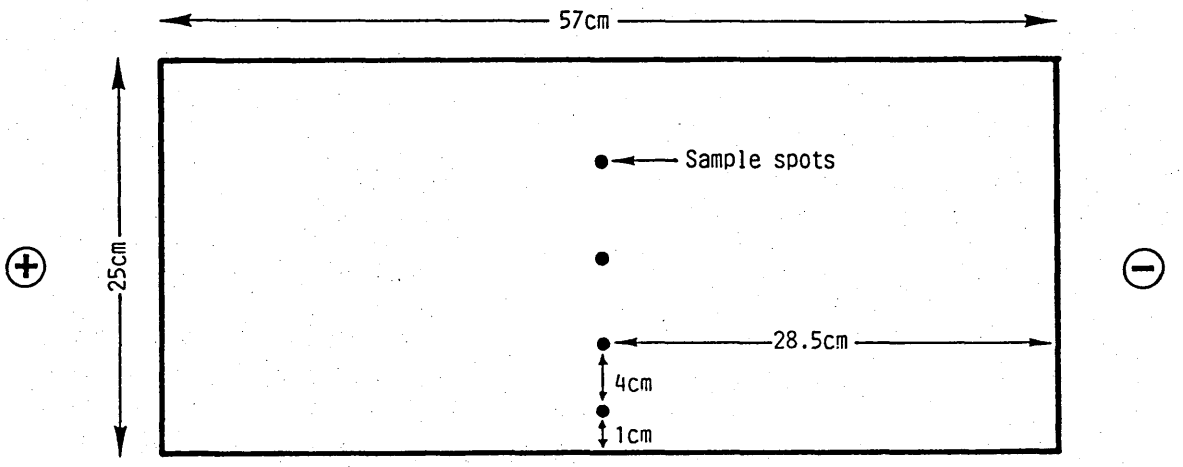


Table 2.1 Preparation of SDS polyacrylamide gels

- quantities and stock solutions used.

Table 2.1

Stock solution	Stacking gel (ml)	Separating gel (ml) 10%	15%	20%
30% acrylamide 0.8% bisacrylamide	1.5	10	15	20
1.5M Tris HCl pH8.8	-	7.5	7.5	7.5
0.5M Tris HCl pH6.8	3.75	-	-	-
H_2O	9.45	12.05	7.05	2.05
10% (w/v) SDS	0.15	0.3	0.3	0.3
10% (w/v) ammonium persulphate (freshly prepared)	0.15	0.15	0.15	0.15
TEMED	0.01	0.01	0.01	0.01

CHAPTER THREE

Chemical Modification of Isocitrate Dehydrogenase with Phenylglyoxal

3.1 Introduction

Chemical modification of amino acid side chains is an important method for identification of residues involved in protein function either at the active site of an enzyme or at points distinct from the active site such as allosteric sites (e.g. Vandenbunder et al., 1981) or areas of subunit interaction (e.g. Perutz, 1978). In order to achieve a complete understanding of how proteins carry out their biological function, we require a detailed knowledge of how their binding sites are constituted. This has become a key problem in the area of protein structure - function analysis.

Escherichia coli isocitrate dehydrogenase, hereafter ICDH, provides a well studied protein which has been shown to be controlled by a reversible phosphorylation of a specific serine residue. It has been suggested that this phosphorylation occurs close to or at the $NADP^+$ binding site in such a way as to prevent binding of co-factor, and that this renders the enzyme inactive. The binding of $NADP^+$ at the active site is believed to produce a charge-charge interaction, between the phosphate group(s) of $NADP^+$ and a positively charged residue at the active site, which produces a conformational change. The introduction of the phosphate group on a serine residue close to the $NADP^+$ binding site could trigger a similar conformational change, and this would be expected to block binding of co-enzyme.

We first addressed the question of the existence and nature of the positively charged group(s) at the co-enzyme binding site of ICDH using chemical modification reagents. Since arginine residues are often found at co-factor binding sites, and there is an arginine residue adjacent to the phosphorylated serine, we decided to choose the arginine-specific dicarbonyl reagent phenylglyoxal.

Results

3.2 Purification of ICDH

Table 3.1 shows a purification table for isolation of ICDH from *E. coli* ML308. The purification procedure was carried out routinely and reproducibly. The average yield was greater than 50% with a 170-190-fold purification. A 10% SDS gel was run after each purification to check the purity of the final product (Fig. 3.1).

3.3 Inactivation of ICDH with phenylglyoxal

Phenylglyoxal was found to inactivate pure ICDH rapidly in a pseudo-first order process (Method 2.7.1) (Fig. 3.2). The half-life of the enzyme activity was in the range 2.4 - 3.4 minutes (10 determinations) at pH7.3 and $0^{o}C$ with 20mM phenylglyoxal. The inactivation reaction was very reproducible with only slight variations between distinct batches of pure ICDH. The rate of inactivation could be increased, as expected, by carrying out the inactivations at higher temperatures.

3.3.1 Effect of phenylglyoxal concentration

When similar inactivations were carried out using two other dicarbonyl reagents. 2,3-butanedione and 1,2-cyclohexanedione it was found that little or no inactivation of ICDH occurred (Fig. 3.3). This gave rise to the question of whether inactivation of ICDH with phenylglyoxal was rapid because this reagent was an affinity label for the enzyme. If this were the case then one would expect the inactivation to show saturation kinetics with respect to phenylglyoxal concentration. Inactivations of ICDH were therefore carried out using a range of phenylglyoxal concentrations (1-30mM) and the half-life of enzyme activity was calculated. Figure 3.4 shows a plot of phenylglyoxal concentration against the reciprocal of $t_{1/2}$. In the case of saturation kinetics, $^{1}/t_{1/2}$ would tend to a maximum as the phenylglyoxal concentration increases. The data obtained gave a

biphasic graph. This suggests that phenylglyoxal has some affinity for ICDH but that it can react with the enzyme without binding to it in advance.

3.3.2 Protection against inactivation

Both $NADP^+$ (50-200μM) and isocitrate (25-100μM) can protect the enzyme against inactivation by phenylglyoxal (Figs. 3.5 and 3.6 respectively). The effect of isocitrate is at first sight surprising in view of the fact that the enzyme obeys an ordered mechanism with $NADP^+$ binding first (Nimmo, 1986). The most likely explanation of this anomaly is that, since the last step of purification involves elution with $NADP^+$, the enzyme preparation actually contains small amounts of the co-enzyme. This possibility is tested in the next section. This may also be responsible for the slight variations seen between batches of ICDH during inactivation with phenylglyoxal. The protection data allowed us to calculate K_s values for these substrates. Figure 3.7 shows the method of graphical determination of K_s for $NADP^+$. The values obtained (16μM and 60μM for isocitrate and NADP respectively) are similar to the K_m values of 8μM and 22.7μM determined under somewhat different conditions (Table 3.2). The data are consistent with the idea that phenylglyoxal affects the active site of the enzyme. The fact that both $NADP^+$ and isocitrate protect against inactivation raises the question of the number of arginine residues that react with phenylglyoxal. While direct measurement of the number of reactive groups would be achieved using radioactively labelled phenylglyoxal (see chapter 4), some useful information could also be gained from protection experiments. For example, if there was an arginine residue at the sub-site for each substrate, one might expect that each substrate alone would only give partial protection. The data drawn in Fig. 3.7 for $NADP^+$ and the corresponding data for isocitrate (Fig. 3.8) show that this is not the case. Experiments in which both isocitrate and $NADP^+$

were present initially would be difficult to interpret because of the possibility that some of the substrates would be converted to products by the high enzyme concentration, even in the absence of added divalent metal ions. We therefore carried out abortive substrate protection experiments which involved protection of the enzyme activity with one substrate and one product e.g. NADPH and isocitrate.

The results obtained showed no increase in protection was achieved, which is consistent with the modification of only one arginine residue by phenylglyoxal (Fig. 3.9). However, these experiments are only indirect and we therefore used [7-^{14}C] phenylglyoxal to look more closely at the stoichiometry of the enzyme inactivation reaction.

3.3.3 Inactivation of ICDH from E.coli TG1/pCK505 with phenylglyoxal

E.coli TG1/pCK505 (Method 2.3.1) carries the plasmid pCK505 which bears the icd structural gene encoding ICDH, and it overexpresses it 10-fold. The purification procedure of ICDH from this strain (Method 2.5.5) does not involve the procion-red sepharose step of the ML308 ICDH purification (Method 2.5.2), and hence eliminates the elution step employing $NADP^+$. Thus the ICDH has been purified without allowing $NADP^+$ to bind at its co-factor binding site. Since the mechanism of ICDH involves a compulsory order process with $NADP^+$ binding first, we asked whether isocitrate would be able to protect the ICDH purified from TG1/pCK505 from inactivation with phenylglyoxal.

TG1/pCK505 ICDH was inactivated with phenylglyoxal as in method 2.7.1. Protection experiments were carried out with either 200μM $NADP^+$ or 100μM isocitrate. Figure 3.10 shows the results obtained. $NADP^+$ protected the enzyme against inactivation with phenylglyoxal, however isocitrate was unable to protect ICDH against loss of activity. This suggested isocitrate was unable to bind at the active site in the absence of $NADP^+$.

3.3.4 Labelling of ICDH with [7-^{14}C]-phenylglyoxal

Inactivation of ICDH using radioactively labelled phenylglyoxal was carried out essentially as with non-radioactive compound (Method 2.7.1). In order to measure the incorporation of label into the protein successfully it was necessary to separate the unreacted label from the protein sample. This was done by precipitating the protein sample onto filter paper discs by immersion into 10% trichloroacetic acid. Any unreacted label was removed by successive washings with TCA (Method 2.7.2). To determine the most suitable washing method several identical samples of either [7-^{14}C]-phenylglyoxal or ICDH reacted with [7-^{14}C]-phenlyglyoxal were spotted onto filter discs and subjected to a variety of washing procedures. It was found that the majority of the unbound label was removed by the first washing and that equilibration between the disc and the solution was achieved within 15 min. Therefore 4x15 min washes gave satisfactory reduction of non-precipitated label to background level, further 15 min washes removed no more label. Under these conditions the modification of ICDH by phenylglyoxal was found to be stable. However instability of the adduct in the long term will be discussed in Chapter 4. We could therefore use this method to monitor the time-course of incorporation of label into ICDH. Incorporation of [7-^{14}C]-phenylglyoxal into ICDH and inactivation of the enzyme were assessed in the following way. At set time intervals samples were removed and assayed for enzyme activity. At the same times samples were taken and used to determine incorporation of ^{14}C-radioactivity into the protein. The results showed that the loss of ICDH activity was linearly related to incorporation of label into the enzyme. Extrapolation of this relationship to 100% inactivation corresponded to incorporation of 0.98nmol phenylglyoxal/nmol ICDH subunit (Mean of 4 experiments ranges 0.87 - 1.06), (Fig. 3.11). Thus complete inactivation of enzyme

activity appeared to be dependent on modification of one arginine residue by one molecule of phenylglyoxal.

Incorporation of radioactive phenylglyoxal into the protein was reduced if the inactivation experiments were carried out in the presence of either 200μM NADP or 100μM isocitrate (Figs.3.12 and 3.13 respectively). The reduction of incorporation correlated with a reduction in loss of enzyme activity. When incorporation of [7-^{14}C]-phenylglyoxal/ICDH subunit was plotted against inactivation, the data from all three conditions (ICDH, ICDH + 200μM $NADP^+$ and ICDH + 100μM isocitrate) fell on the same line. This indicated the same arginine was modified in all three cases. (Fig. 3.14).

3.3.5 Labelling of phosphorylated ICDH with [7-^{14}C]-phenylglyoxal

In order to test accessibility of arginine in ICDH-P we measured the incorporation of radioactivity from [7-^{14}C]-phenylglyoxal. Samples could not be assayed for activity since phosphorylated ICDH is already inactive. Results were very striking; little or no [7-^{14}C]-phenylglyoxal was incorporated into the protein over the time course of the experiment (Fig. 3.13., Table 3.3). The experiment was carried out four times and always gave the same result. This indicated that the arginine modified in active ICDH was in some way protected by phosphorylation and hence inaccessible to the phenylglyoxal.

This result was so striking that it became necessary to rule out the possibility that it was caused by some non-specific effect of the phosphorylation process. We therefore investigated whether ICDH that had been first phosphorylated and then dephosphorylated could still be inactivated by phenylglyoxal. We prepared phosphorylated ICDH as in method 2.4.2.c), using either unlabelled ATP or [γ-^{32}P]-ATP. The dephosphorylation, and hence reactivation of the ICDH, was monitored either using release of ^{32}P from the protein by precipitation of the protein in a similar manner to ICDH phosphatase assays (Method 2.4.2.c))

or by recovery of enzyme activity. After complete reactivation and dephosphorylation of ICDH was obtained phenylglyoxal was added to a final concentration of 10mM and again the activity loss monitored. The initial results showed that, surprisingly, the ICDH could not be inactivated by phenylglyoxal. However on examination of the effects of the individual components of the ICDH-P reactivation mix on the inactivation of ICDH by phenylglyoxal it was found that several components, $MgCl_2$ and ADP in particular, could protect the ICDH activity from inactivation with phenylglyoxal. (Fig. 3.15). In an experiment in which the dephosphorylated reactivated sample was dialysed against 50mM Mops-NaOH/, 1mM EDTA pH7.3, and then treated with phenylglyoxal, rapid inactivation of the ICDH was observed (Fig. 3.16). This confirms that phosphorylation has a specific effect on the accessibility of ICDH to phenylglyoxal.

3.4. Effect of phenylglyoxal on chymotryptic digestion of ICDH and ICDH-P

The effect of phosphorylation on the accessibility of ICDH to phenylglyoxal was also examined in experiments using limited proteolysis. Partial chymotryptic digestion of ICDH produced a very distinctive pattern when fragments were run on an SDS gel. (Fig. 3.17a)) Digestion in the presence of either substrate, NADP or isocitrate, had no effect on the digestion pattern obtained. ICDH-P proved to be much more resistant to digestion but the same digestion pattern was obtained (Fig. 3.18a). However when ICDH was first inactivated with phenylglyoxal then digested with chymotrypsin the pattern obtained was very different (Fig. 3.17b)). In this experiment there was a time dependent further degradation of the initial digestion peptides to much smaller peptide material. However when phosphorylated ICDH, which had been pre-incubated with phenylglyoxal, was digested with chymotrypsin the normal ICDH chymotrypsin digestion pattern was obtained

(Fig. 3.18b)). Comparison of Figs. 3.17 and 3.18 shows that phenylglyoxal affects active ICDH but not phosphorylated ICDH.

3.5 Discussion

Selective chemical modification of arginine residues in proteins under mild conditions is desirable for a number of purposes, especially in connection with the definition of the active sites of enzymes. The group of reagents used to selectively modify arginine residues in proteins are the ∝-dicarbonyl compounds, particularly phenylglyoxal, 2,3-butanedione and 1,2-cyclohexanedione. Using such reagents arginines have been identified as essential residues in the function of many enzymes (Riordan, 1979). In each of these enzymes, the essential arginine is though to interact with a negatively charged group of a substrate or co-factor molecule, especially the phosphate groups of NAD^+ and $NADP^+$ co-enzymes. This idea has been confirmed by X-ray crystallographic studies, for example of lactate dehydrogenase (Adams *et al.*, 1973).

In virtually all of the examples of enzyme modification with dicarbonyl reagents, the chemical modification is not only limited to arginyl residues but to a selected few of the total arginines present per molecule (Table 3.4). Since as a rule hydrophilic residues are located at the surface of proteins, and therefore accessible to the solution, this would suggest that factors other than simply accessibility affect how the arginines react. A good example of this selective modification is found with creatine kinase (Borders and Riordan, 1975). Only one of the 18 arginyl residues per subunit reacts with phenylglyoxal. It reacts with phenylglyoxal 10-15 times faster than does the free amino acid. Several factors could be involved in determining the reaction selectivity. Since most polar groups would be exposed on the surface, a few may be involved in polar interactions with other amino side chains, others may be solvated and hence their reaction is slow. Some, however,

may be in a chemical environment that enhances their activity and that may be essential to their biological function. Thus the very properties of an arginine residue at an active site, produced by its local environment, also seem to increase its susceptibility to dicarbonyl reagents.

Phenylglyoxal proved to be a good choice of chemical modification reagent to investigate the active site of ICDH. It inactivated ICDH in a rapid and reproducible manner. Table 3.5 shows inactivation data for phenylglyoxal with a variety of enzymes. ICDH appears to be fairly rapidly inactivated as compared to the other examples quoted. Inactivation of ICDH with either 2,3-butanedione or 1,2-cyclohexanedione was very poor. Little inactivation was observed with cyclohexanedione and butanedione appeared to inactivate ICDH but at a very slow rate. A comparison of the inactivation of three different enzymes with the three dicarbonyl reagents is shown in Table 3.6. The data indicate that the three reagents are of broadly comparable reactivity.

This suggested that phenylglyoxal might be acting as an affinity label for ICDH. However the results obtained were ambiguous. Saturation kinetics were not observed. However the apparent pseudo-first order rate constant clearly did not increase linearly with the concentration of phenylglyoxal. One possible explanation is that two effects are being observed. Phenylglyoxal may be able to bind to the NADP site of ICDH before reacting but can also react directly without binding. It might be easier to observe saturation kinetics if the arginine residue in ICDH was less reactive.

Protection against inactivation by phenylglyoxal could be achieved with either $NADP^+$ or isocitrate. The fact that both substrates protect suggests that phenylglyoxal modification occurs at the active site of the enzyme. The results from inactivation experiments using

ICDH purified from TG1/pCK505 indicate that protection by isocitrate is related to the small amounts of $NADP^+$ associated with the ICDH as a result of the purification step involving $NADP^+$ elution from procion-red sepharose (Method 2.5.2). The increased protection observed with isocitrate may be as a result of a tighter binding to the active site of the two substrates together. The possibility of a conformational change on binding of both substrates, hence increasing the protection, cannot be ruled out. The results shown in Figure 3.9. of "dual substrate" protection experiments confirm the result that both substrates did not significantly increase the protection achieved with isocitrate alone.

However our data do not prove conclusively that phenylglyoxal reacts at the active site. We cannot rule out the possibility that on substrate binding a conformational change occurs which adventitiously protects an arginine residue distinct from the active site from modification.

Addition of both substrates in an inactivation experiment did not increase the protection achieved. This is consistent with the view that modification of only one arginine residue is involved in inactivation. However, it may be the case that there are two arginines which can be modified at this site, but reaction of either one results in complete inactivation of the enzyme. If so, the stoichiometry experiments would indicate that the labelling of these residues must be mutually exclusive.

Use of [7-^{14}C]-phenylglyoxal provided a convenient tool to investigate the inactivation reaction in greater depth. A correlation between incorporation with complete inactivation corresponding to incorporation of 1nmol phenylglyoxal/nmol ICDH subunit. The stoichiometry and the reaction mechanism have not been conclusively determined for the arginine-phenylglyoxal reaction, and this will be discussed more fully in Chapter 4. However reports have indicated two

different possible stoichiometries for the reaction of 2:1 and 1:1 for phenylglyoxal and arginine respectively.

Takahashi (1968) reported a 2:1 adduct for Ribonuclease A whereas Borders & Riordan (1975) reported a 1:1 adduct for creatine kinase. ICDH appears to fall into the second class. This suggested modification of only 1 arginine residue per enzyme subunit. A reduction in incorporation of radioactive label was achieved by inclusion of one or other of the substrates in the incubation, and this was accompanied by a reduction in the loss of activity. Graphical comparison of the results from the three labelling experiments showed a linear relationship between label incorporated and inactivation which is also good evidence for the existence of only one modified residue. The ability to reduce the label by presence of substrate gave a potentially useful method for isolating a specifically labelled peptide.

The hypothesis that phosphorylation occurs close to or at the $NADP^+$ binding site was supported by the result that phosphorylation of ICDH prevented incorporation of phenylglyoxal into the protein. This suggested the active site of ICDH contained one particularly reactive arginine which can be protected against modification either by binding of $NADP^+$ or by phosphorylation. The amino acid sequence round the site of phosphorylation is already known (Borthwick et al., 1984b). There is an arginine residue immediately adjacent to the phosphorylatable serine. It is tempting to speculate that this is the residue modified by phenylglyoxal.

During the course of work with phenylglyoxal we also considered the effect phenylglyoxal modification had on the overall conformation of the protein. Protease digestion was used as the analysis method. Phosphorylation of ICDH is known to increase its resistance to protease digestion (Garland & Nimmo, 1984). Phenylglyoxal was also found to affect protease digestion but in the opposite manner. Phenylglyoxal

caused the digestion to proceed further. Degradation to smaller peptide material suggested the availability of more sites for protease action. This may occur if some sites were made accessible due to a conformation change, such as perhaps a general opening out of protein structure. Another possibility is the phenylglyoxal-arginine adduct itself provided another recognition site for the action of chymotrypsin. Phosphorylation of ICDH prevented the increased susceptibility to protease digestion after inactivation with phenylglyoxal. The results suggested phosphorylation and phenylglyoxal action occur at a common site in ICDH, the active site.

Attempts to use phenylglyoxal as a tool to isolate a specifically labelled active site peptide are described in the next chapter.

Figure 3.1 Purification of isocitrate dehydrogenase from E.coli ML308

10% polyacrylamide gel run in the presence of SDS monitors the purification of ICDH (Methods 2.5.2)

Track A Crude extract

B Protamine sulphate supernatant

C Ammonium sulphate supernatant 60-75% pellet

D Gel filtration pool

E DEAE - cellulose pool

F Procion red pool

G Molecular weight markers

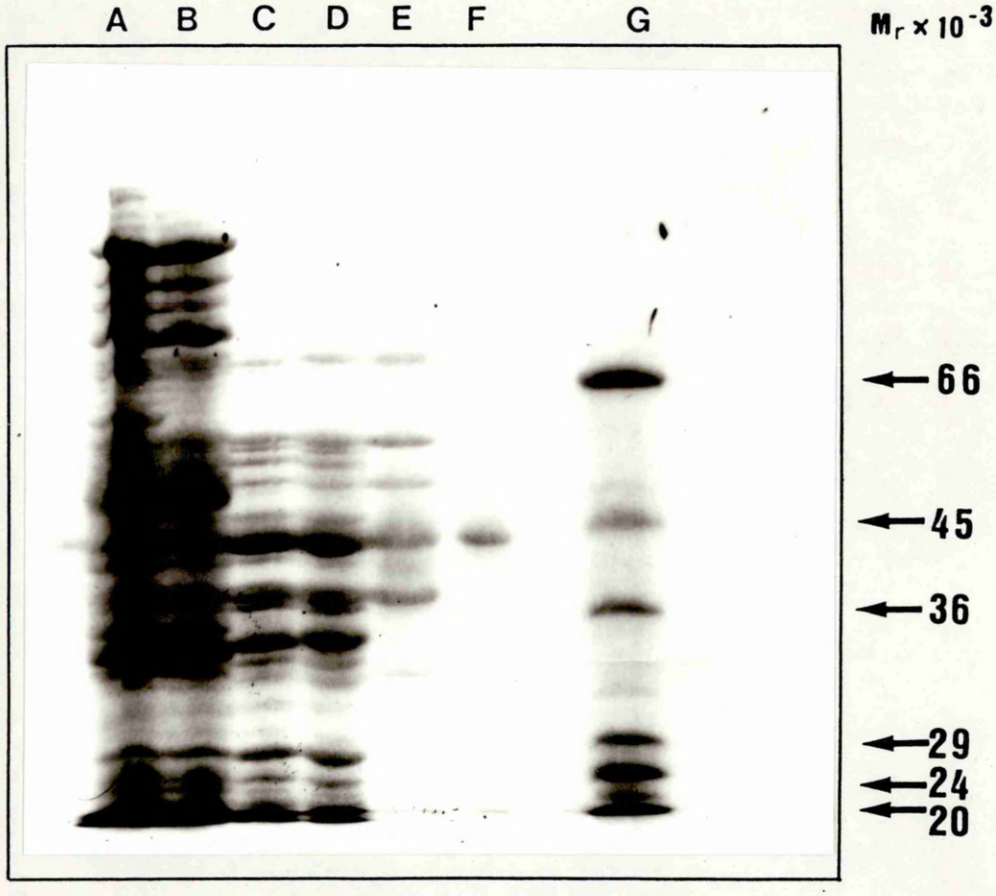

A
B
C
D
E
F
G
$M_r \times 10^{-3}$
66
45
36
29
24
20

Figure 3.2 Inactivation of isocitrate dehydrogenase with 20mM phenylglyoxal

ICDH was inactivated with a solution of 20mM phenylglyoxal as in Methods 2.7.1.

The activity at each time point is expressed as a percentage of the initial activity before the addition of phenylglyoxal. The percentage activity remaining was then plotted on a logarithmic scale vs time (mins).

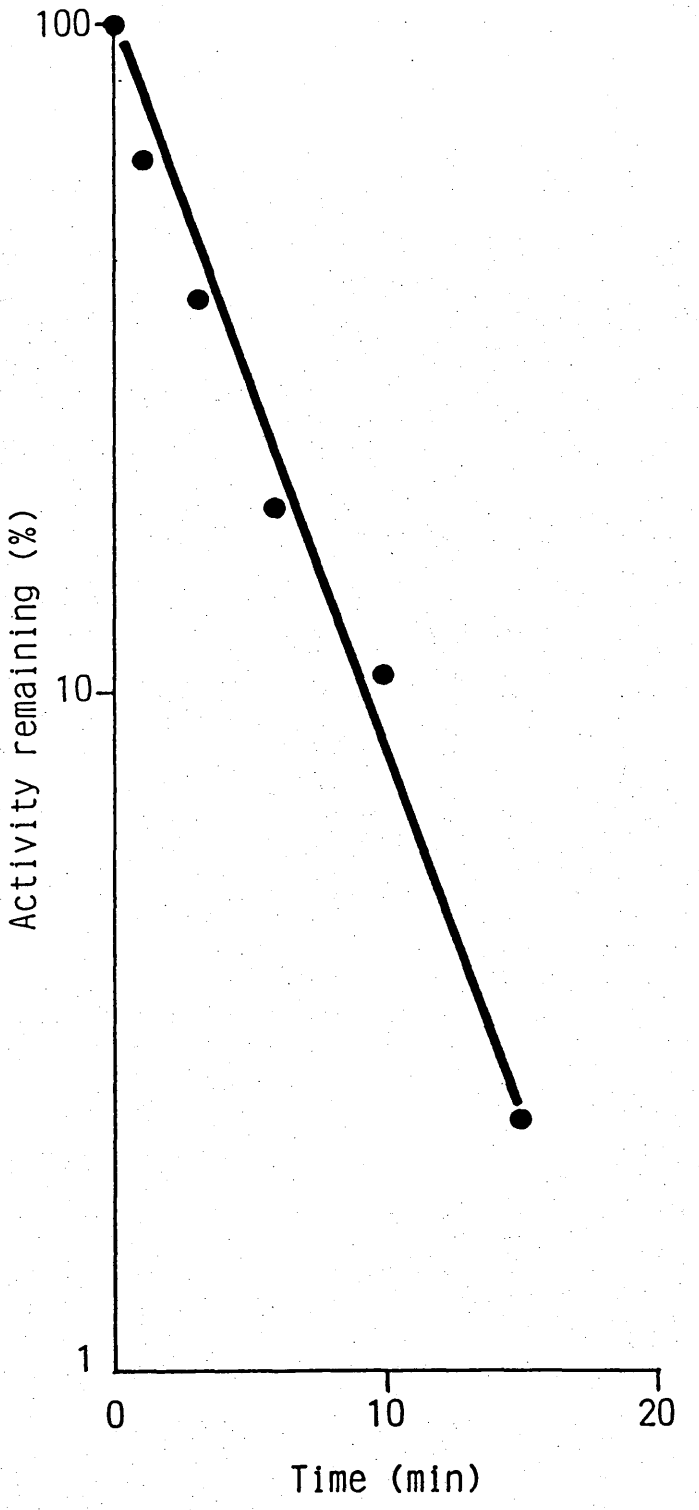

100
10
1
Activity remaining (%)
0
10
20
Time (min)

Figure 3.3 Inactivation of isocitrate dehydrogenase with the dicarbonyl reagents 2,3-butanedione and 1,6-cyclohexanedione

ICDH was inactivated with either 150mM 2,3-butanedione or 50mM 1,6-cyclohexanedione as in Methods 2.7.1.

Results were plotted as for Figure 3.2.

● 150mM 2,3-butanedione

▲ 50mM 1,2-cyclohexanedione

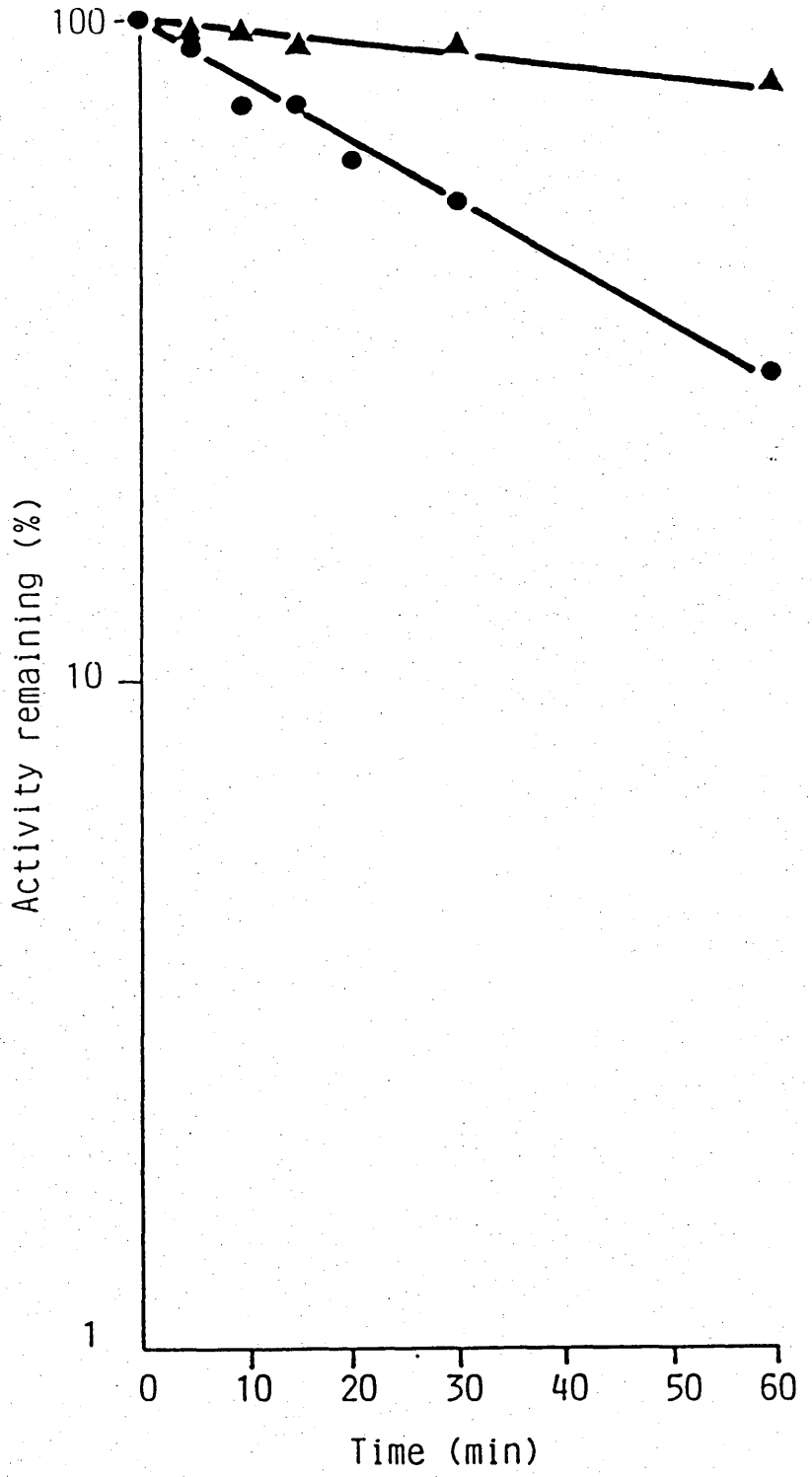

100
10
1
Activity remaining (%)
0
10
20
30
40
50
60
Time (min)

Figure 3.4 Effect of phenylglyoxal concentration on the inactivation of isocitrate dehydrogenase

ICDH was inactivated with a range of phenylglyoxal concentrations (1-30mM) as in Methods 2.7.1. These data were used to determine the half-life for inactivation, and the results plotted against the corresponding phenylglyoxal concentration.

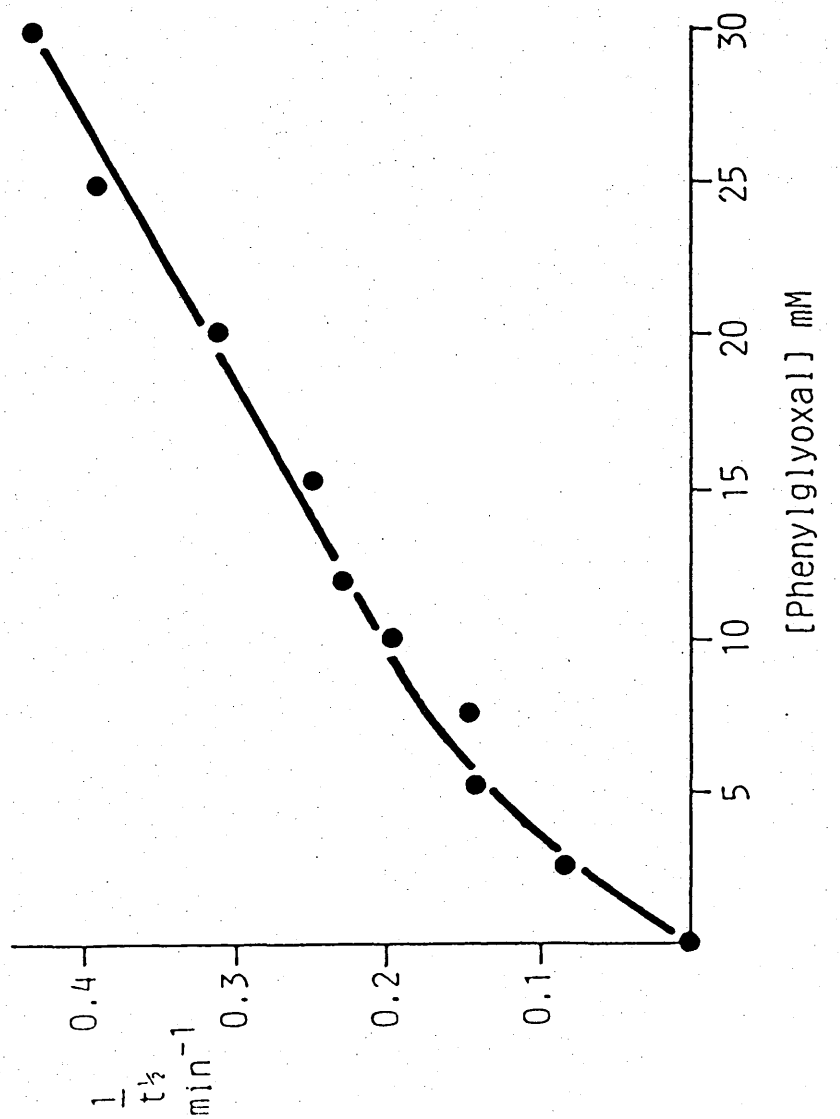

0.4
0.3
0.2
0.1
$\frac{1}{t^{\frac{1}{2}}}$ min^{-1}
5
10
15
20
25
30
[Phenylglyoxal] mM

Figure 3.5 Inactivation of isocitrate dehydrogenase with 20mM phenylglyoxal - protection with $NADP^+$

ICDH was inactivated with 20mM phenylglyoxal as in Methods 2.7.1 in the presence of increasing concentrations of $NADP^+$.

- X no additions
- △ 50μM $NADP^+$
- ○ 100μM $NADP^+$
- ▲ 150μM $NADP^+$
- ● 200μM $NADP^+$

Results were plotted as for Figure 3.2.

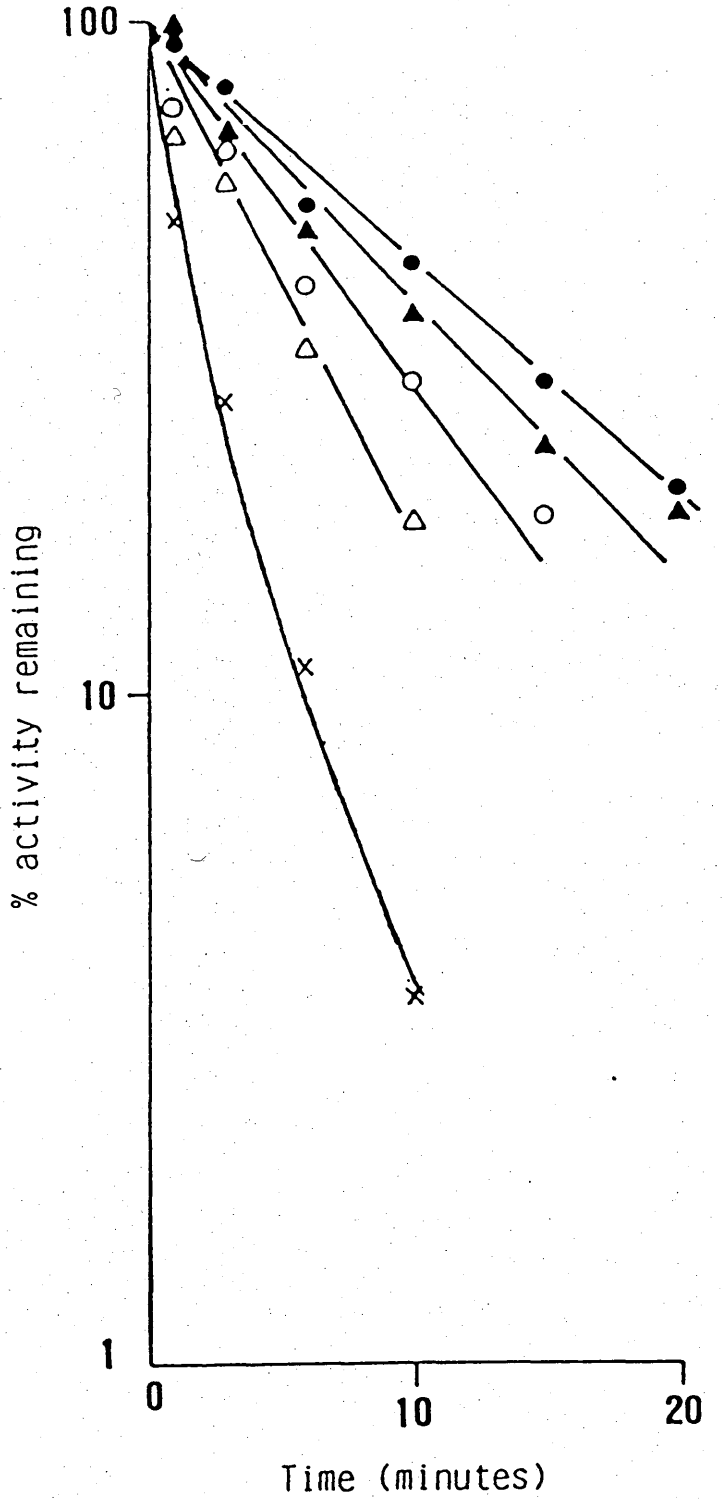

100
10
1
% activity remaining
0
10
20
Time (minutes)

Figure 3.6 Inactivation of isocitrate dehydrogenase with 20mM phenylglyoxal - protection with isocitrate

ICDH was inactivated with 20mM phenylglyoxal as in Methods 2.7.1 in the presence of increasing concentrations of isocitrate.

- X no additions
- △ 25μM isocitrate
- ○ 50μM isocitrate
- ▲ 75μM isocitrate
- ● 100μM isocitrate

Results were plotted as in Figure 3.2.

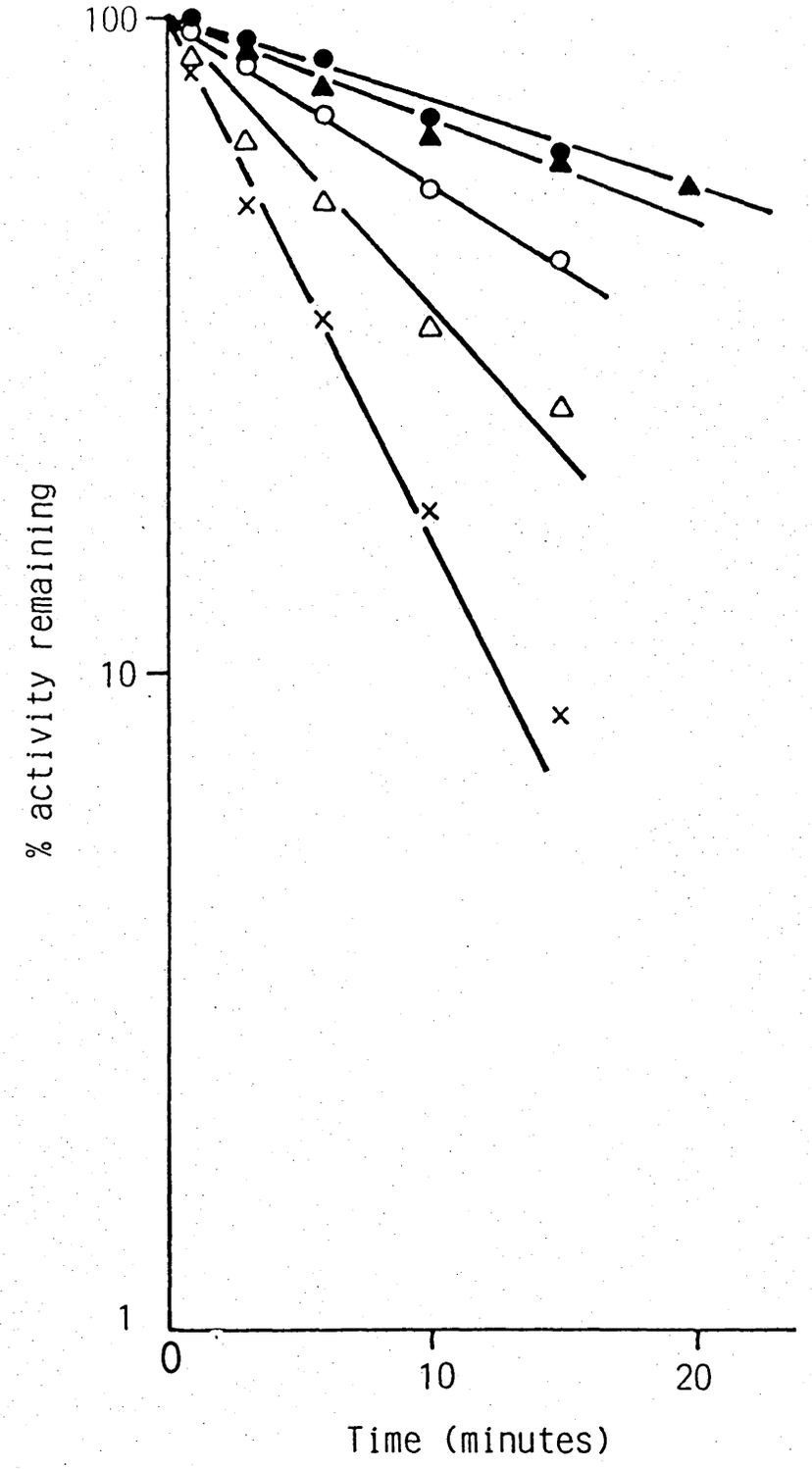

100
10
1
% activity remaining
0
10
20
Time (minutes)

Figure 3.7 Determination of K_S for $NADP^+$ in the presence of 20mM phenylglyoxal

The half-lives for ICDH inactivation were determined from each of the $NADP^+$ incubations in Figure 3.5. The half-life values determined were then replotted against the corresponding $NADP^+$ concentration.

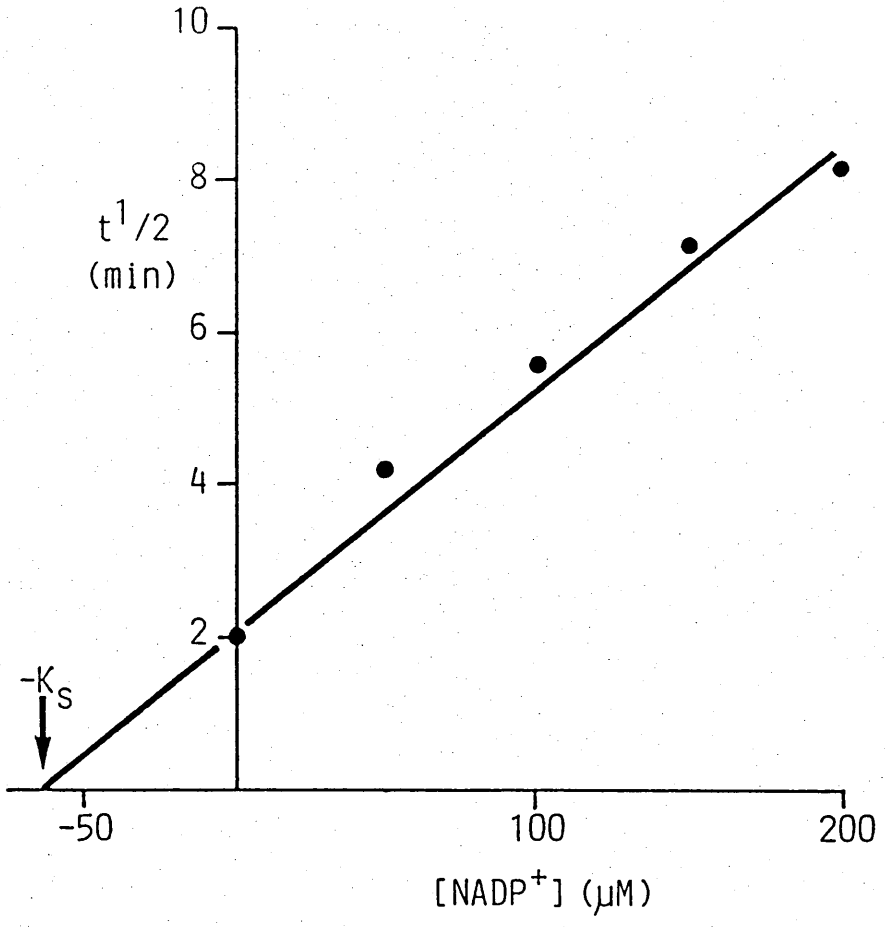

10
8
6
4
2
$t^1/2$
(min)
$-K_s$
-50
100
200
[NADP$^+$] (μM)

Figure 3.8 Determination of the K_S value for isocitrate in the presence of 20mM phenylglyoxal

The half-lives for ICDH inactivation were determined from each of the isocitrate incubations in Figure 3.6. The results were then replotted against the corresponding isocitrate concentration.

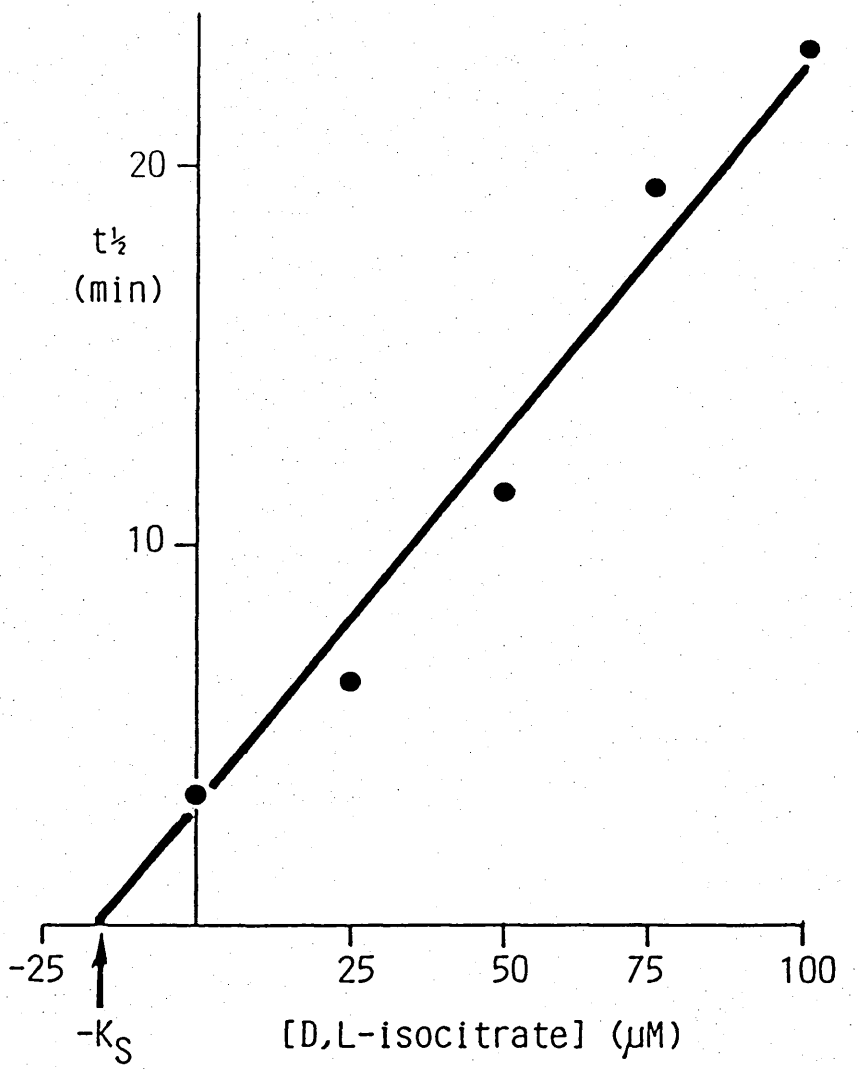

20
t½
(min)
10
-25
25
50
75
100
-Ks
[D,L-isocitrate] (μM)

Figure 3.9 Abortive substrate protection experiments

ICDH was inactivated with 20mM phenylglyoxal as in Methods 2.7.1 in the presence of the following substrates and products.

- ■ no additions
- ● 200μM alpha-ketoglutarate
- ▲ 100μM NADPH
- △ 200μM $NADP^+$
- □ 200μM $NADP^+$ + 200μM alpha-ketoglutarate
- ○ 100μM isocitrate
- ▽ 100μM isocitrate + 100μM NADPH

Results were plotted as for Figure 3.2.

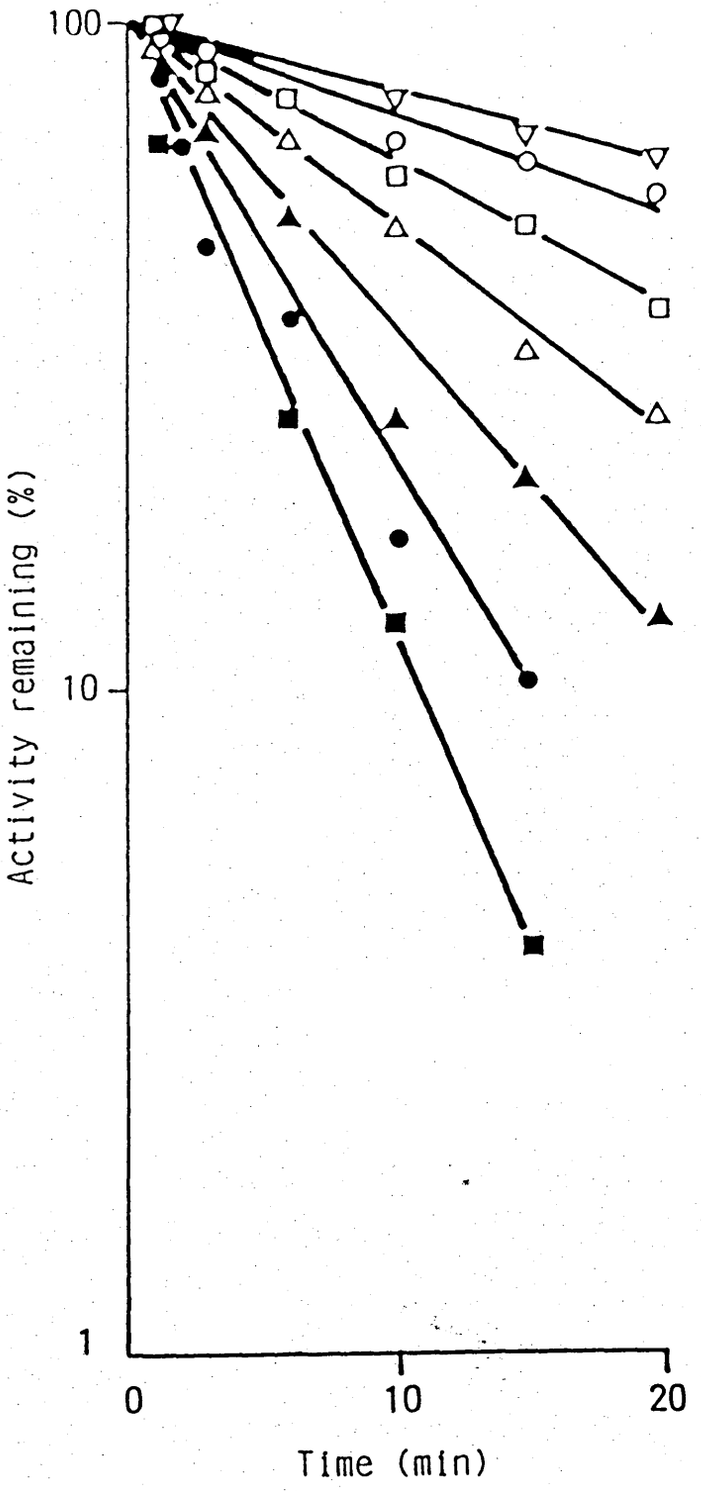

100
10
1
Activity remaining (%)
0
10
20
Time (min)

Figure 3.10 Inactivation of isocitrate from E.coli TG1/pCK505 with phenylglyoxal

ICDH purified from E.coli TG1/pCK505 was inactivated with a solution of 10mM phenylglyoxal as in Methods 2.7.1. Results were plotted as for Figure 3.2.

○ no additions

■ + 200μM $NADP^+$

▲ + 100μM isocitrate

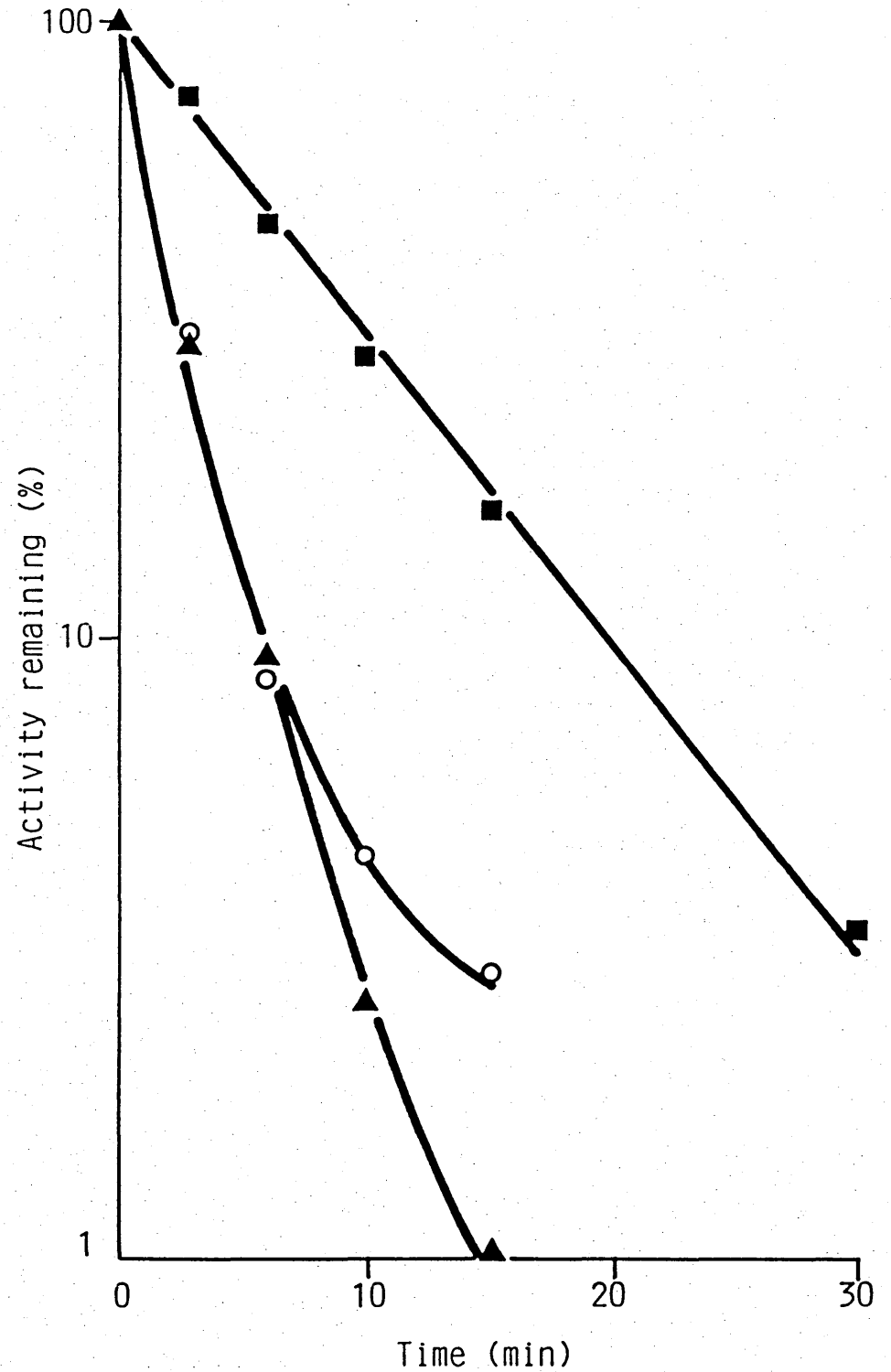

100
10
1
Activity remaining (%)
0
10
20
30
Time (min)

Figure 3.11 Incorporation of [7-^{14}C]-phenylglyoxal into isocitrate dehydrogenase- % Inactivation vs Incorporation

ICDH was inactivated with [7-^{14}C]-phenylglyoxal and the label incorporated determined as in Methods 2.7.2. The amount of label incorporated was determined as nmol phenylglyoxal/nmol ICDH subunit for each time point and the results plotted against percentage inactivation of the ICDH at the corresponding time point.

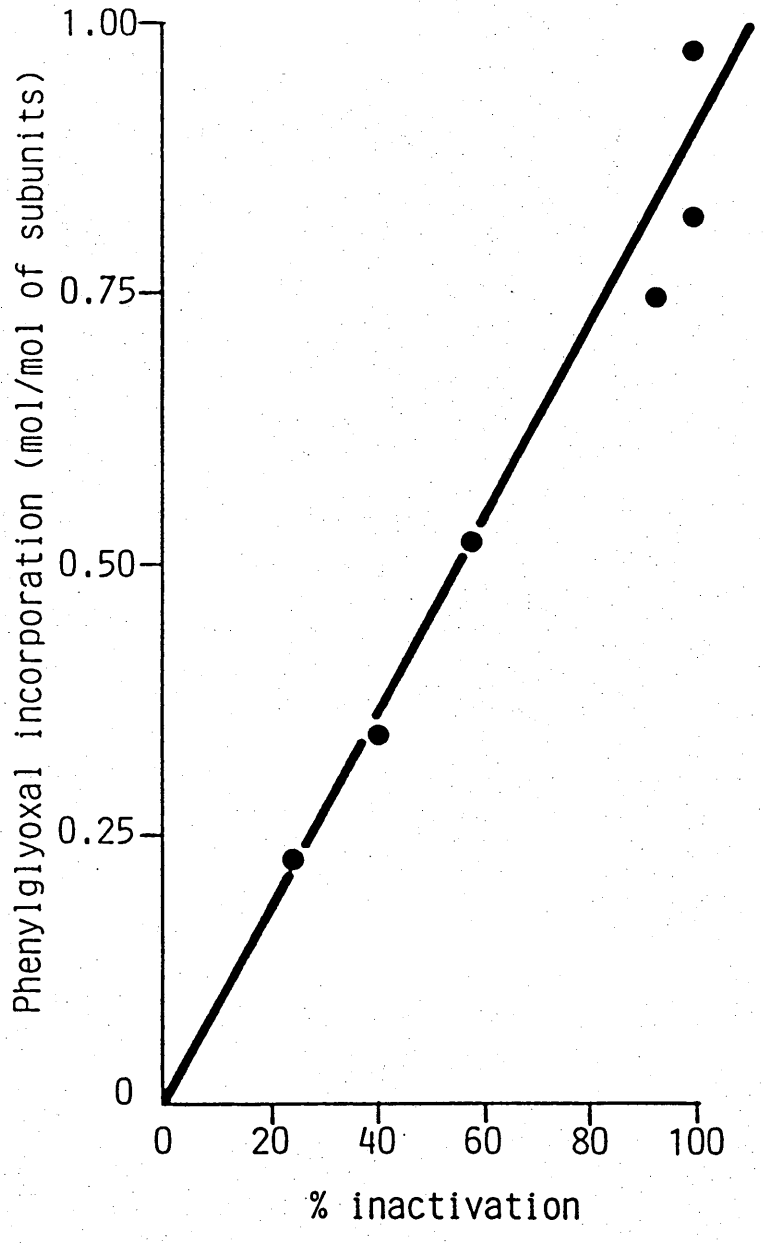

1.00
0.75
0.50
0.25
0
Phenylglyoxal incorporation (mol/mol of subunits)
0
20
40
60
80
100
% inactivation

Figure 3.12 Incorporation of [7-^{14}C]-phenylglyoxal into isocitrate dehydrogenase - Effect of 200μM $NADP^+$

ICDH was inactivated with [7-^{14}C]-phenylglyoxal. Activity loss and incorporation of label were monitored as in Methods 2.7.1 and 2.7.2, respectively. The experiment was also carried out in the presence of 200μM $NADP^+$.

Activity loss

▲ no additions

△ + 200μM $NADP^+$

Incorporation of label

○ no additions

● + 200μM $NADP^+$

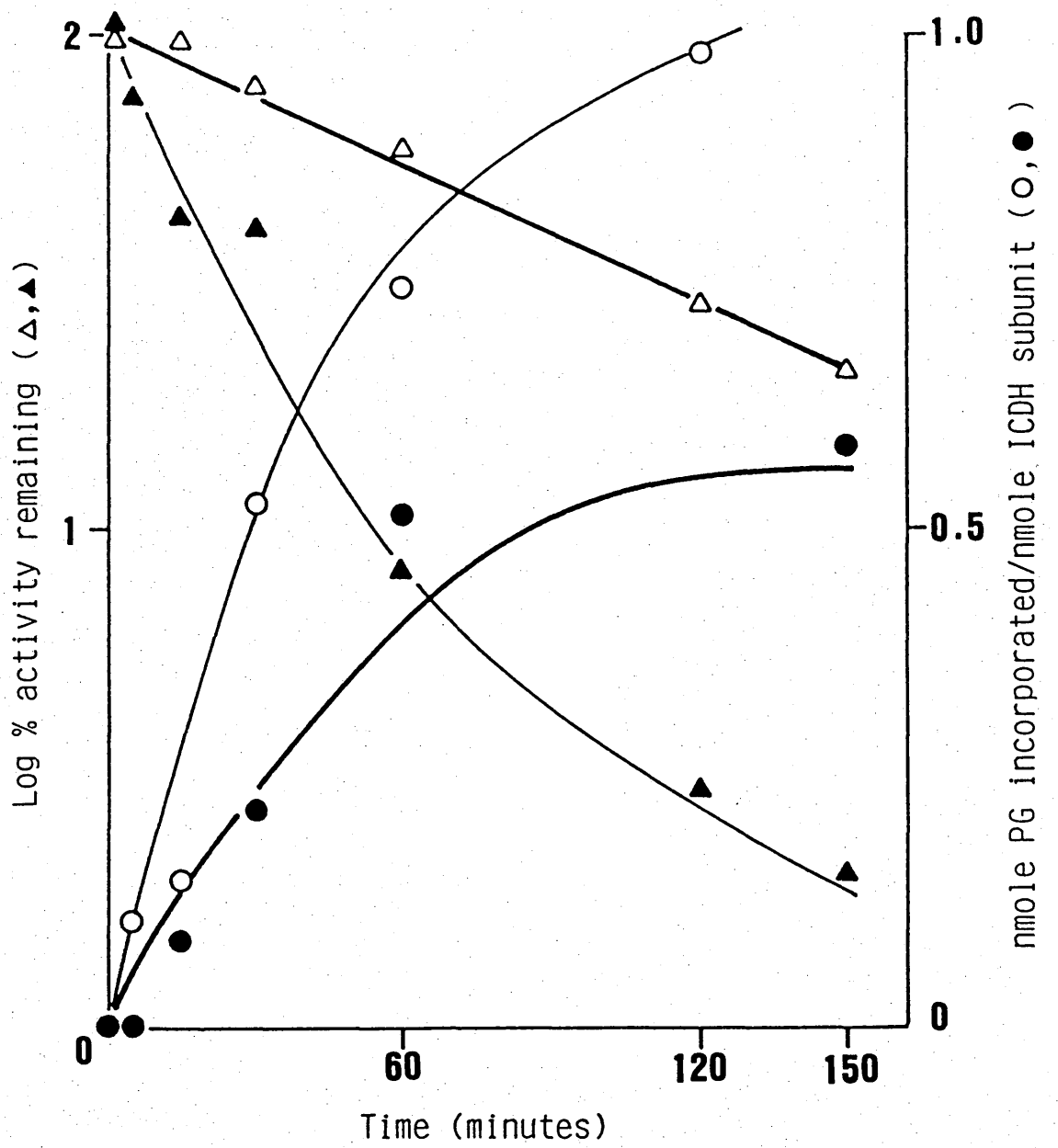

2
1
0
1.0
0.5
0
60
120
150
Time (minutes)
Log % activity remaining (△,▲)
nmole PG incorporated/nmole ICDH subunit (○,●)

Figure 3.13 Incorporation of [7-^{14}C]-phenylglyoxal into isocitrate dehydrogenase - Effect of isocitrate and phosphorylation

ICDH was inactivated with [7-^{14}C]-phenylglyoxal in the presence of 100μM isocitrate. Activity loss and incorporation of label were monitored as in Methods 2.7.1 and 2.7.2, respectively. The data were plotted against time.

Phosphorylated ICDH was also incubated with [7-^{14}C]-phenylglyoxal and incorporation of label determined as in Methods 2.7.2. Again the results were plotted against time.

Activity loss

▲ + 100μM isocitrate

Incorporation of label

● + 100μM isocitrate

○ ICDH-P

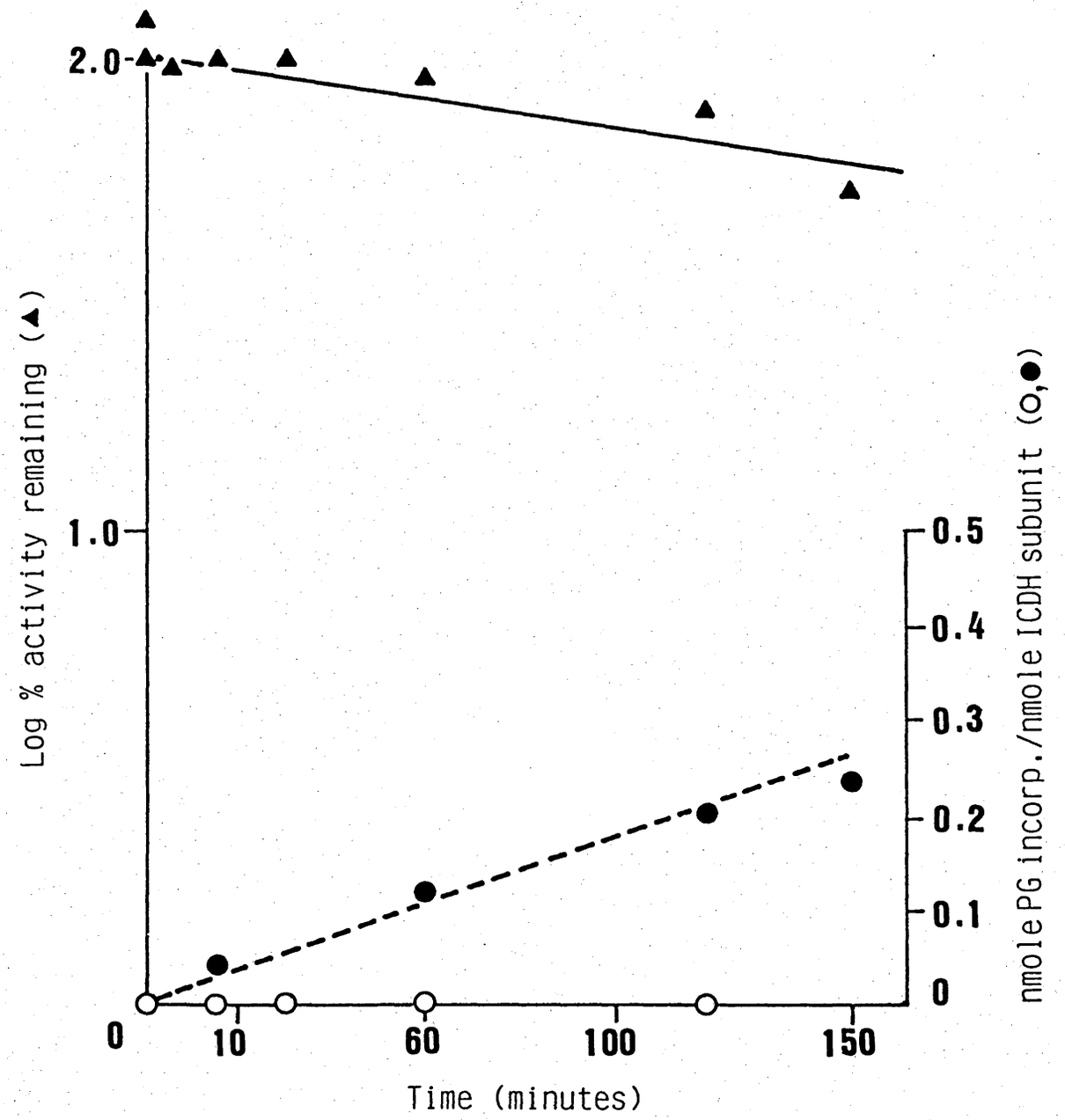

2.0
1.0
0
Log % activity remaining (▲)
0.5
0.4
0.3
0.2
0.1
0
nmole PG incorp./nmole ICDH subunit (O,●)
0
10
60
100
150
Time (minutes)

Figure 3.14 Correlation between incorporation of label into isocitrate dehydrogenase and loss of enzyme activity

The data from Figures 3.11 and 3.12 were replotted as nmol phenylglyoxal incorporated/nmol ICDH subunit vs % inactivation.

- ● no additions
- ▲ + 200μM $NADP^+$
- ○ + 100μM isocitrate

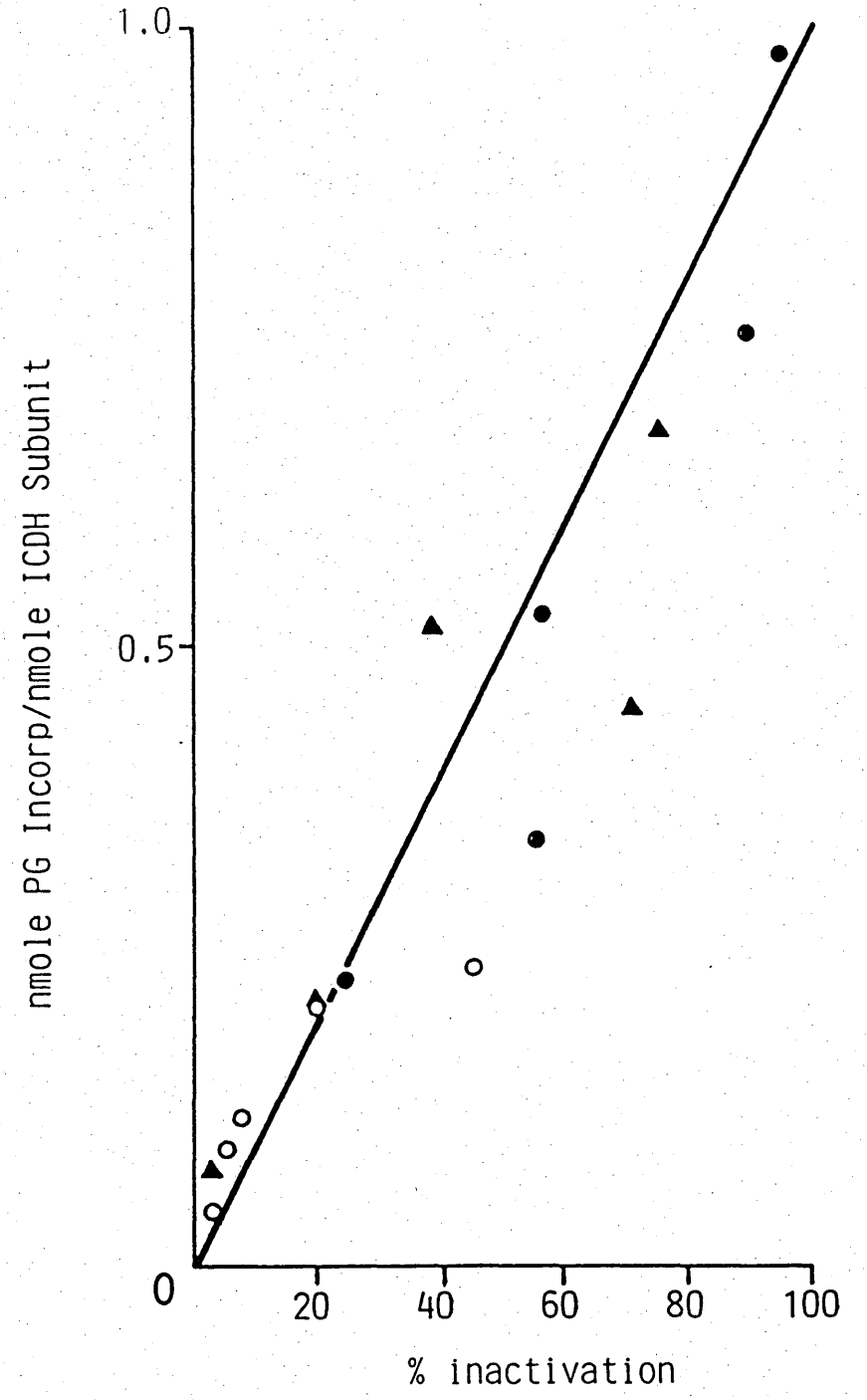

1.0
0.5
0
20
40
60
80
100
% inactivation
nmole PG Incorp/nmole ICDH Subunit

Figure 3.15 Effect of phosphorylated isocitrate dehydrogenase reaction mix components on phenylglyoxal inactivation of isocitrate dehydrogenase

ICDH was inactivated with 10mM phenylglyoxal as in Methods 2.7.1, in the presence of the following metabolites

- ● no additions
- ▽ 0.5mM ADP
- △ 1mM PEP
- ▲ ICDH kinase/phosphatase
- ○ 10mM $MgCl_2$

Results were calculated and plotted as for Figure 3.2.

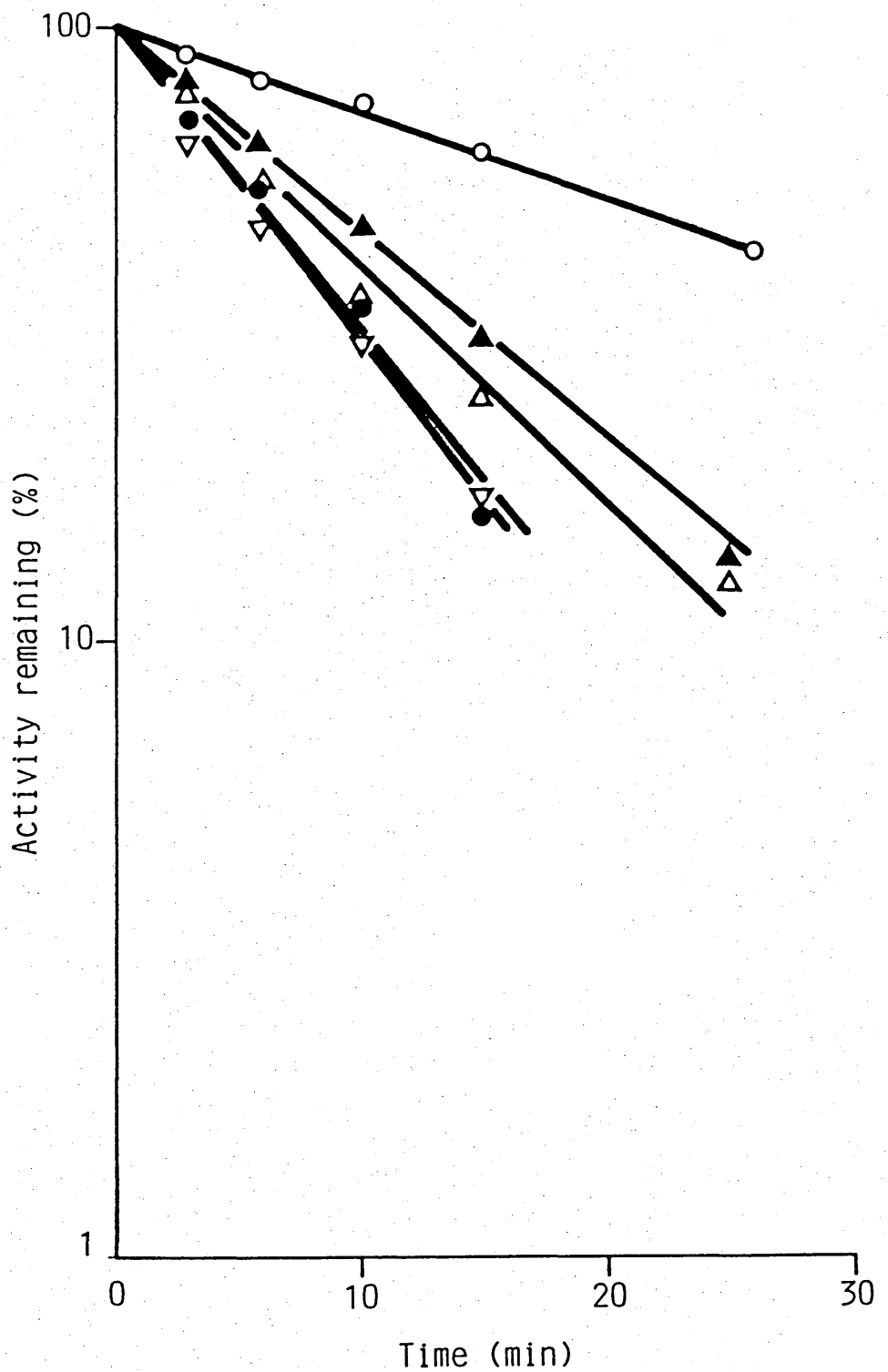

100
10
1
Activity remaining (%)
0
10
20
30
Time (min)

Figure 3.16 Reactivation of phosphorylated isocitrate dehydrogenase followed by phenylglyoxal inactivation

A. Shows the dephosphorylation of phosphorylated ICDH, according to the Methods 2.4.2(c). This was followed by overnight dialysis of the reactivated sample against 50mM MOPS 1mM EDTA pH7.3.

B. Shows inactivation of the dialysed sample with 10mM phenylglyoxal as in Methods 2.7.1.

Results were plotted as units of ICDH activity vs time.

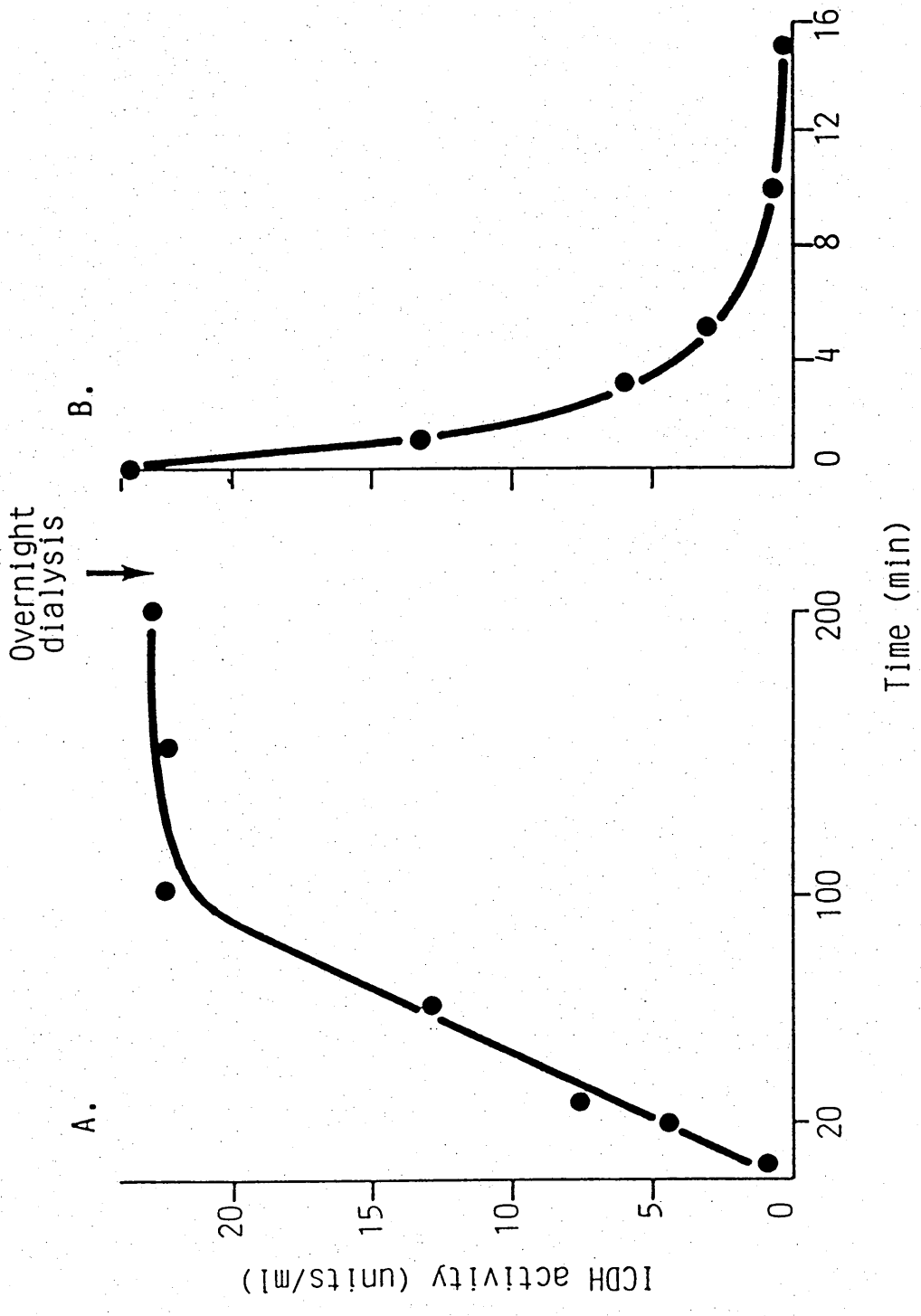
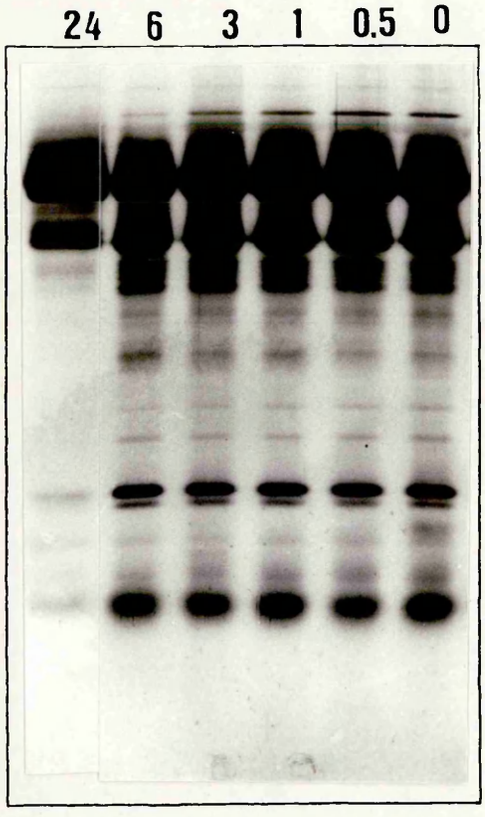

A.
B.
Overnight dialysis
ICDH activity (units/ml)
Time (min)
0
5
10
15
20
20
100
200
0
4
8
12
16

Figure 3.17 Digestion of isocitrate dehydrogenase with chymotrypsin - Effect of phenylglyoxal

A. Shows a time course of digestion of ICDH with chymotrypsin. Digestion was carried out as in Methods 2.8. The digested samples were run on a 15% SDS gel and stained with silver.

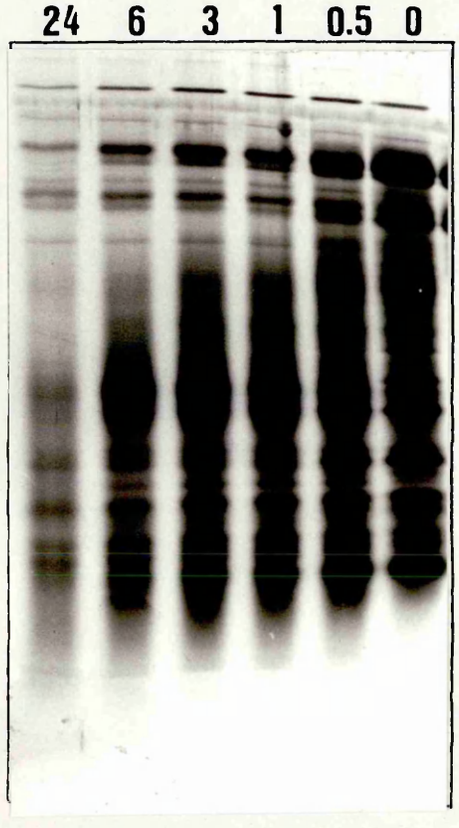
B. Shows a time course of digestion of ICDH, first inactivated with 10mM phenylglyoxal (Methods 2.7.1), then digested with chymotrypsin. Digestion was carried out as in Methods 2.8. The digested samples were run on a 15% SDS gel and stained with silver.

TIME (hr)



A

TIME (hr)

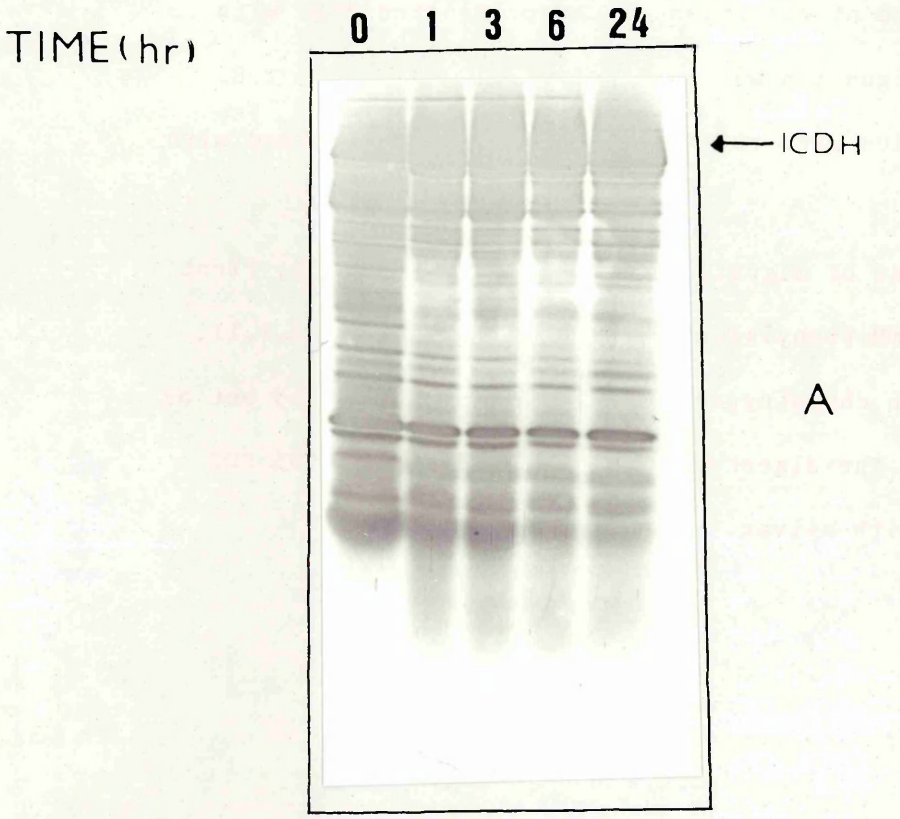


B

Figure 3.18 Digestion of phosphorylated isocitrate dehydrogenase with chymotrypsin - Effect of phenylglyoxal

A. Shows a time course of digestion of phosphorylated ICDH with chymotrypsin. Digestion was carried out as in Methods 2.8. The digested samples were run on a 15% SDS gel and stained with silver.

B. Shows a time course of digestion of phosphorylated ICDH, first incubated with 10mM phenylglyoxal for 30 min (Methods 2.7.1), then digested with chymotrypsin. Digestion was carried out as in Methods 2.8. The digested samples were run on a 15% SDS gel and stained with silver.


TIME(hr)
0 1 3 6 24
ICDH
A

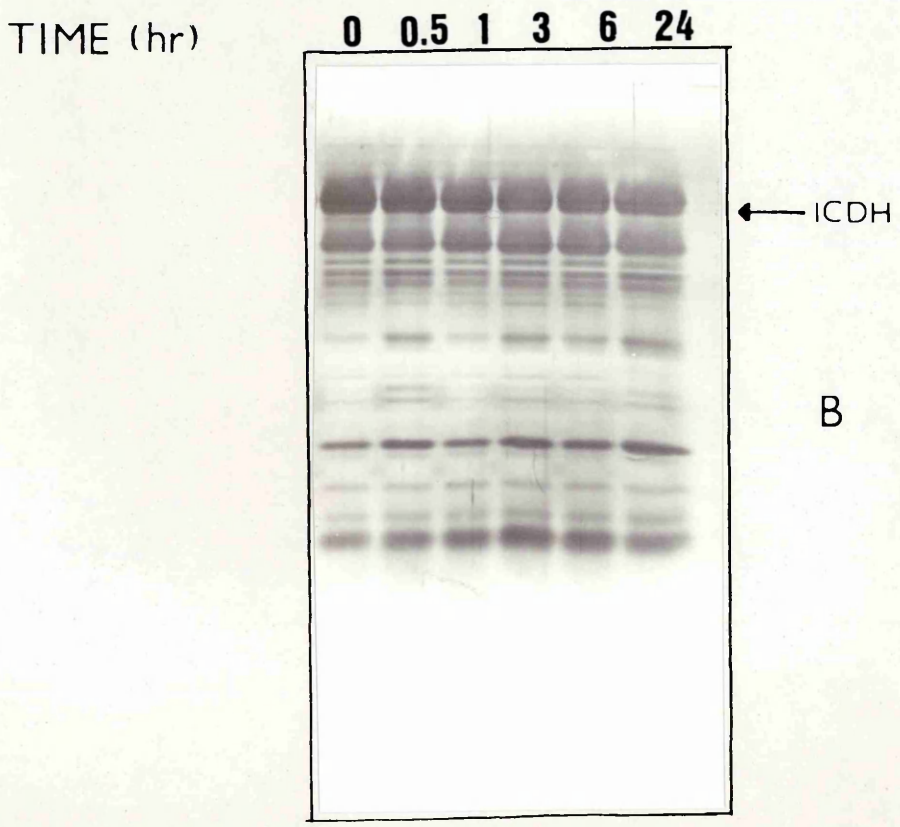

TIME (hr)
0 0.5 1 3 6 24
ICDH
B

Table 3.1 Purification of isocitrate dehydrogenase from E.coli ML308 grown on 20mM glycerol.

Details of each stage of the purification are outlined in Methods 2.5.2 (a) to (e).

Table 3.1

Step	Total Protein (mg)	Activity units	Specific Activity units/mg	Yield %	Purification -fold
Extract	2575	2508	0.97	100	1
60-70% $(NH_4)_2SO_4$	216	1698	7.86	67.7	8.2
DEAE-cellulose	58.2	1627	27.9	64.9	28.8
Procion red sepharose	8.2	1447	176.5	57.7	182

Table 3.2 Comparison of K_s and K_m values of isocitrate dehydrogenase for $NADP^+$ and isocitrate

Table 3.2

Ligand	K_s	K_m
$NADP^+$	60μM	22.7μM
Isocitrate	16μM	8μM
Citrate	3.6mM	–

Table 3.3 Incorporation of [7-^{14}C]-phenylglyoxal into isocitrate dehydrogenase and phosphorylated isocitrate dehydrogenase.

- Effect of $NADP^+$ and isocitrate.

ICDH and ICDH-P were incubated with [7-^{14}C]-phenylglyoxal in the presence or absence of $NADP^+$ or isocitrate as in Methods 2.7.2. This table shows the incorporation obtained after 150 min, expressed as nmoles phenylglyoxal incorporated/nmol ICDH subunit.

Table 3.3

Incubation	nmol PG incorp./nmole ICDH subunit
ICDH	0.977
ICDH+200μM $NADP^+$	0.589
ICDH+100μM isocitrate	0.239
ICDH-P	0.01

Table 3.4 Selective modification of arginyl residues

(Taken from Riordan, 1979)

Table 3.4

Enzyme	Reagent	Arg Modified	Total Arg
Creatine kinase	Phenylglyoxal	1	18
Aldolase	Phenylglyoxal	1	14
Malate dehydrogenase	Butanedione	1	8
Aspartate transcarbamylase	Phenylglyoxal	1	24
Glutamic dehydrogenase	Cyclohexanedione	1	17
Phosphoglycerate mutase	Butanedione	1	16
Adenylate kinase	Phenylglyoxal	1	11
Alcohol dehydrogenase	Butanedione	2	11
Carboxypeptidase	Butanedione	2	10

Table 3.5 Comparison of the inactivation of three enzyme activities with different dicarbonyl reagents

(a) Bovine carboxypeptidase A

(b) Beef liver glutamate dehydrogenase

(c) Pig heart aspartate aminotransferase

a Riordan (1979)

b David *et al.* (1976)

c Blumenthal & Smith (1974)

d Riordan & Scandurra (1975)

e Gilbert & O'Leary (1975)

Table 3.5

(a)

Reagent	Reaction time (min)	Molar excess reagent	% activity remaining peptidase	% activity remaining esterase
Phenylglyoxal[a]	60	150	37	64
2,3-butanedione[a]	15	150	14	290
1,2-cyclohexanedione[a]	30	150	99	108

(b)

Reagent	Reaction time (min)	Molar excess reagent	% activity remaining
Phenylglyoxal[b]	90	150	0
2,3-butanedione[b]	150	150	15-20
1,2-cyclohexanedione[c]	5	750	45

(c)

Reagent	Reaction time (min)	Molar excess reagent	% activity remaining
Phenylglyoxal[d]	30	175	15
2,3-butanedione[d]	20	550	5
1,2-cyclohexanedione[e]	100	15600	0

Table 3.6 Comparison of the inactivation of a selection of enzyme activities with phenylglyoxal

1. Cole *et al*. (1986)
2. Koland *et al*. (1982)
3. Borders & Riordan (1975)
4. Rogers *et al*. (1978)
5. Powers & Riordan (1975)
6. Lobb *et al*. (1976)
7. This work, Chapter 3

Table 3.6

Enzyme	Reaction time (min)	Phenylglyoxal conc. (mM)	Enzyme conc. (μM)	% Activity remaining
Wheat germ aspartate transcarbamoylase[1]	4	5	0.1	31
E.coli pyruvate oxidase[2]	20	5	3.5	40
Rabbit muscle creatine kinase[3]	70	0.2	2.5	10
Chicken egg white ovotransferrin[4]	50	17	65	40
Ovine brain glutamate synthetase[5]	20	4	0.75	20
Rabbit muscle aldolase[6]	30	4	12	20
E.coli isocitrate dehydrogenase[7]	15	20	4.4	5

CHAPTER FOUR

Attempts to Isolate a Peptide Labelled with [7-^{14}C]-Phenylglyoxal from the Active Site of Isocitrate Dehydrogenase

4.1. Introduction

The complete amino acid sequence of E. coli ICDH was recently deduced from the nucleotide sequence of the icd gene (Thorsness and Koshland, 1987). The sequence round the phosphorylation site was determined by conventional means (Borthwick et al., 1984; Malloy et al., 1984). These known sequences are of great value in that they provide a basis for studies of other structural features of ICDH, such as the active site of the enzyme.

Inactivation of ICDH with phenylglyoxal is believed to result in modification of an active site arginine residue, as judged by protection experiments (see Chapter 3). The initial objective of the work described in this chapter was to isolate peptides from ICDH that were labelled with [7-^{14}C]-phenylglyoxal. Sequencing would then allow comparison with the whole primary sequence and the phosphorylation site. If the modified arginine proved to be adjacent to the phosphorylatable serine residue, this would provide evidence to support the proposed theory that phosphorylation occurs close to or at the $NADP^+$ binding site. Of course. the tertiary structure of an enzyme's active site is normally constructed from residues separated in the primary sequence, brought together to form the active site by protein folding. It is quite possible that the arginine residue at the co-enzyme binding site, i.e. the residue labelled by phenylglyoxal, is not the arginine which is adjacent to the phosphorylatable serine in the primary sequence but a residue spatially close to the serine residue as a result of tertiary structure. If this were the case, identification of the arginine residue modified by phenylglyoxal would neither confirm nor deny the hypothesis about phosphorylation given the absence of a high resolution 3-dimensional structure of the enzyme. However any attempt to identify this arginine residue would prove useful in that an active site residue would be defined.

It has been claimed that arginine modification by phenylglyoxal is more stable under acid conditions (Patthy and Thesz, 1980), and that labelled peptides can be isolated. For example, arginine residues have been identified in rabbit muscle aldolase (Patthy et al., 1979), glucose 1-phosphatase (Vandenbunder et al., 1981) and lysozyme (Patthy and Smith, 1975). Therefore we intended to do digestion and peptide purification in acid conditions, of ICDH which had been labelled with phenylglyoxal in the presence or absence of $NADP^+$ or isocitrate. We hoped that these compounds might protect specific peptides, thus allowing identification of an active site peptide.

4.2 Results

4.2.1 Large scale labelling of ICDH with [7-^{14}C] phenylglyoxal

4.2.2 Labelling

Large scale labelling of ICDH involved some alterations to the conditions used in previous labelling experiments (Method 2.7.2), as well as development of a suitable method to remove unreacted label. Incorporation experiments had been carried out with an enzyme concentration of 0.2 mg/ml and a phenylglyoxal concentration of 10-20mM. To save on radioactive reagent, and also to reduce the reaction volume, the concentration of the ICDH was increased to 1 mg/ml. However, trial experiments with this enzyme concentration and non-radioactive phenylglyoxal resulted in very little enzyme inactivation over a two hour time course. Due to the increase in ICDH concentration, the concentration of the ICDH storage buffer (10mM potassium phosphate, 5mM potassium citrate, 2mM Magnesium chloride 40% glycerol pH6.5) components also increased greatly. Citrate in particular was found to protect the enzyme against inactivation with phenylglyoxal. This allowed a K_s of 3.6mM for binding of citrate to ICDH to be determined (Fig. 4.1). $MgCl_2$ at this higher concentration was also known to affect the rate of inactivation with phenylglyoxal (see Fig. 3.15).

Dialysis of the ICDH against 20mM potassium phosphate, 2mM $MgCl_2$, 10% glycerol, pH6.5 to remove the citrate overcame the problem and allowed inactivation of 1 mg/ml ICDH using 10mM phenylglyoxal.

Large scale labelling of ICDH was carried out with enzyme alone or in the presence of 200μM $NADP^+$ of 100μM isocitrate. Activity was monitored at the start and at the end of a 2-1/2 hour incubation. Each reaction was stopped by loading the sample on to a Sephadex G-50 column equilibrated in 10% formic acid. The labelled fractions were pooled, freeze dried and resuspended in a smaller volume of 10% formic acid for digestion with pepsin. At each stage a small sample was monitored for presence of ^{14}C-radioactivity. After overnight digestion the samples were freeze dried and then dissolved in a small volume of 0.1% TFA (100-200μl). A small sample was counted for presence of ^{14}C-radioactivity still associated with the peptides.

4.2.3 HPLC Analysis of peptide samples

^{14}C-labelled peptides were separated by reverse-phase chromatography on a C_{18} column, equilibrated in 0.1% TFA and eluted with a gradient of acetonitrile in 0.1% TFA. Figure 4.2 shows a trace of peptide separation of labelled ICDH which had been digested with pepsin. A sample from each fraction was counted for ^{14}C-radioactivity and these data are also shown. There was one major peak of radioactivity eluted early on in the run (Peak 1) Table 4.1 shows the radioactivity associated with this peak for the three samples ICDH, ICDH + 200μM $NADP^+$ and ICDH + 100μM isocitrate. The ^{14}C- radioactivity incorporated for the protected samples was reduced as compared to the ICDH alone, as we would expect from the protection experiments (Chapter 3).

The ^{14}C- radioactivity associated with the ICDH sample after HPLC separation (Pool 1, Fig. 4.2) was 71% of the initial radioactivity associated with the peptide sample before the protease digestion step.

The radioactive fractions from this HPLC separation were pooled and freeze dried and rerun on a reverse - phase column with a shallower acetonitrile gradient (Fig. 4.3). Only 39% of the initial ^{14}C was found to be still associated with the peptide fractions after this run (Fig. 4.3). The two radioactive peaks were pooled independently, freeze dried and run on an HPLC gel filtration column (TSK, G2000 SW column (7.5 x 600mm), LKB) equilibrated in 0.1% TFA. Analysis of the fractions obtained showed that only 15% of the initial ^{14}C- radioactivity was recovered. Only one radioactive peak was observed but it did not correspond to any of the peptide peaks as judged by the A_{215} trace (Fig. 4.4). Any further analysis of the peptide sample failed because of the loss of ^{14}C- radioactivity at each step (Table 4.2).

The data thus suggested an instability of the arginine-phenylglyoxal adduct in acid conditions which were previously thought to promote stability. We therefore decided to assess the stability of the adduct more carefully.

4.3 Stability of the phenylglyoxal-arginine adduct

The stability of the adduct was studied in the following ways:

4.3.1 Dialysis

ICDH was inactivated with phenylglyoxal (Method 2.7.1). The mixture was then dialysed against 50mM Mops-NaOH, 1mM EDTA pH7.3 for long time intervals and the mix assayed for regain of the ICDH activity. Data showed that regeneration of ICDH activity could be achieved with long periods of dialysis, over 24 hours, and indicated an instability of the ICDH-phenylglyoxal adduct (Table 4.3).

4.3.2 Washing with trichloroacetic acid

ICDH was inactivated with [7-^{14}C]-phenylglyoxal, 10µl samples were spotted onto filter paper discs and washed for 4x1 hour periods in 10% TCA (Method 2.7.2). At this point one disc was removed and counted for ^{14}C- radioactivity. This washing period was known to remove any

unreacted label from the discs, but not protein-associated label. (Chapter 3). The remaining discs were left in 10% TCA and washed for increasing lengths of time, up to 48 hours. Discs were removed at set times over this period and counted for protein-associated label. Figure 4.5 shows that label is lost from ICDH over the time course. It appears to be a biphasic decay with the initial phase corresponding to rapid loss of unreacted label followed by a slow decay of protein-associated label.

4.3.3 Stabilisation with $NaBH_4$

It has been suggested that the reaction of arginine with phenylglyoxal may proceed by formation of a Schiff-base (Takahashi, 1968). It is possible to trap a Schiff-base, and hence stabilize the structure, by addition of $NaBH_4$ (e.g. Chaudhuri et al., 1986). It was found that $NaBH_4$ could inactivate ICDH in the absence of phenylglyoxal. As a consequence we could not test whether $NaBH_4$ stabilised the ICDH-phenylglyoxal adduct by monitoring any regain of activity after dialysis. However, incorporation of label, and loss of label, could be monitored by precipitating the protein with TCA. Table 4.4 shows the ^{14}C- radioactivity associated with ICDH $\pm$ $NaBH_4$ treatment for several washing conditions (Method 2.7.4) ICDH that had been treated with phenylglyoxal and then $NaBH_4$ contained no more ^{14}C-radioactivity than control enzyme treated with phenylglyoxal alone. Thus treatment with $NaBH_4$ did not affect the stability of the phenylglyoxal-arginine adduct indicating that Schiff-base formation in the reaction between phenylglyoxal and ICDH was unlikely.

4.4 Comparison of thermolytic digests of ^{32}P- ICDH and [7-^{14}C]-phenylglyoxal ICDH

These results indicated that the arginine-phenylglyoxal adduct was unstable over long periods of washing or dialysis. I therefore decided to reduce the time between labelling of ICDH and analysis of the peptide digests. To do this I digested labelled ICDH and used SDS-PAGE

separation of the peptides directly after labelling and without removing unbound label by dialysis or precipitation (Method 2.7.6).

The first requirement was to find a suitable protease for digestion. The objective was to compare peptides from ^{32}P- phospho ICDH and ^{14}C- phenylglyoxal inactivated ICDH in order to establish whether the same peptide was labelled in each case. Therefore the prerequisite was that the protease produced the same digestion pattern with P-ICDH and phenylglyoxal-inactivated ICDH. Chymotrypsin, for example, would not be suitable (Section 3.4). Several proteases, and various digestion conditions were tested before thermolysin was found to be the most suitable (Figure 4.6).

ICDH was inactivated with [7-^{14}C]-phenylglyoxal and immediately digested with thermolysin, without removing unbound label. ^{32}P- ICDH was similarly digested with thermolysin. Duplicate samples were run on either side of a 15% SDS gel. This enabled one half to be stained for protein while the other half was dried down immediately after electrophoresis for autoradiography. The results are shown in Figure 4.7. At least one radioactive band appears common to both digests, and corresponds to a protein staining band for both samples. The radioactive band runs above the gel dye front. SDS PAGE of the radioactive fragments and comparison with myoglobin fragments indicated that the labelled peptide was less than 2.5kDa in size.

This is good, albeit indirect, evidence that the arginine residue at the active site of ICDH is in the same thermolytic fragment as the phosphorylatable serine residue.

4.5 Discussion

The stability of the arginine-phenylglyoxal adduct has been a well documented problem, with reports of both stable and unstable adducts being formed. Takahashi (1968) reported that the inactivation of arginine residues with phenylglyoxal had a stoichiometry of two phenylglyoxal to one arginine. He reported that the product was stable under acidic conditions but decomposed slowly at neutral or alkaline

pH. The stability was such that a labelled peptide could be isolated. Borders & Riordan (1975) reported a 1:1 stoichiometry for the phenylglyoxal arginine reaction for creatine kinase. Peters et al. (1981) suggested that the question of irreversibility and stoichiometry were not unrelated. They stated that formation of a stable 2:1 adduct proceeds via a 1:1 adduct that is formed reversibly (Fig. 4.8). It has been suggested that the formation of the 2:1 adduct is in some cases prohibited by the steric constraints of protein structure, accounting for the frequent observation of 1:1 adducts.

The reaction products of the arginine-dicarbonyl reaction have caused much discussion. No reaction mechanism has yet been established, although many possible schemes have been put forward. Patthy and Thesz (1980) proposed that the reaction of the guanidino group with dicarbonyl reagents proceeds via a set pathway, the rate limiting step being essentially identical with an amine-carbonyl addition reaction. They described the obligatory step of the reaction as the formation of a double carbinolamine a compound containing vicinal cis-hydroxyl groups. In the case of the 1,2 cyclohexanedione-arginine reaction they established the formation of such a compound (Patthy et al., 1979). Stabilization of the butanedione-arginine reaction can be achieved by inclusion of borate in the inactivation mixture. However, addition of borate to an ICDH-phenylglyoxal inactivation experiment had no such effect on the stability. Incorporation of [7-^{14}C]- phenylglyoxal into ICDH (Section 3.3.3) indicated that incorporation of 1 phenylglyoxal molecule per enzyme subunit resulted in complete inactivation of enzyme activity. This obviously suggests that an active site arginine reacts with one phenylglyoxal. However it does not rule out the possibility that two phenylglyoxal residues have reacted with one arginine residue but at only half of the sites still resulting in inactivation of the dimeric ICDH.

The lack of stability is not, perhaps, surprising in view of the results of Peters et al.(1981) data. However our initial experiments had shown that the modification was stable for up to four hours in TCA. Our decision to isolate a labelled peptide using acidic conditions, was based on this observation and the fact that others had shown these to be the most suitable for stabilization of the inactivation product. In retrospect the nature of the observed 1:1 adduct with ICDH indicated that the inactivation product would prove to be of an unstable nature. HPLC analysis of labelled peptides proved unsuccessful. The modification proved to be unstable in the longterm when unreacted label was removed in acid or neutral conditions. My more detailed analysis of the stability confirmed this. As a consequence of the instability, we failed to achieve our initial objective of isolating a ^{14}C- labelled peptide. Attempts to stabilize the inactivation product with $NaBH_4$ proved unsuccessful.

We therefore looked for an alternative method which would enable us too compare the site(s) labelled by phenylglyoxal with the phosphorylation site. The thermolytic digestion experiment shows the existence of at least one common radioactively labelled peptide between ^{32}P- ICDH and [7-^{14}C]-phenylglyoxal ICDH. In the primary sequence of ICDH, excluding the adjacent arginine residue, the nearest arginine to the phosphorylatable serine residue is 14 residues away. Although we have no conclusive evidence it seems most likely that the arginine modified by phenylglyoxal is the residue adjacent to the phosphorylatable serine. This supports the idea that phosphorylation of ICDH occurs at its co-enzyme binding site.

Figure 4.1 Determination of the K_S value for citrate in the presence of 10mM phenylglyoxal

ICDH was inactivated with 10mM phenylglyoxal in the presence of increasing concentrations of citrate (Methods 2.7.1). The half-life for inactivation was calculated for each citrate concentration and plotted against the citrate concentration.

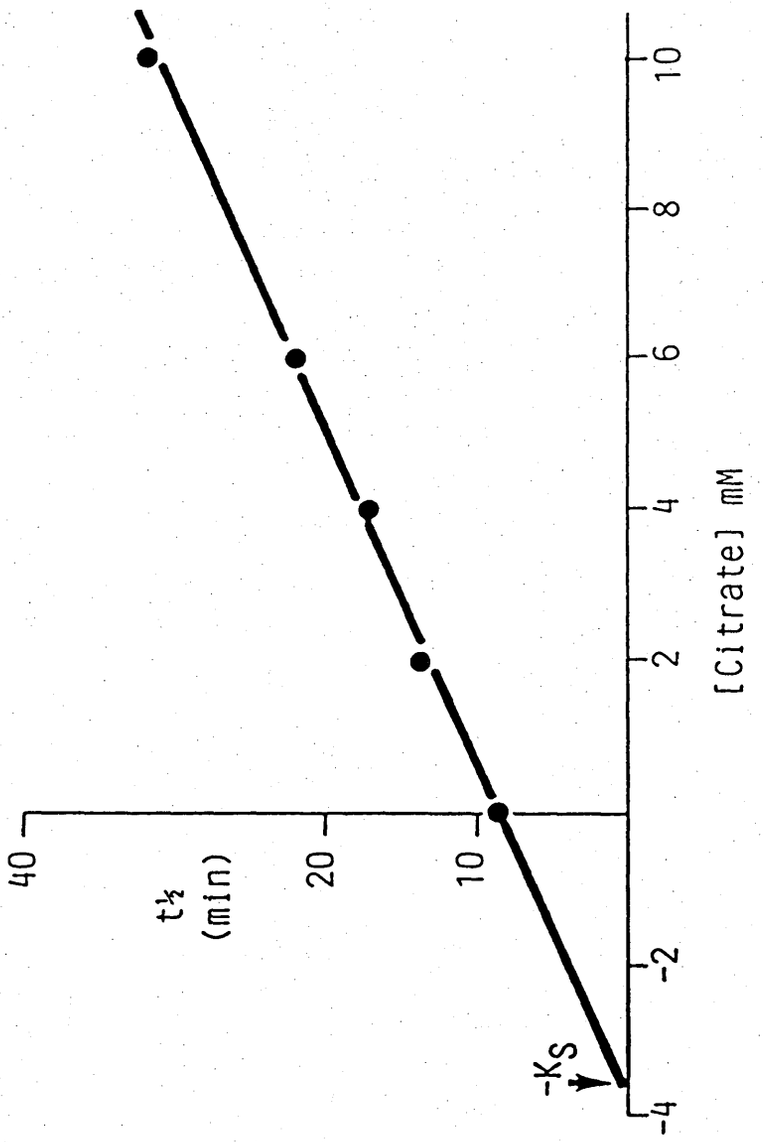

40
20
10
t½
(min)
-Ks
-4
-2
2
4
6
8
10
[Citrate] mM

Figure 4.2 HPLC separation of isocitrate dehydrogenase peptic digests - (A)

ICDH labelled with [7-^{14}C]-phenylglyoxal (Methods 2.7.3a) was digested with pepsin (Methods 2.7.3b) and loaded onto a μ bondapak C18 reverse-phase HPLC column. The peptides were eluted with a linear gradient of acetonitrile. The flow rate was 1ml/minute. 1ml fractions were collected. A sample of each fraction (50μl) was counted for radioactivity and this data is also shown.

Fractions 5-10 were pooled and freeze-dried for further purification (Pool 1).

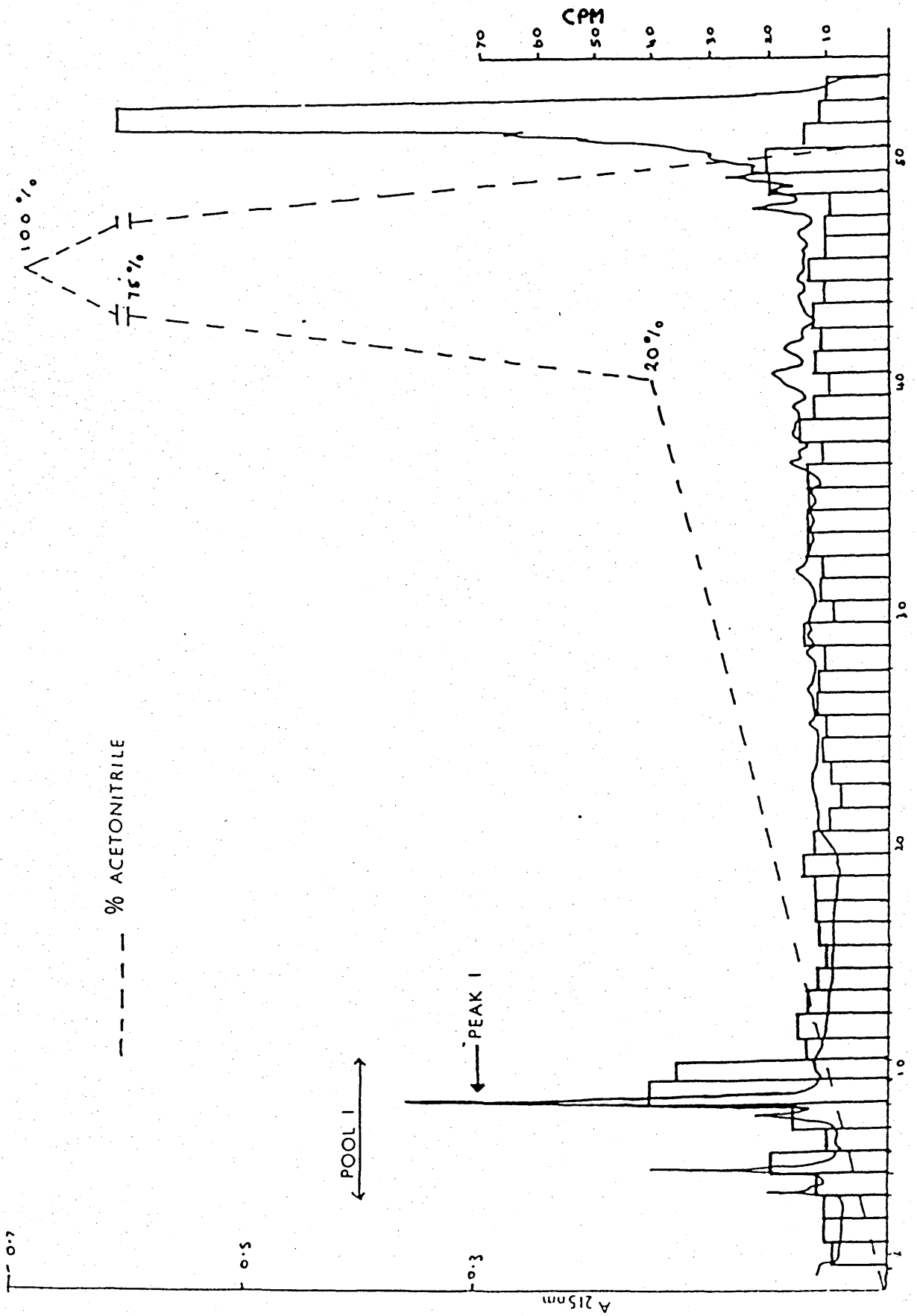

CPM
70
60
50
40
30
20
10
100%
75%
20%
% ACETONITRILE
PEAK 1
POOL 1
A 215nm
0·7
0·5
0·3
60
50
40
30
20
10
1

Figure 4.3 HPLC separation of isocitrate dehydrogenase peptic digests - (B)

The pooled fractions from Figure 4.2 (Pool 1) were further purified by a second run on a μ bondapak C18 reverse-phase HPLC column. The peptides were eluted with a shallower acetonitrile gradient. The total running time for the gradient was 60 min. Only the 5-34 min section of the chromatogram is shown. The flow rate was 1ml/minute. 1ml fractions were collected. A sample of each fraction (50μl) was counted for radioactivity and this data is also shown.

The following fractions were pooled:

Pool 1 - Fractions 10-12

Pool 2 - Fractions 16-17

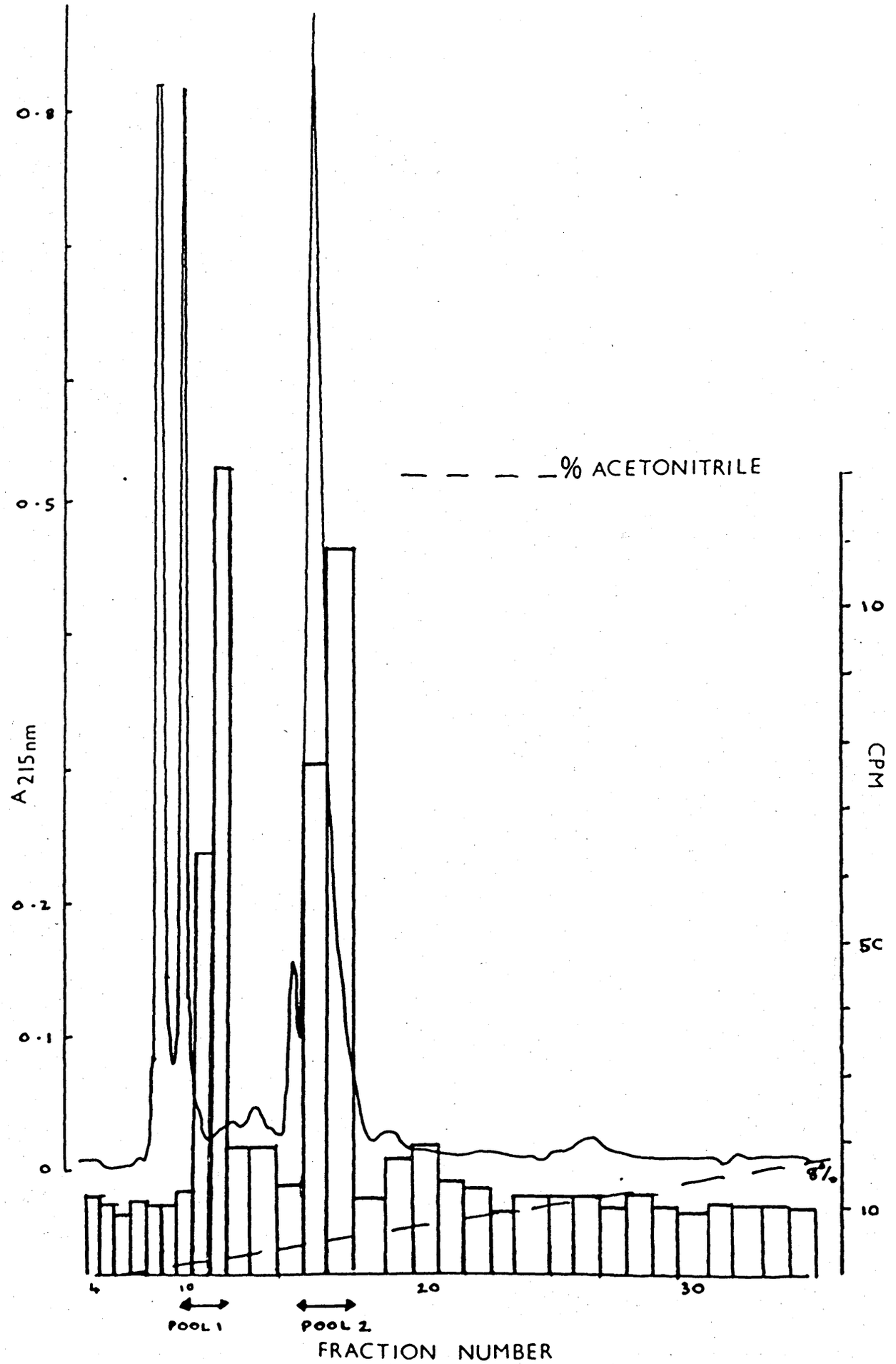

0·8
0·5
0·2
0·1
0
A215nm
10
5C
CPM
10
8%
4
10
20
30
POOL 1
POOL 2
FRACTION NUMBER
% ACETONITRILE

Figure 4.4 HPLC separation of isocitrate dehydrogenase peptic digests - (C)

Pool 2 from Figure 4.3 was further purified by separation on an HPLC gel filtration column (TSK G2000 SW column, 7.5 x 600mm). The flow rate was 0.5ml/minute. One minute fractions were collected. A sample of each fraction (50µl) was counted for radioactivity and this data is also shown.

Only the 40-68 minute section of the chromatogram is shown.

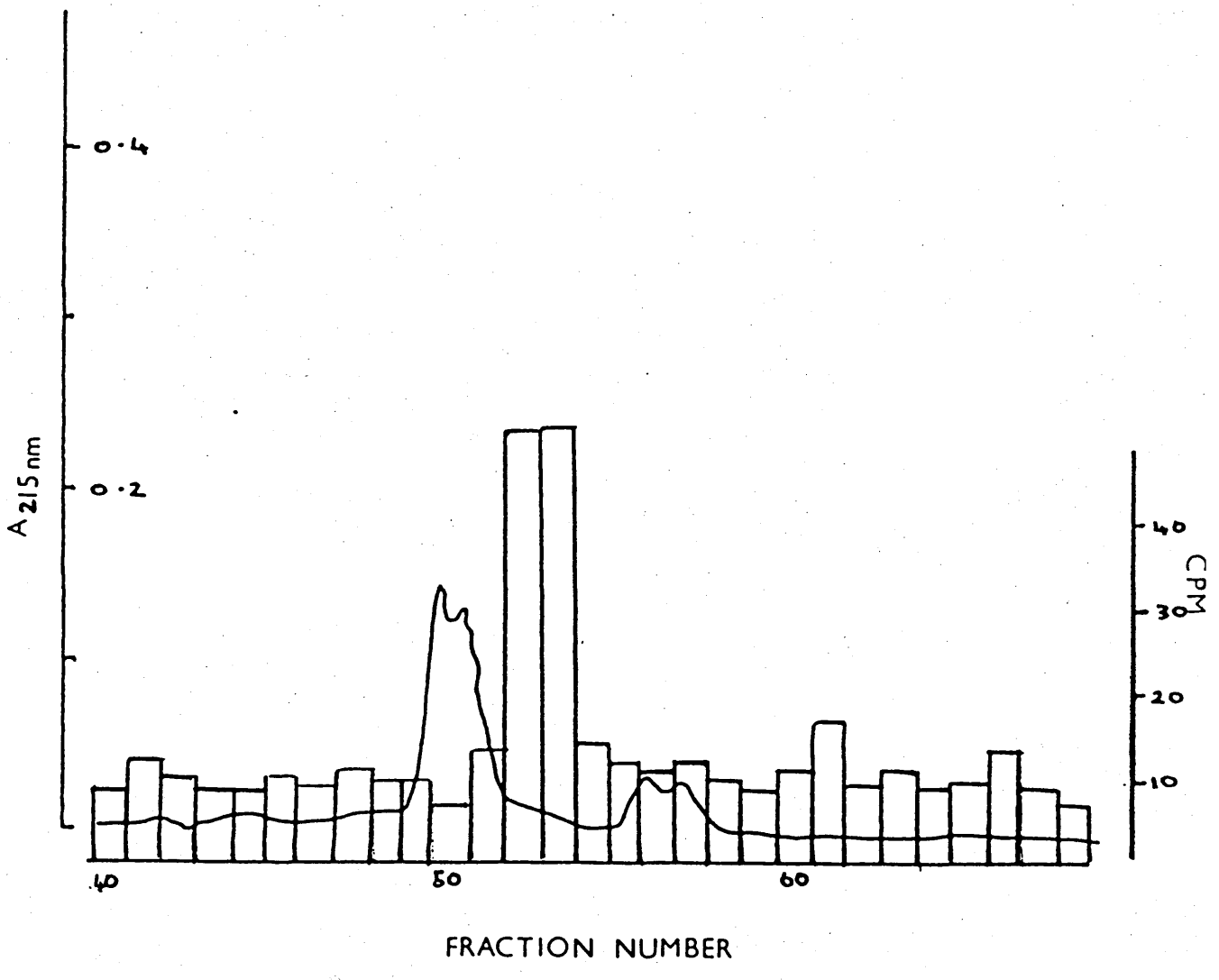

0·4
0·2
A215nm
40
30
20
10
CPM
40
50
60
FRACTION NUMBER

Figure 4.5 Loss of [7-^{14}C]-phenylglyoxal from filter paper discs

ICDH was inactivated with 10mM [7-^{14}C]-phenylglyoxal as in Methods 2.7.1. At 0 min and 120 min during the inactivation 8 x 10µl samples were removed and spotted onto separate filter paper discs. The discs were washed for increasing time periods in 10% TCA (Methods 2.7.2). The TCA solution was changed at 1, 2, 3, 4, 16, 24, 36 hours. At set times the discs were removed and counted for protein associated label.

A control experiment was set up using 8 x 10µl samples of 10mM [7-^{14}C]-phenylglyoxal alone spotted onto filter discs and washed as above.

△ 10µl 10mM [7-^{14}C]-phenylglyoxal

■ 0 min incubation

● 60 min incubation

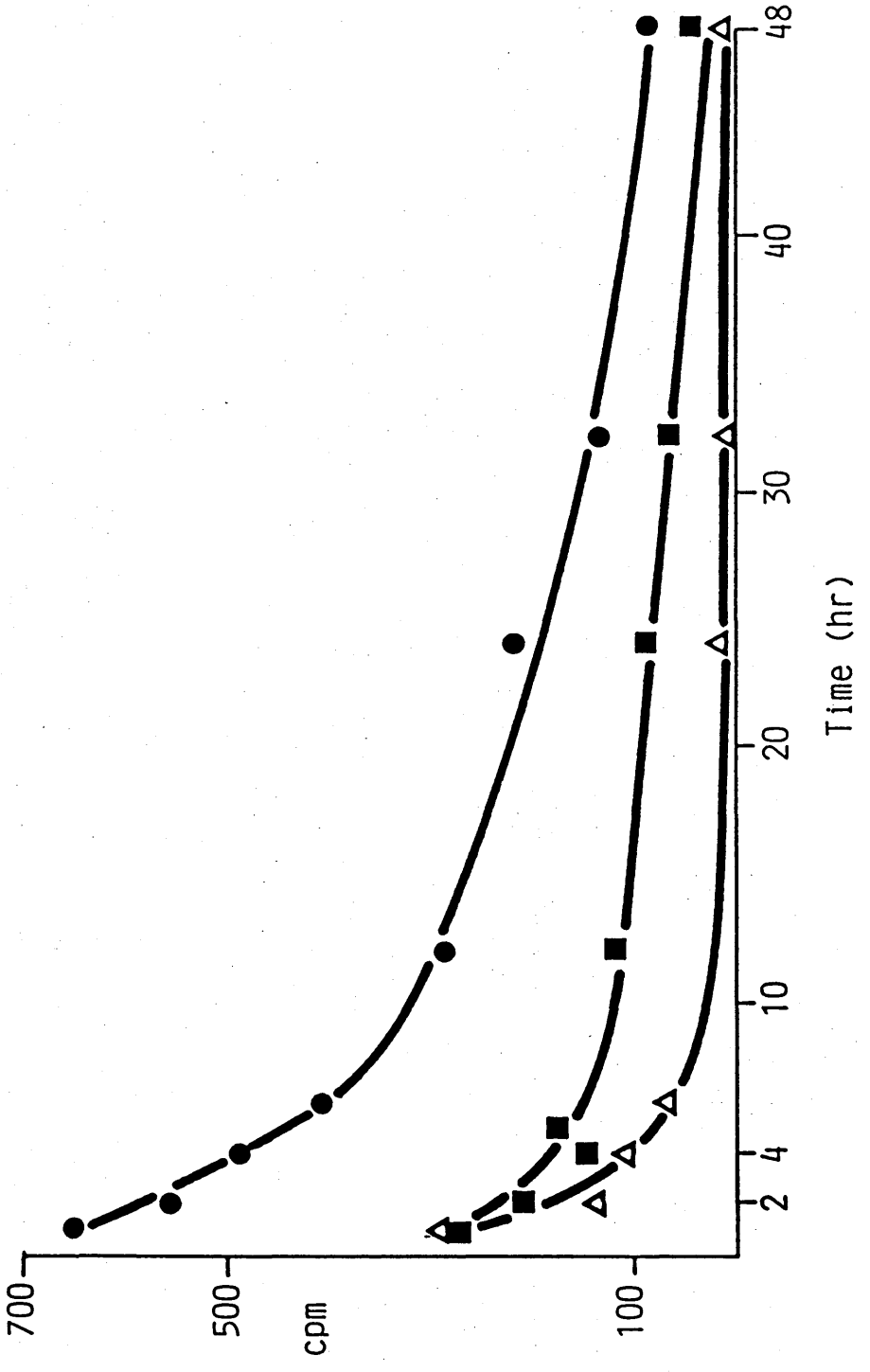

700
500
100
cpm
2
4
10
20
30
40
48
Time (hr)

Figure 4.6 Digestion of [^{32}P]-phospho-isocitrate dehydrogenase and [7^{14}C]-phenylglyoxal-isocitrate dehydrogenase with thermolysin

Phosphorylated ICDH and ICDH labelled with phenylglyoxal were digested with thermolysin as in Methods 2.7.6. The digested samples were run on a 15% SDS gel and stained with silver (Methods 2.6.4b).

Tracks A,B - [7-^{14}C]-phenylglyoxal-ICDH thermolytic digests

Tracks C,D - [^{32}P]-phospho ICDH thermolytic digests

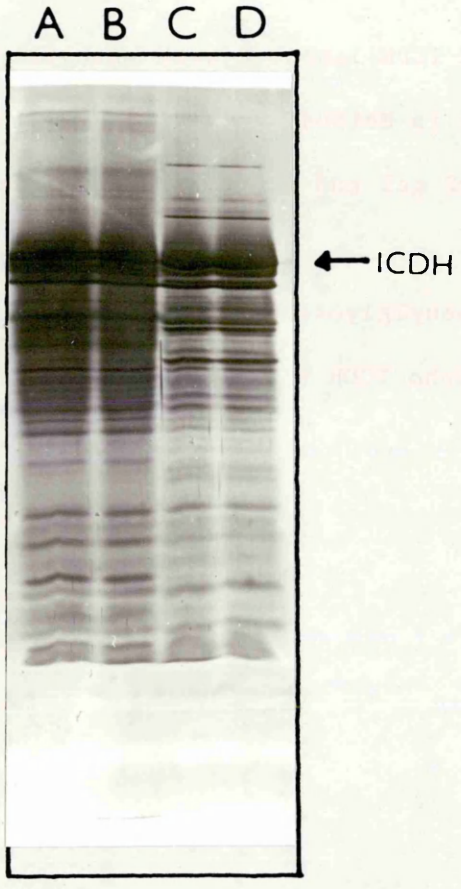

A B C D
ICDH

Figure 4.7 Digestion of [^{32}P]-phospho-isocitrate dehydrogenase and [7-^{14}C]-phenylglyoxal-isocitrate dehydrogenase with thermolysin

Samples were prepared as in Figure 4.6. The digested samples were run on a 15% SDS gel, the gel was immediately dried down and autoradiographed (Methods 2.7.6).

Tracks A,B - Autoradiograph of [^{32}P]-phospho ICDH thermolytic digests

Tracks C,D - Autoradiograph of [7-^{14}C]-phenylglyoxal ICDH thermolytic digests

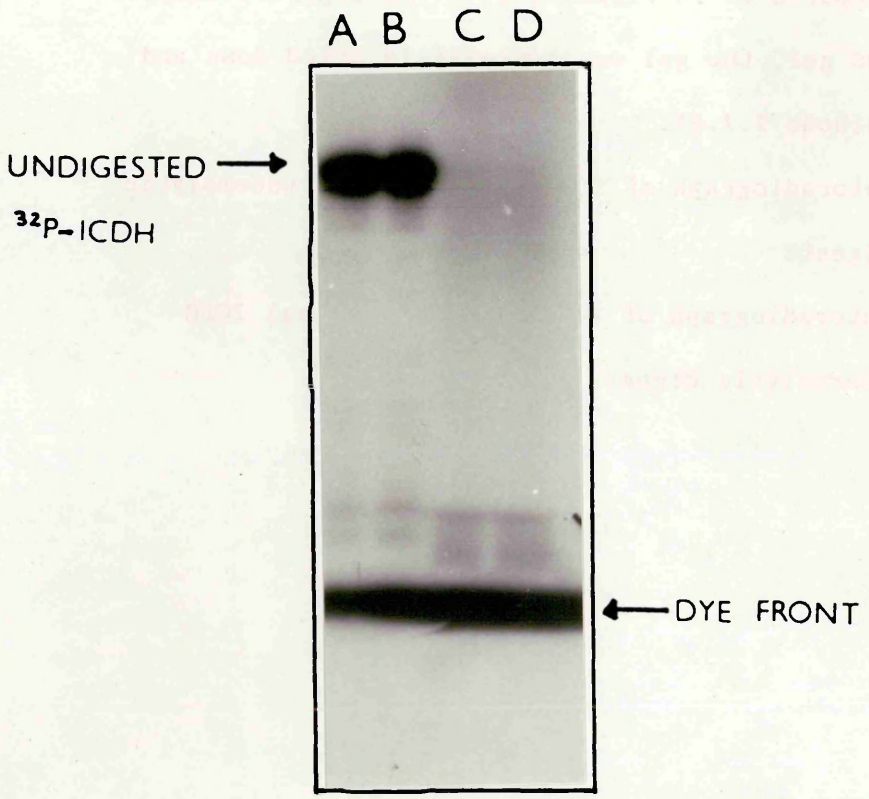

A B C D
UNDIGESTED
^{32}P-ICDH
DYE FRONT

Figure 4.8 Scheme proposed for the reaction of arginine residues with dicarbonyl reagents

(Taken from Peters *et al.*, 1981).

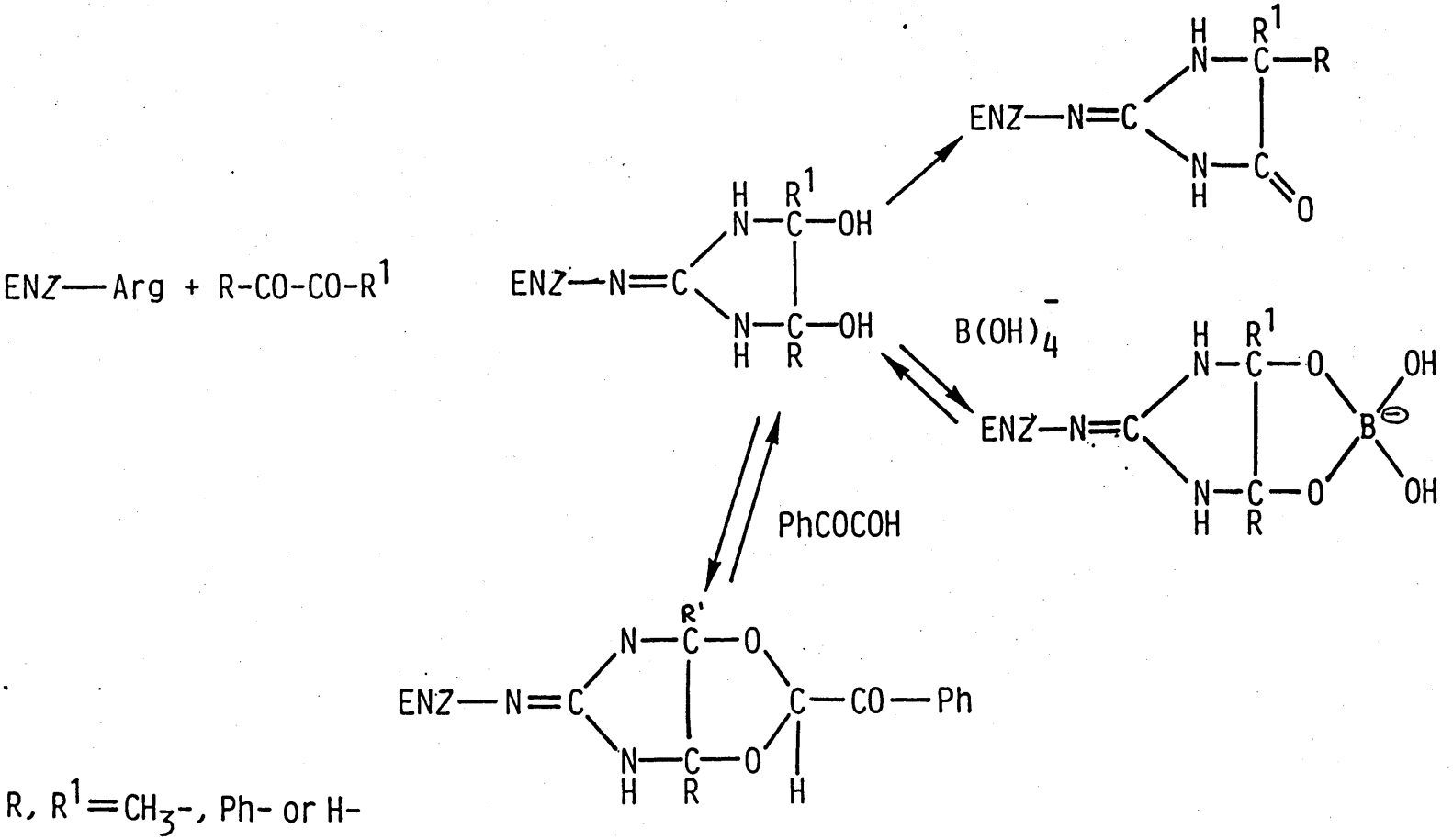

ENZ—Arg + R-CO-CO-R1
B(OH)4
PhCOCOH
R, R1=CH3-, Ph- or H-

Table 4.1 ^{14}C radioactivity associated with Peak 1 from Figure 4.2 for three isocitrate dehydrogenase-phenylglyoxal incubations

ICDH was labelled with [7-^{14}C] phenylglyoxal in the presence or absence of 200μM $NADP^+$ or 100μM D,L-isocitrate (Methods 2.7.3), digested with pepsin and separated on an HPLC reverse phase column (Methods 2.7.3b-c).

The radiocativity was determined for each of the three incubations for the Peak corresponding to PEAK 1 in Figure 4.2.

Table 4.1

Sample	CPM Associated with Radioactive Peak 1
ICDH	5300
ICDH + 200μM $NADP^+$	2000
ICDH + 100μM isocitrate	1100

Table 4.2 ^{14}C-radioactivity associated with the [7-^{14}C]-phenylglyoxal-isocitrate dehydrogenase peptic digests during the purification procedures employed

100% corresponds to radioactivity associated with the labelled ICDH sample before peptic digestion.

Table 4.2

Stage	[^{14}C]-CPM Incorporated	% Initial Incorp.
Before peptic digestion	55400	100
After peptic digestion	51700	93
1st HPLC C18 column	39530	71
2nd HPLC C18 column	21675	39
HPLC gel filtration	8425	15

Table 4.3 Effect of dialysis on the reactivation of isocitrate dehydrogenase inactivated with phenylglyoxal

ICDH was inactivated with 10mM phenylglyoxal (Methods 2.7.1). The activity was measured at zero time before the addition of the phenylglyoxal and this was taken as 100% activity. The activity was measured after 50 min and the sample then dialysed against 50mM MOPS 1mM EDTA pH7.3 for 17 and 24 hours and reassayed for ICDH activity.

Table 4.3

Phenylglyoxal inactivation	units/ml	% Act. remaining
0 time	28.9	100
50 min	0.48	1.6
17 hr dialysis of final inactivated product	4.82	16.7
24 hr dialysis of final inactivated product	9.12	31.6

Table 4.4 Effect of $NaBH_4$ on the stability of the isocitrate dehydrogenase - phenylglyoxal adduct

ICDH was inactivated with [7-^{14}C]-phenylglyoxal (5μCi/μmol) as in Methods 2.7.1. The inactivated sample was then treated with $NaBH_4$ as in Methods 2.7.4. The incubation mix was then split and the stability of the inactivation product analysed using TCA washing (Methods 2.7.2) and dialysis.

As a comparison, control ICDH was inactivated with [7-^{14}C]-phenylglyoxal and the label associated with the protein determined by TCA washing (Methods 2.7.2).

Table 4.4

ICDH Sample	CPM
Phenylglyoxal inactivated + 4 x 15 min TCA washes	827
Phenylglyoxal inactivated + $NaBH_4$ treatment + 4 x 15 min TCA washes	245
Phenylglyoxal inactivated + $NaBH_4$ treatment + 4 x 15 min TCA washes $\pm$ overnight TCA washes	168
Phenylglyoxal inactivated + $NaBH_4$ treatment + overnight dialysis + 4 x 15 min TCA washes	41

CHAPTER FIVE

Substrate Recognition of ICDH Kinase

5.1 Introduction

In the mid - 1970's it became clear that many eukaryotic protein kinases recognise elements of primary structure. Two lines of evidence suggest this. First, the phosphorylation site sequences were determined for various protein substrates and a number of protein kinases. It was found that the various sites phosphorylated by any one kinase showed some similarity to each other. Secondly it was found that synthetic peptides can be used as substrates and this has resulted in the elucidation of a number of the structural features of the substrates of the various protein kinases. It has become clear that although the primary structure of the phosphorylation site is not the only factor involved, it is usually possible to distinguish different groups of protein kinases as a result of their ability to phosphorylate specific peptide sequences. Protein kinases can be classed according to their regulatory agent. For example cAMP-dependent protein kinase is activated by cyclic AMP and it will phosphorylate an exposed serine residue located in a sequence preceded by two basic amino acid residues. If the serine is preceeded by only one basic residue then it will be a poor substrate (review Cohen, 1988). One substrate for this enzyme is glycogen synthase (1A) its phosphorylation sequence is Arg-Arg-Ala-Ser (e.g. Cohen, 1988), this is just one of the many sites phosphorylated in glycogen synthase. Other kinases can be activated by calcium and calmodulin for example phosphorylase kinase (e.g. Krebs and Fischer, 1956), or there are protein kinases which are independent of any regulatory agent. For example Casein kinase II phosphorylates exposed serine (or threonine) residues followed by one or more glutamic acid residues the first of which occurs one position removed from the serine. It has a broad substrate specificity *in vitro*, but it is known to phosphorylate glycogen synthase *in vivo* (e.g. Singh *et al.*, 1982). However, its role *in vivo* is uncertain.

More recently there has been the recognition of tyrosine phosphorylation as an important modification in the action of some hormones and growth factors. They are phosphorylated by protein kinases which are regulated by first messengers. Examples of these are the receptors for insulin (Ullrick et al., 1985) and epidermal growth factor (Cohen et al., 1982; Staros et al., 1985). The binding of the ligand appears to activate the intrinsic protein kinase activity of the receptor, which is manifested experimentally as autophosphorylation of the receptor. It is at present uncertain whether the chain of intracellular events elicited by the hormone is a consequence of a conformational change in the receptor resulting from this phosphorylation, or whether it results from the phosphorylation of an extrinisic substrate, the identity of which is still unknown.

Eukaryotic proteins whose activities are affected by phosphorylation are normally allosteric proteins and phosphorylation frequently results in changes in affinity of the target enzyme for a substrate or an allosteric cofactor. For example phosphorylation of glycogen phosphorylase at serine - 14 reduces the K_a for AMP very considerably and the inhibition by metabolites that bind at the activator site, namely ATP, ADP and glucose - 6- phosphate (e.g. Graves and Wang, 1972; Sprang et al., 1988).

In contrast to eukaryotic phosphorylation is the phosphorylation of ICDH from E. coli ML308. Phosphorylation of this enzyme on a single serine residue per subunit, almost totally inactivates it, (Borthwick et al., 1984a). The phosphorylation sequence of ICDH has been determined and it is unlike any known phosphorylation sequences (Borthwick et al. 1984c). The proposed hypothesis of how this phosphorylation reaction affects enzymatic activity states that phosphorylation occurs close to or at the $NADP^+$ binding site and hence prevents cofactor binding (Nimmo, 1984). The proposed location of the phosphorylation site differs from

the eukaryotic proteins whose phosphorylation residues normally occur at highly accessible points on the protein surface (e.g. phosphorylase, see Fig. 5.12) and raises the question of how ICDH is recognised by its kinase. We therefore synthesized a 14- residue peptide corresponding to the region round the phosphorylation site of ICDH to study the substrate specificity of ICDH kinase.

5.2 Preparation of synthetic peptides

A 14- residue synthetic peptide. hereafter HN2, was synthesized. It corresponded to the first fourteen residues of the chymotryptic peptide isolated from ^{32}P- labelled ICDH digests and, contains the phosphorylatable serine as residue 10, (Fig. 5.1). HPLC analysis using a reverse-phase column of the peptide sample revealed 1 major and 4 minor peaks (Fig. 5.2) amino acid analysis of the individual peaks showed they all contained the 14 desired residues (not shown). Possible explanations of the multiple peaks would include incomplete deprotection of side chain groups or deamination of the asparagine side group. Amino acid analysis (Table 5.1) and sequencing (Table 5.2) of the HN2 peptide showed that it contained the desired residues on the correct order. The major peak shown in Figure 5.2 constituted 72% of the peptide sample as judged by absorbance at 215nm. Since this peak contained material of the correct amino acid composition and was the major component no further purification of the peptide was carried out.

5.3 Phosphorylation of synthetic peptides

Phosphorylation experiments were carried out in a similar way to ICDH phosphorylation experiments (Method 2.9.3). The ICDH kinase used in these experiments completely inactivated ICDH in less than 8 minutes (Fig. 5.3).

Two methods were used to detect phosphorylation. In both cases the method was tested by carrying out controls in which the synthetic peptide kemptide was phosphorylated by cAMP-dependent protein kinase catalytic subunit.

5.3.1. Electrophoretic analysis of peptides

Phosphorylation of the synthetic peptides could only be detected if the phospho peptide could be fully separated from unreacted ATP. HN2 contained only one charged amino acid residue, arginine, and so at neutral pH it would have an overall positive charge, while the ATP would have a net negative charge of nearly three. Electrophoresis at pH6.5 should therefore result in migration of the ATP and peptide HN2 in opposite directions thus achieving separation. If phosphorylation of the peptide occurred the positive charge would be cancelled out and the peptide would have an overall charge close to zero. This would still allow separation of peptide and [γ-^{32}P]-ATP.

Initially, trial electrophoretic separations of [γ-^{32}P]ATP and synthetic peptide were carried out using thin layer electrophoresis at pH6.5. The thin layer plates were stained with ninhydrin to locate peptide samples, and autoradiographed to detect [γ-^{32}P]ATP. Comparison of the peptide spot and autoradiograph indicated that sufficient separation of the ATP and peptide samples had not occurred. In a kinase assay it would be difficult to distinguish [γ-^{32}P]ATP from phosphorylated peptide using this method.

I therefore tried an alternative method of electrophoresis, high voltage paper electrophoresis. This has been successfully used to separate [γ-^{32}P]ATP from peptides and phosphorylated peptide samples (e.g. Daile and Carnegie, 1974). Using this technique I achieved efficient separation of [γ-^{32}P]ATP from peptide HN2. Time course ICDH kinase assays were then carried out using HN2 as a substrate (Method 2.9.4b). At set time points samples were removed and spotted onto the electrophoresis paper. Similar time course experiments were carried out with cAMP-dependent protein kinase catalytic subunit and kemptide. Figures 5.4 and 5.5 show phosphorylation of kemptide occurred with the appearance of a second ninhydrin staining spot of lower mobility

(Fig.5.4). This corresponded to a radioactive spot on the autoradiograph (Fig.5.5). No phosphorylation was observed with ICDH kinase and peptide HN2 (Figs.5.6 and 5.7). The data suggest that recognition of elements of primary structure does not play a major role in the recognition of ICDH by ICDH kinase.

5.3.2 Anion exchange resin analysis

The second method used to monitor the possible phosphorylation reaction employed the anion exchange resin AG- 1 X8. The resin binds unreacted [γ-^{32}P]ATP under the conditions used (Method 2.9.4.c) whereas the peptide and phosphorylated peptide will not bind to the column. Incubations of peptide HN2, at a range of concentrations 1-10 mg/ml with ICDH kinase were set up as for the electrophoretic analysis, and the samples taken at different times were analysed using AG-1 X8 resin as in (Method 2.9.4.c). The eluate fractions were collected and counted for radioactivity. The data obtained were plotted as cpm vs time of incubation. Figure 5.8 shows the graph obtained ^{32}P was incorporated into kemptide in the presence of cAMP dependent protein kinase catalytic subunit in a time dependent manner. However no phosphorylation of peptide HN2 by ICDH kinase was observed.

Anion exchange resin analysis of kinase incubations containing ICDH kinase and kemptide or cAMP-dependent protein kinase catalytic subunit and HN2 showed neither kinase activity could phosphorylate the other synthetic peptide (Fig.5.9).

Quantitative analysis of the results obtained from anion exchange was carried out to determine how poor a substrate peptide HN2 was for ICDH kinase. The ICDH kinase used in the assay had the ability to phosphorylate ICDH at a rate of 0.056 nmol/min. Assuming that HN2 was as good a substrate as ICDH I calculated how many cpm would be incorporated into the peptide sample after a 60 min kinase incubation. Calculations showed that less than 0.74% of the expected cpm value

was associated with the peptide sample. Similar analysis of a 120 min kinase incubation indicated less than 0.4% of the expected ^{32}P had been incorporated into the peptide.

5.4 Phosphorylation of chymotryptic digests of ICDH

The apparent inability of ICDH kinase to phosphorylate the peptide HN2 raises the question of what ICDH kinase does recognise. Can it only phosphorylate intact ICDH? I decided to test this by assessing whether ICDH kinase could phosphorylate ICDH that had been digested with chymotrypsin. It seemed possible that the digest (Fig.5.10) might contain fragments of ICDH that retained enough secondary or tertiary structure to be recognised by the kinase. ICDH was first digested overnight with chymotrypsin. Before proceeding with the kinase assays chymostatin, an inhibitor of chymotrypsin, was added to prevent chymotryptic digestion of the added kinase.

Partially purified ICDH kinase was used in this experiment. Therefore, we could not assign any of the protein-staining bands to ICDH fragments. However, a sample of the digested material was run separately and stained with silver to check that digestion had occurred (Fig.5.10). No autophosphorylation of the kinase sample was observed, hence any radioactive bands observed with the ICDH digests could be attributed to phosphorylation of ICDH or its fragments. However the results showed that no phosphorylation, other than undigested ICDH, was detected for the chymotryptic digests (Fig.5.11). This suggested ICDH kinase recognised only intact ICDH as a phosphorylatable substrate.

5.5. Discussion

The work in this chapter addressed the question of how ICDH kinase recognises its protein substrate. No evidence was obtained that ICDH kinase could phosphorylate a 14- residue peptide corresponding to the sequence round the phosphorylation site. Of the two methods used to

detect phosphorylation, only the anion-exchange analysis allowed a quantitative determination of how good a substrate HN2 was for the kinase. The quantitative results from the ion-exchange analysis showed that HN2 was a very poor substrate for the ICDH kinase being phosphorylated at less than 0.4% of the rate of ICDH. It is however important to note that a range of peptide concentrations were tested in the kinase assay, but this was limited by the peptide solubility in the buffer used (maximum concentration 12mg/ml). It may be the situation then that HN2 is a substrate for ICDH kinase but, if so the K_m value must be very high well above the concentrations we have used.

This apparent inability of ICDH kinase to phosphorylate a short peptide sequence distinguishes it from most eukaryotic kinases which recognise and phosphorylate synthetic peptides corresponding to regions of primary sequence.

Since ICDH kinase appears not to recognise the primary sequence of its phosphorylation site, it must recognise some other feature(s) of the enzyme structure such as the overall enzyme conformation or two distinct separated areas of the protein structure. Analysis of phosphorylation of chymotryptic digests of ICDH also proved negative. The fragments generated by partial protease digestion were of greater size than the peptide HN2 and it was thought that some of these fragments might have retained regions of secondary structure which could be recognised by the kinase. However, it appeared from the experiment that ICDH kinase only recognised intact ICDH as a phosphorylatable substrate. However, the conclusion from this experiment cannot be definitive. I cannot rule out the possibility that a potential substrate was generated by the digestion, but only at a very low concentration so that its phosphorylation was not detectable.

The lack of ability of ICDH kinase to phosphorylate either HN2 or proteolytic fragments may not be quite so surprising as the properties

of eukaryotic protein kinases might imply. Phosphorylation of eukaryotic proteins by their protein kinases usually alters the allosteric properties of the enzyme, whereas phosphorylation of ICDH totally inactivates the enzyme. Differences between the eukaryotic kinase substrates and ICDH can also be observed in the effects of proteolysis. For many eukaryotic phosphoproteins, proteolytic digestion removes the phoshphorylation site and generates a form whose biological properties resemble those of the dephosphoprotein. For example phosphorylase b is phosphorylated by phosphorylase kinase to the active a form. Trypsin digestion of phosphorylase a produces a pseudophosphorylase b which has the same properties as phosphorylase b but has lost its first 16 amino acid residues including the phosphorylated serine. Thus reversal of the phosphorylation, and its effects, have been mimicked by proteolytic cleavage (Cori and Cori, 1945; Keller, 1955).

In contrast the kinetic effects of phosphorylation on ICDH cannot be reversed by proteolytic removal of the phosphoserine residue (Garland and Nimmo, 1984). This is understandable in view of the hypothesis that phosphorylation of ICDH occurs close to or at its $NADP^+$ binding site. If this idea is correct then proteolytic removal of the phosphoserine residue would destroy the $NADP^+$ binding site and irreversibly inactivate the enzyme.

Phosphorylation sequences of eukaryotic kinase substrates are often located at one end or the other of the primary sequence, and can form "arm like" structures which are very accessible to the cellular components. The structure of phosphorylase shows this very well (Fig.5.12) (Sprang and Fletterick, 1979; Sprang et al., 1988). Proteolytic cleavage of such a structure may in no way affect the enzyme structure and activity, only removing the possibility of controlling its activity by phosphorylation, and would reverse the effects of phosphorylation on the biological properties of the protein.

It seems from my results that ICDH kinase recognises the conformation of intact ICDH rather than an element of primary structure at the phosphorylation site. The kinase presumably recognises either the molecule as a whole or several distinct parts of it, the phosphorylation site being an integral part of the enzyme strucure. This together with the proposed site of phosphorylation indicates that ICDH control by phosphorylation occurs by a mechanism quite different from those involved in most other phosphorylation systems studied to date.

Figure 5.1 Sequence of peptide HN2

(See Methods 2.9.1 for preparation details)

N-Thr-Thr-Pro-Val-Gly-Gly-Gly-Ile-Arg-Ser(P)-Leu-Asn-Val-Ala-C

Figure 5.2 HPLC analysis of peptide HN2

The purity of the deprotected synthetic peptide was checked on an HPLC reverse phase column (Methods 2.9.1).

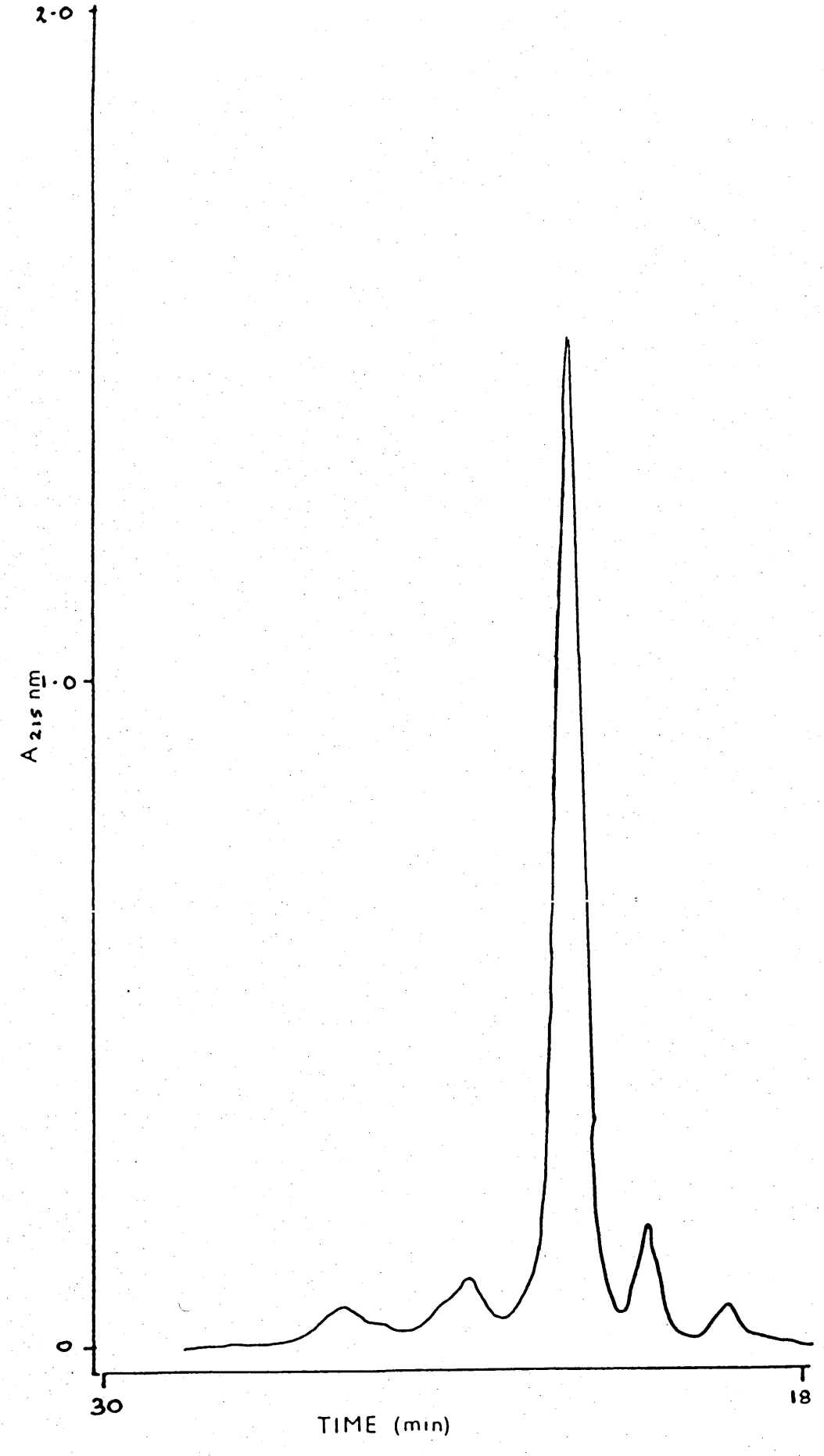

2·0
1·0
0
A 215 nm
30
18
TIME (min)

Figure 5.3 Inactivation of isocitrate dehydrogenase with ICDH kinase

ICDH was inactivated with a purified sample of ICDH kinase as in Methods 2.4.2b. The results were plotted as activity remaining (%) vs time (min).

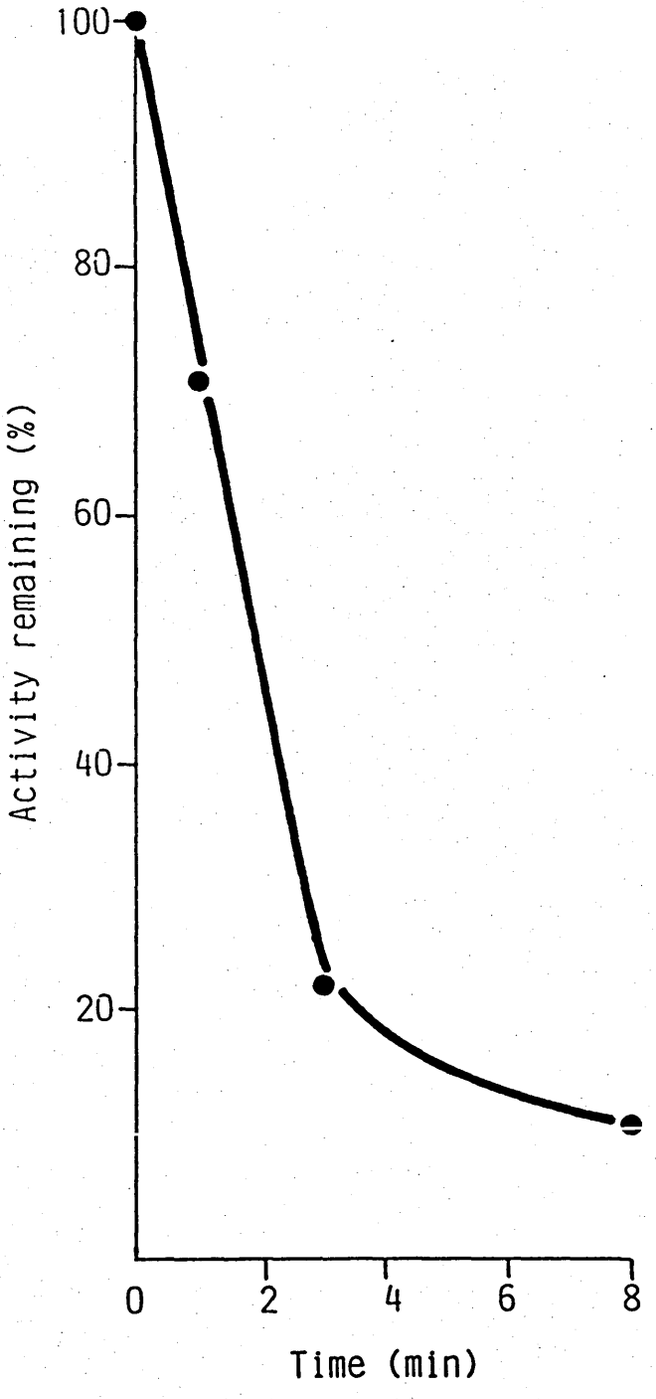

100
80
60
40
20
Activity remaining (%)
0
2
4
6
8
Time (min)

Figure 5.4 Phosphorylation of kemptide by the catalytic subunit of cAMP-dependent protein kinase. Electrophoretic analysis-ninhydrin stain.

Kinase assays were carried out using kemptide and cAMP-dependent protein kinase catalytic subunit as described in Methods 2.9.3. Samples were removed at set times and 15-20µl was rapidly spotted onto the electrophoretogram as in Methods 2.9.4b. Control kinase incubations were set up which omitted either the peptide or the kinase.

The electrophoretogram was dried and stained with ninhydrin.

Control one - 30 min kinase assay/no kinase added

Control two - 30 min kinase assay/no peptide added

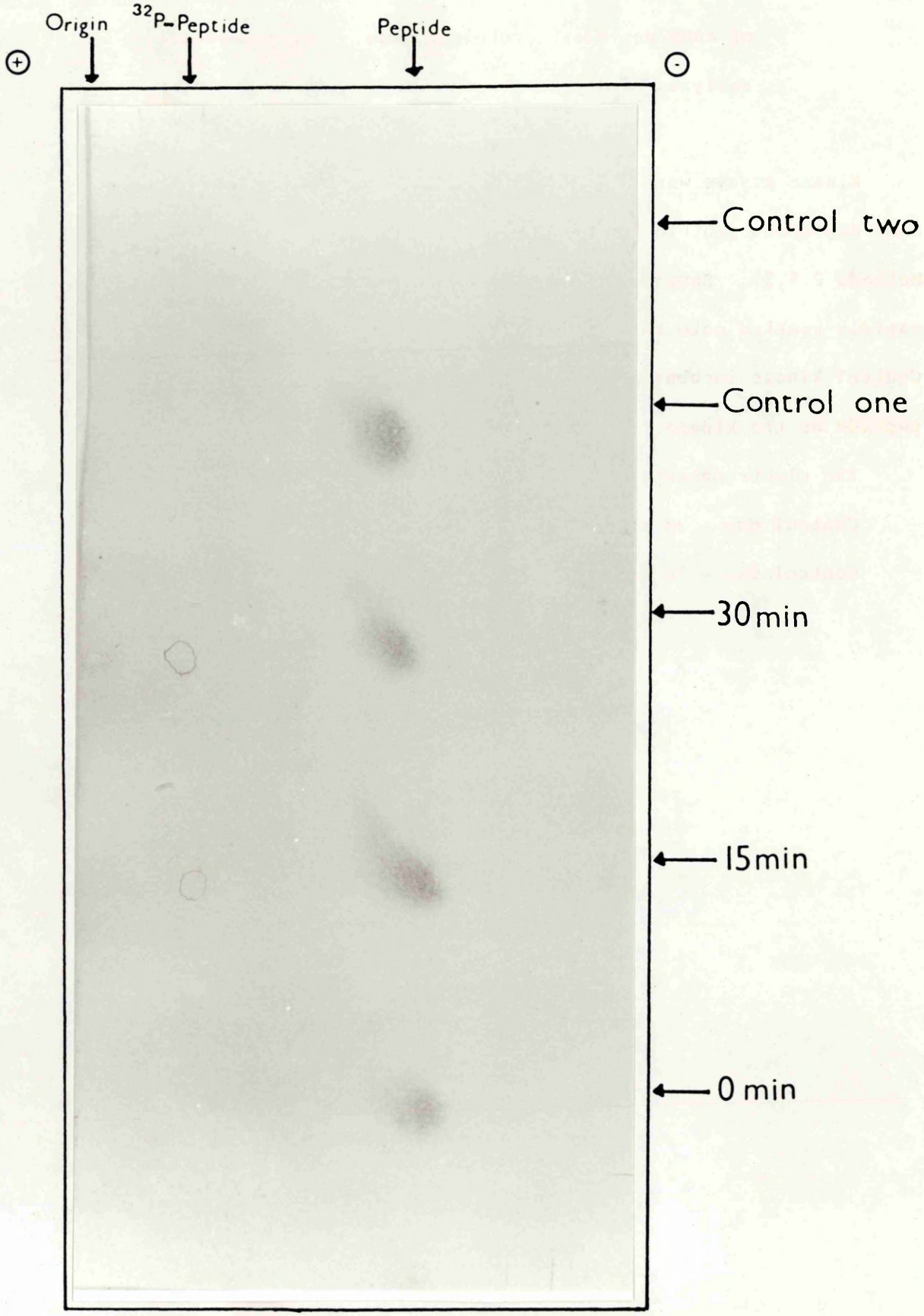

Origin
32P-Peptide
Peptide
⊕
⊖
Control two
Control one
30 min
15 min
0 min

Figure 5.5 Phosphorylation of kemptide by the catalytic subunit of cAMP-dependent protein kinase. Electrophoretic analysis - autoradiogram.

Autoradiograph of ninhydrin stained electrophoretogram from Figure 5.4.

Control one - 30 min kinase assay/no kinase added

Control two - 30 min kinase assay/no peptide added

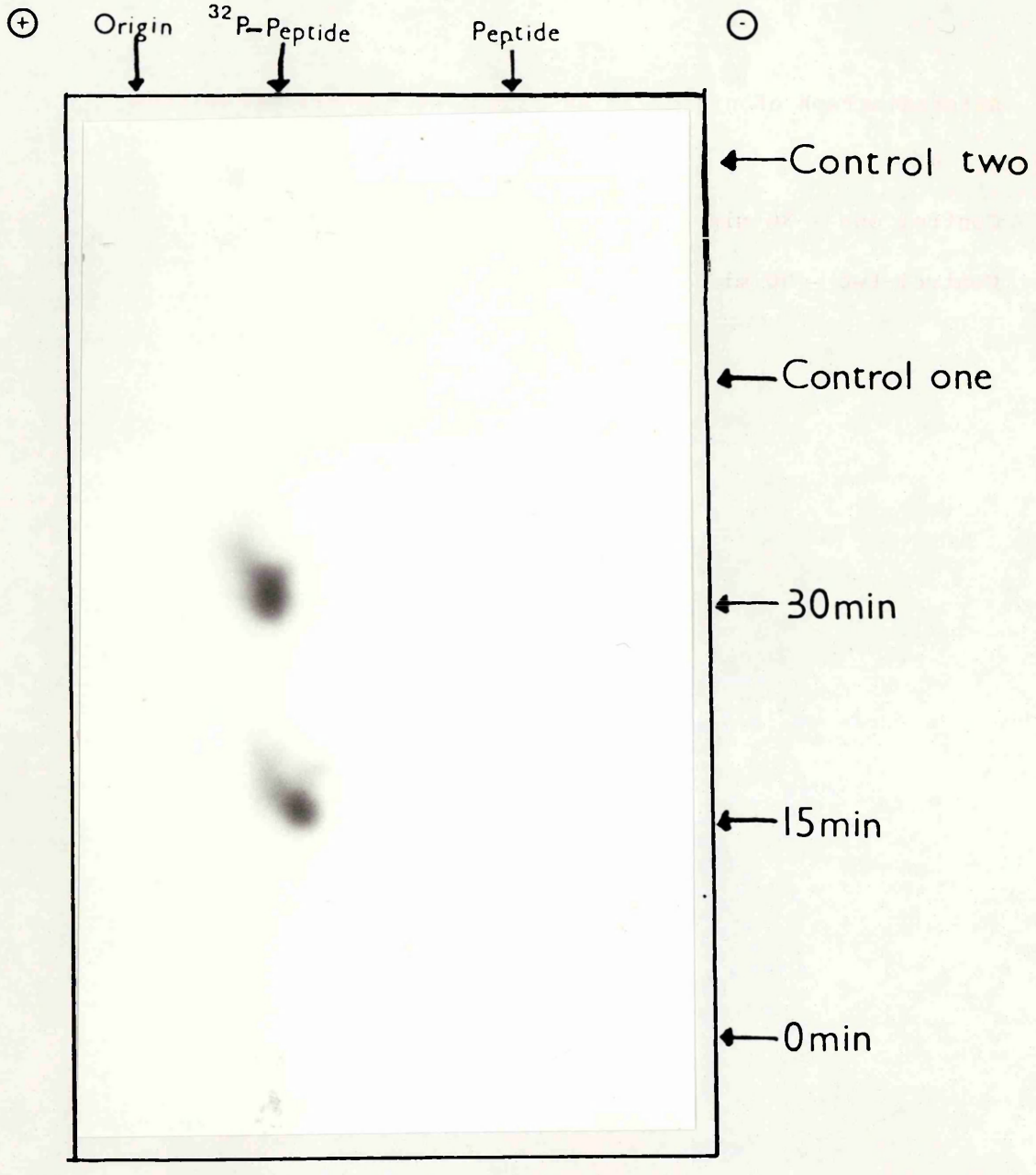

⊕
Origin
32P-Peptide
Peptide
⊖
Control two
Control one
30min
15min
0min

Figure 5.6 Phosphorylation of HN2 with ICDH kinase.

Electrophoretic analysis - Ninhydrin stain

Kinase assays were carried out using the synthetic peptide HN2 and ICDH kinase/phosphatase as described in Methods 2.9.3. Samples were removed at set times and 15-20µl rapidly spotted onto the electrophoretogram as in Methods 2.9.4b. Control kinase incubations were set up which omitted either the peptide or the kinase. The electrophoresis was run for 90 min, the paper dried and stained with ninhydrin.

Control one - 50 min kinase assay/no kinase

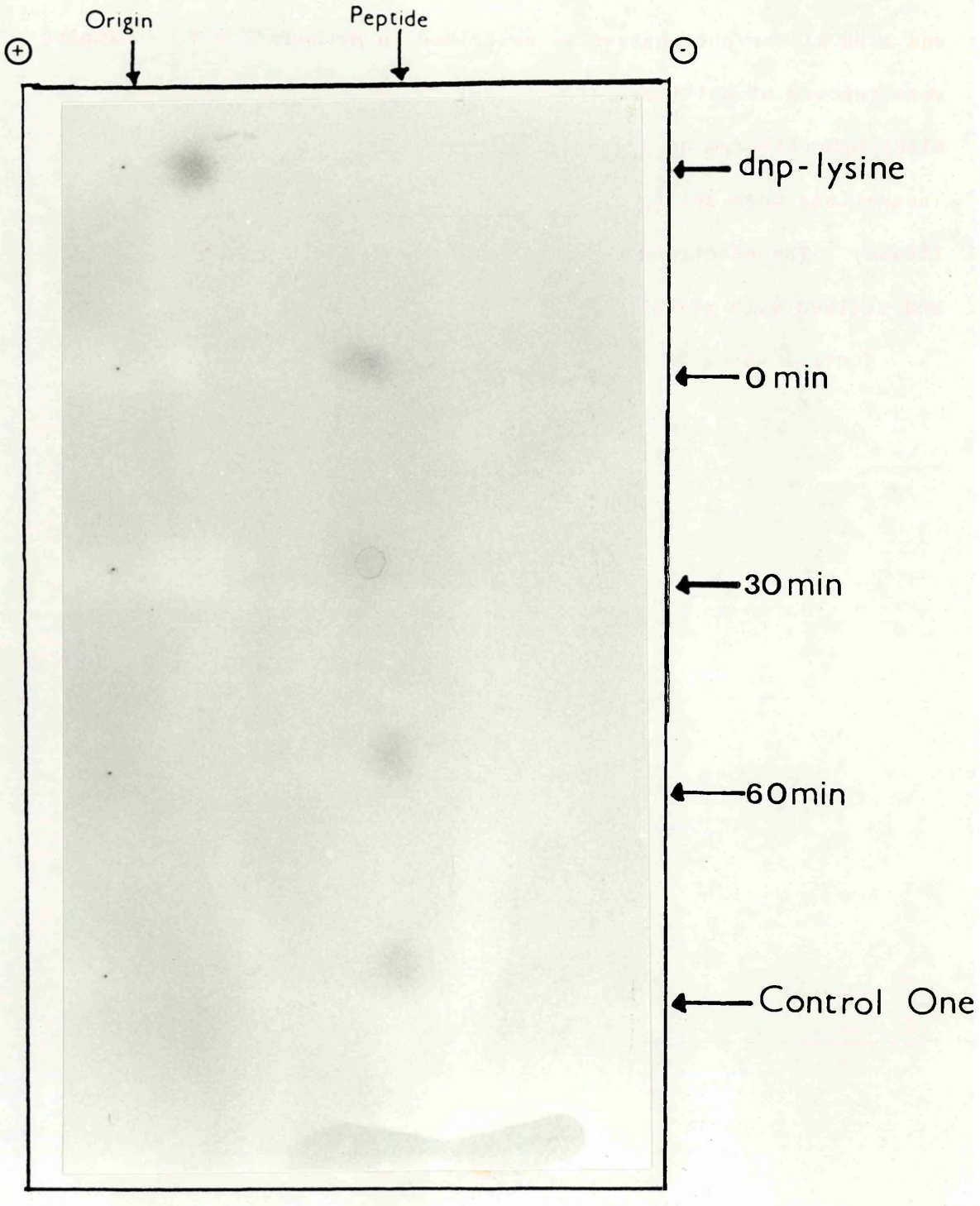

Origin
Peptide
⊕
⊖
dnp-lysine
0 min
30 min
60 min
Control One

Figure 5.7 Phosphorylation of HN2 with ICDH kinase.

Electrophoretic analysis - autoradiogram

Autoradiograph of ninhydrin stained electrophoretogram from Fig.5.6.

Control one - 60 min kinase assay/no kinase added

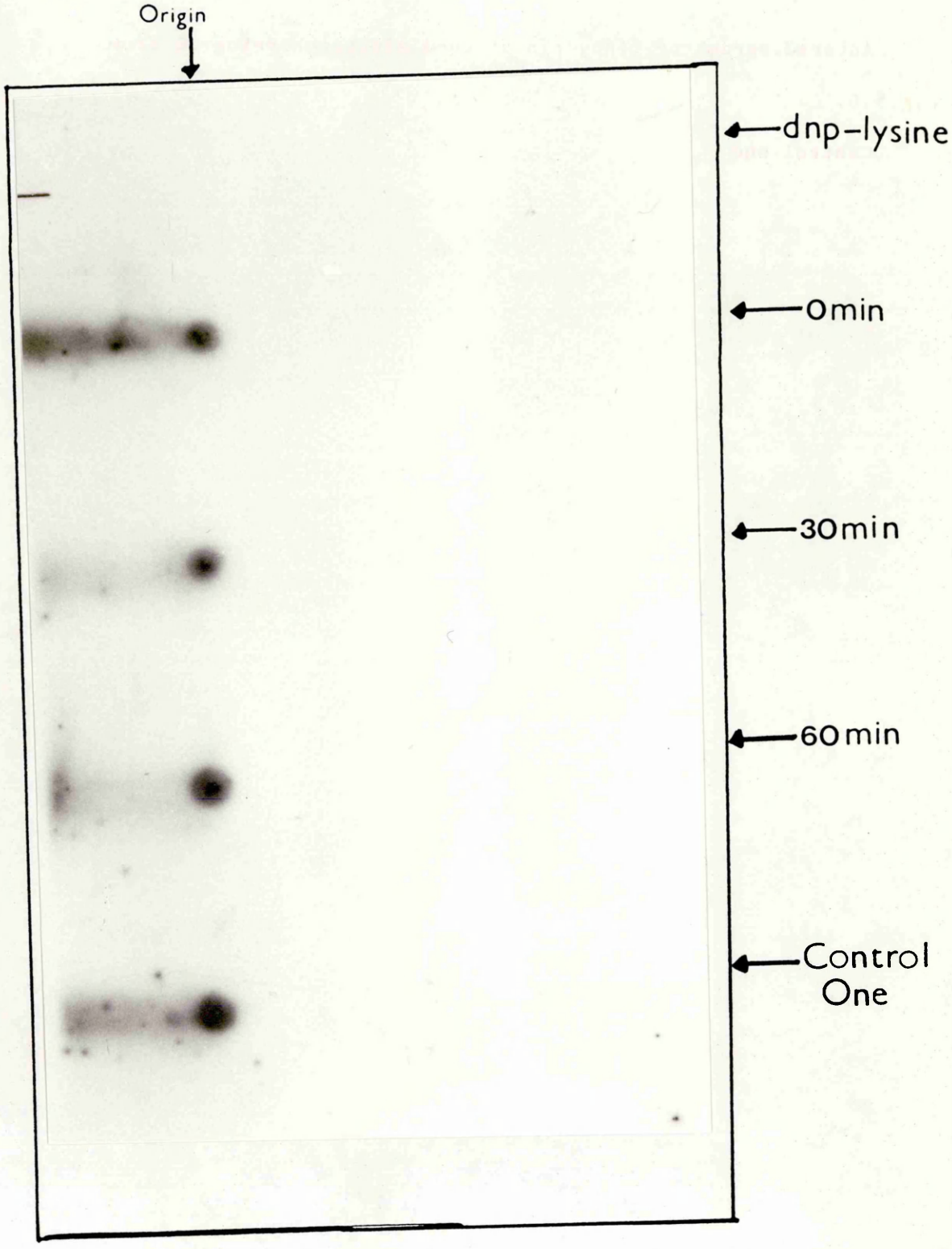

Origin
dnp-lysine
0min
30min
60min
Control
One

Figure 5.8 Incorporation of ^{32}P into synthetic peptides - anion exchange resin analysis

Kinase assays were carried out as in Methods 2.9.3. HN2 and ICDH kinase/phosphatase incubations and kemptide and cAMP dependent protein kinase catalytic subunit were set up and samples taken over a time course experiment. Reactions were stopped by the addition of acetic acid to a final concentration of 30%. Samples were analysed by anion exchange chromatography as in Methods 2.9.4c. The peptide samples collected were counted for radioactivity and the results plotted against the corresponding time point.

▲ Kemptide/cAMP dep protein kinase catalytic subunit

● HN2/ICDH kinase/phosphatase

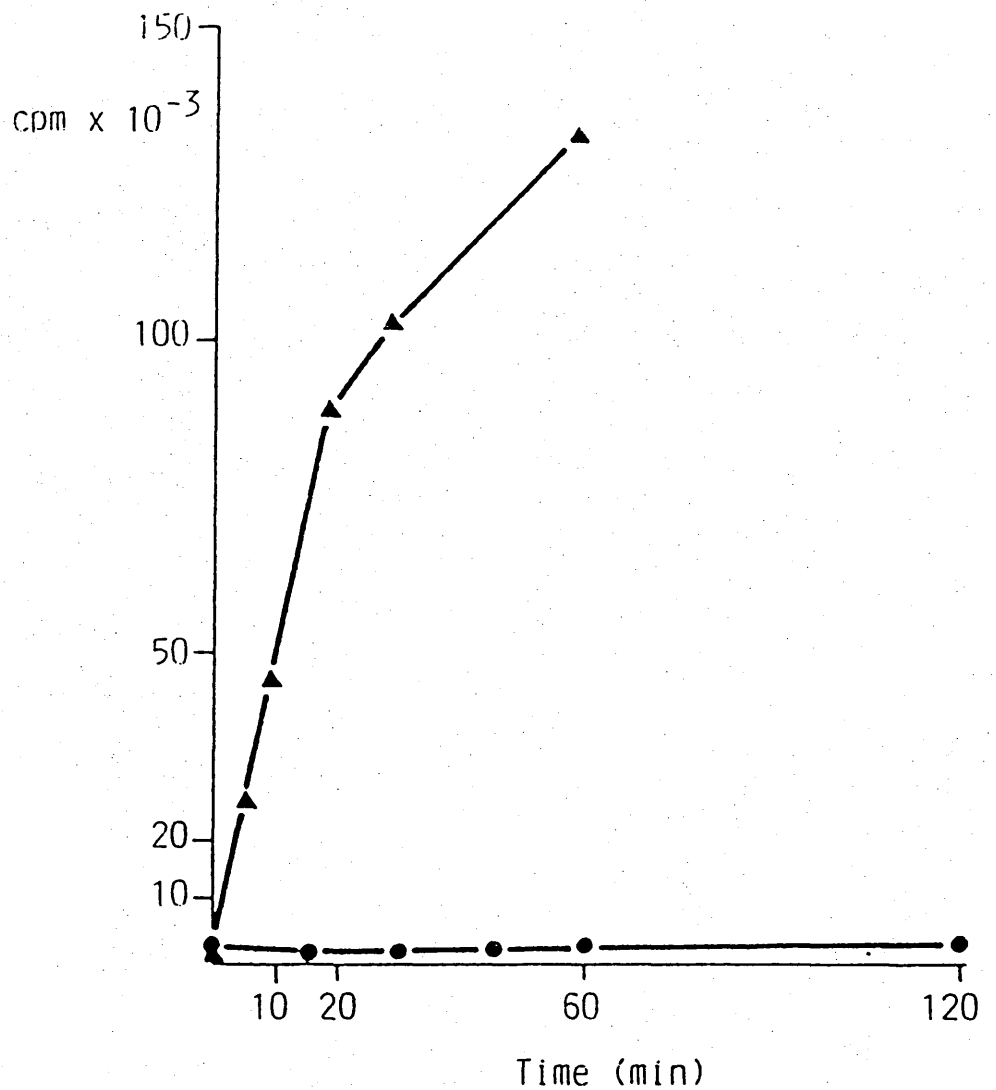

150
cpm x 10^{-3}
100
50
20
10
10
20
60
120
Time (min)

Figure 5.9 Incorporation of ^{32}P into synthetic peptides - anion exchange resin analysis

The experiment was carried out as in Figure 5.8 except the kinase incubations contained HN2 and cAMP dependent protein kinase catalytic subunit or kemptide and ICDH kinase/phosphatase.

- ● HN2/cAMP dep protein kinase catalytic subunit
- △ Kemptide/ICDH kinase/phosphatase
- ■ Kemptide/cAMP dep protein kinase catalytic subunit

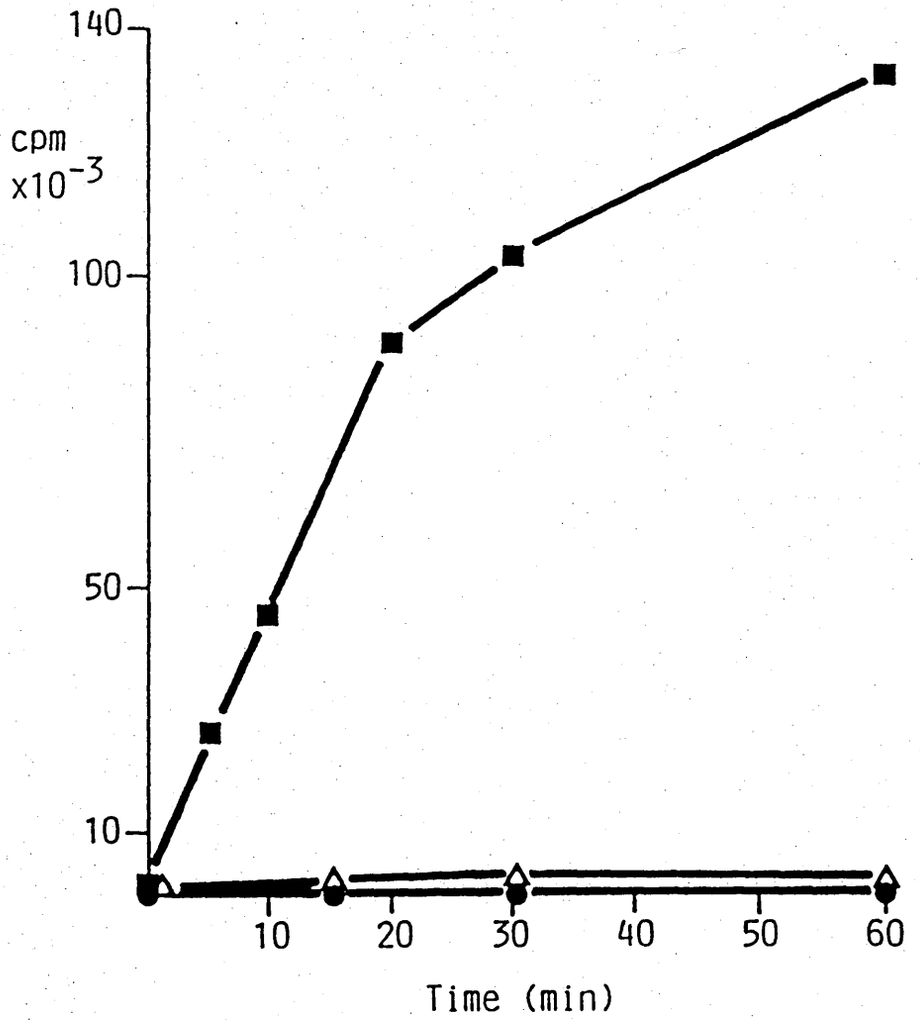

140
cpm
x10^{-3}
100
50
10
10
20
30
40
50
60
Time (min)

Figure 5.10 SDS analysis of peptide HN2 and chymotryptic digests of ICDH

10μg of ICDH was digested overnight with chymotrypsin (ICDH:chymotrypsin, 72:1) and the digest run on a 20% SDS gel.

Track A - ICDH chymotryptic digest

Track B - 5μg peptide HN2

Track C - 10μg peptide HN2

Track D - 25μg peptide HN2

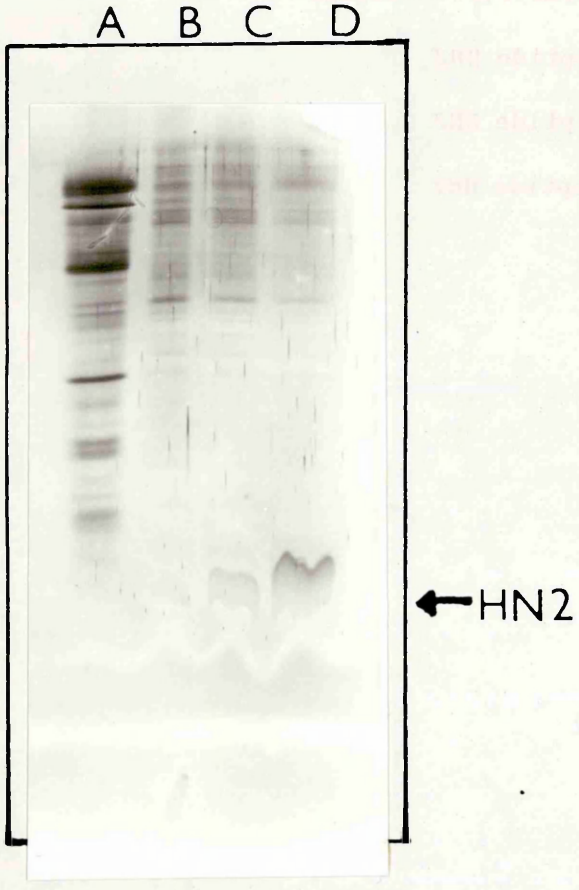

A
B
C
D
HN2

Figure 5.11 Autoradiograph of isocitrate dehydrogenase chymotryptic digest kinase assays

ICDH was digested with chymotrypsin, treated with chymostatin and used in a time course ICDH kinase experiment as in Methods 2.10. Control kinase incubations were carried out with undigested ICDH, no added ICDH digests and without treatment with chymostatin. Samples were run on a 15% SDS gel and autoradiographed.

Track A - undigested ICDH

Track B - No ICDH chymotryptic digest

Track C - no chymostatin treatment

Track D - 0 min)

Track E - 15 min)

Track F - 30 min) Kinase incubation time points

Track G - 60 min)

Track H - 90 min)

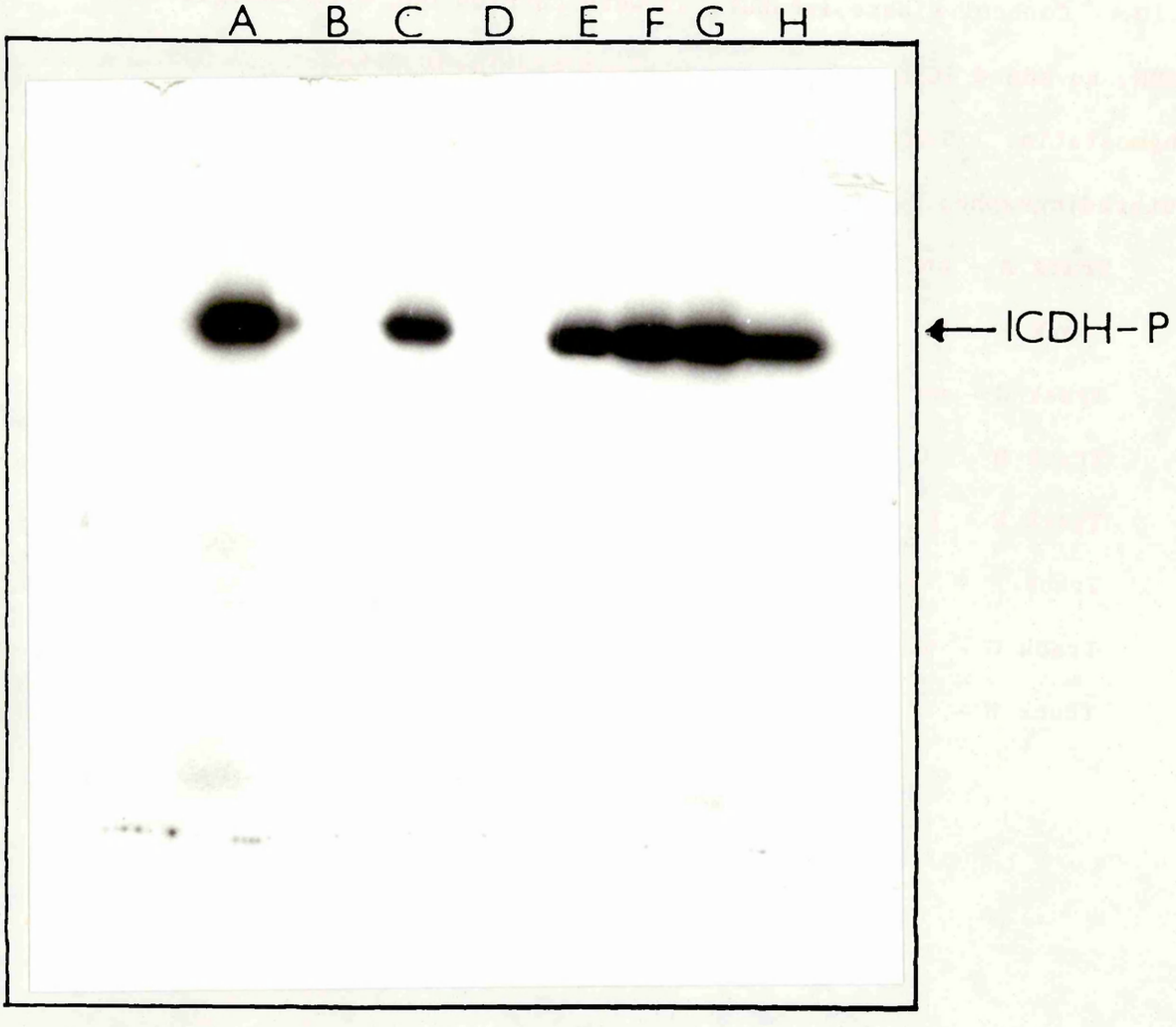

A B C D E F G H
ICDH-P

Figure 5.12 Structure of phosphorylase *a*

A 'barrel and arrow' schematic diagram of phosphorylase *a* illustrating the course and folding of the polypeptide backbone. Skeletal inserts are shown at the pyridoxal phosphate covalently bound through lysine 679, the Ser-14-P moiety and the bound ligands AMP, glucose, caffeine and maltopeptose.

(Taken from a review by Fletterick, 1980)

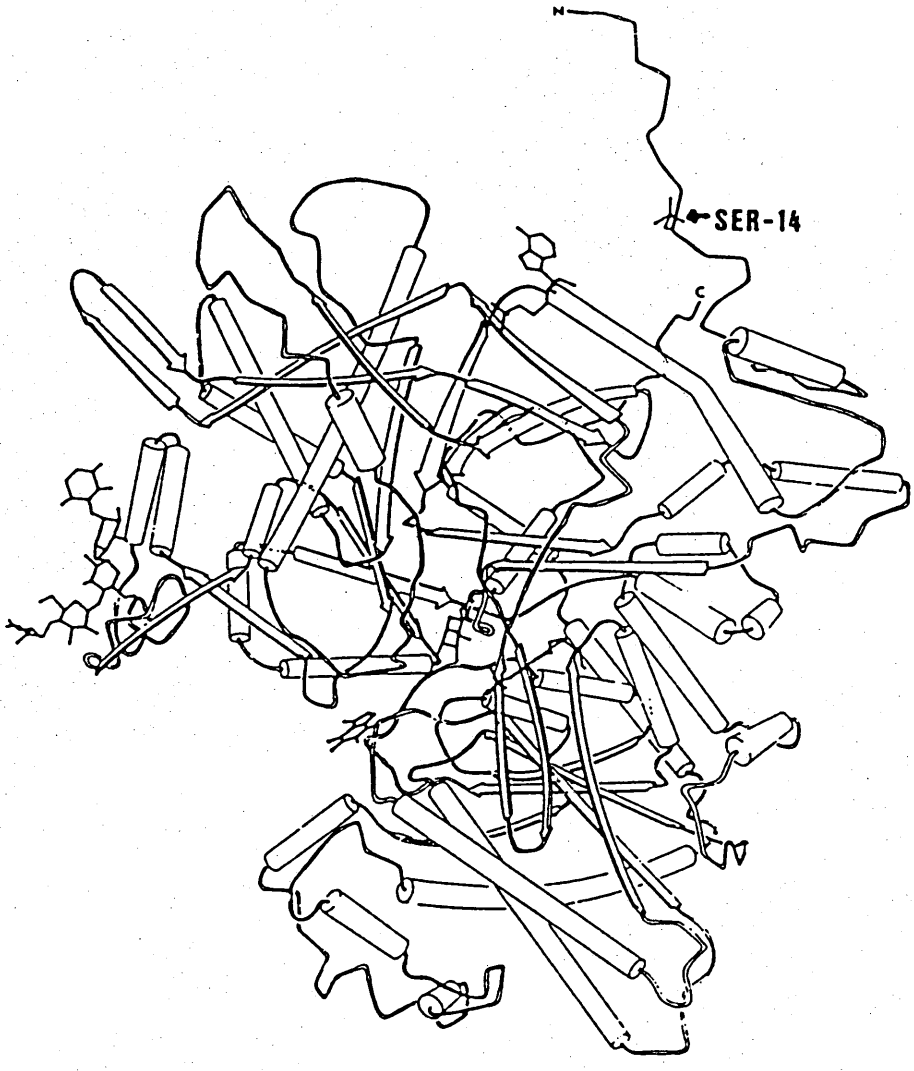

N
SER-14
C

Table 5.1 Amino acid analysis of peptide HN2.

Amino acid analysis was carried out as in Methods 2.9.2 (a).

Table 5.1

Amino acid	pmol recovered	No.of residues	% composition
ASX	5759	1.0	7.0
GLX	49	0	0.1
SER	5279	0.9	6.4
GLY	19612	3.3	23.9
HIS	689	0.1	0.8
ARG	5018	0.9	6.1
THR	8527	1.4	10.4
ALA	7007	1.2	8.5
PRO	6043	1.0	7.3
TYR	29	0	0
VAL	12432	2.1	15.1
MET	516	0.1	0.6
ILE	5234	0.9	6.4
LEU	5935	1.0	7.2
PHE	99	0	0.1
LYS	0	0	0

Table 5.2 Gas phase sequencing of peptide HN2

Sequence analysis was carried out as in Methods 2.9.2 (b).

Table 5.2

Sequence No.	PTH amino acid	pmol recovered
1	THR	25.0
2	THR	22.5
3	PRO	31.0
4	VAL	25.0
5	GLY	20.7
6	GLY	27.0
7	GLY	24.5
8	ILE	16.0
9	ARG	8.8
10	SER	4.6
11	LEU	7.8
12	ASN	7.8
13	VAL	4.5
14	ALA	3.0

CHAPTER SIX

Conformational Analysis of Isocitrate Dehydrogenase with Circular Dichroism

6.1 Introduction

The knowledge of the three-dimensional structures of a number of enzymes and other proteins, derived from X-ray crystallographic studies has greatly enhanced our understanding in structural terms of the properties of these molecules. However, it is also important to establish structural features of these molecules in solution and, in particular, to monitor changes in structure which may occur as a result of changes in environment or of interactions with various ligands such as substrates, inhibitors or activators. Solution studies do not have the degree of resolution afforded by crystallographic techniques; they can, however, lead to the description of the general structural features of a biological macromolecule and pinpoint some particular details about the location and interaction of various specific groups on proteins.

For the purpose of this study Circular Dichroism (CD) was chosen as the technique best suited to look at our system. While CD spectra in the far ultraviolet reflect principally peptide bOnd transitions, and thus the secondary structure of a protein, important information on enzyme structure may be derived also from transitions of the side chains. These may reflect features of both the secondary and tertiary structures. CD bands characteristic of amino acid side chains occur in the spectral region below 330nm. Above 240-250nm there is no overlap with peptide bond transitions so that this region may be used to follow changes in side chain conformation and environment. The principal contributions are made by tryptophan and tyrosine residues, as well as by cystine disulphide bridges (see Timasheff, 1970).

We hoped, therefore, to use CD analysis in the near U.V. region to detect conformational changes in the structure of ICDH on

either $NADP^+$ binding to the enzyme or on phosphorylation of the ICDH. Any results obtained using this technique might help to substantiate the proposed model of how phosphorylation affects ICDH activity (see Fig. 1.5).

6.2 Analysis of ICDH and ICDH-P conformations

Analysis of protein conformation by circular dichroism requires quite large quantities of protein material. Usually a solution with an A_{280} of approximately 1 is required. In the case of ICDH this corresponded to a solution of approximately 1mg/ml. The construction of the ICDH overproducing strain E.coli TG1/pCK 505 which overproduces ICDH by a factor of 10-fold allowed large quantities of ICDH to be purified with relative ease (Methods 2.5.4).

All protein CD analysis were carried out in 50mM MOPS 1mM EDTA pH7.3. A blank CD spectrum was run for the MOPS buffer and this was subtracted from the protein analysis runs. We wished to establish if a conformational change occurred on $NADP^+$ binding to ICDH and phosphorylation of the ICDH and to ascertain if the same conformational change occurred for both cases.

The following CD analysis were carried out in the near U.V. region. The results obtained are plotted as ellipticity per mean residue weight against wavelength (nm) (Methods 2.12).

i) ICDH (Fig.6.1)

ii) ICDH + 150μM $NADP^+$ (Fig.6.2)

iii) ICDH + 50μM NADPH (Fig.6.4)

iv) phospho-ICDH (Fig.6.6)

v) phospho-ICDH + 150μM $NADP^+$ (Fig.6.7)

vi) phospho-ICDH + 50μM NADPH (Fig.6.9)

These initial spectra were then used to determine difference spectra for ICDH on cofactor binding and phosphorylation and for phospho-ICDH in the presence of cofactors. The spectra obtained were as follows:-

vii) ICDH - (ICDH + 150μM $NADP^+$) (Fig.6.3)

viii) ICDH - (ICDH + 50μM NADPH) (Fig.6.5)

ix) ICDH-P - (ICDH-P + 150μM $NADP^+$) (Fig.6.8)

x) ICDH-P - (ICDH-P + 50μM NADPH) (Fig.6.10)

xi) ICDH - (ICDH-P) (Fig.6.11)

xii) (ICDH + 150μM $NADP^+$) - (ICDH-P + 150μM $NADP^+$) (Fig.6.12)

xiii) (ICDH + 50μM NADPH) - (ICDH-P + 50μM NADPH) (Fig.6.13)

From these results the following points can be taken. Firstly this method has indicated that phosphorylation of ICDH induces no detectable conformational change (Fig.6.11). This result, at first, appears to contradict the proposed effect of ICDH phosphorylation. However, the results obtained suggest a second line of thinking which is still consistent with our model.

The complex difference spectrum in the region of 250-280nm in Figure 6.3 can be attributed to the spectrum of $NADP^+$ in this region. However, this figure also shows a clear trough at 292nm which represents a change in the spectrum contributed by the protein. While this might reflect a conformational change in the protein, it is quite possible that this results from displacement of a solvent molecule from a partially exposed tryptophan residue by $NADP^+$. The corresponding difference spectrum for phospho-ICDH (Fig.6.8) shows no such feature at 292nm, consistent with the view that $NADP^+$ cannot interact with this form of the enzyme. As a result, when the spectra of the active and phosphorylated forms of ICDH in the presence of $NADP^+$ are compared the difference spectrum shows a peak at 292nm (Fig.6.12). Similar results were obtained

with NADPH. As shown in Figure 6.5, NADPH induces a spectral change at 292nm in active ICDH, but only a very minor change is seen with phosphorylated ICDH (Fig.6.10). Again, when the active and phospho forms of the enzyme are compared in the presence of NADPH, the difference spectrum shows a positive feature around 290nm (Fig.6.13).

One conclusion can be taken from this set of data. If, as seems likely, phosphorylated ICDH cannot bind $NADP^+$, the lack of binding is due to the presence of the phosphate group as a block rather than to a change in the conformation of ICDH. Taking this point into consideration, our model (Fig.1.5) is still relevant. Consider if phosphorylation occurs near to the $NADP^+$ binding site of ICDH. If the phosphorylation occurs at the phosphate binding site, but is sufficiently removed from the aromatic binding subsite of the $NADP^+$ molecule so as not to affect the ellipticity of the tryptophan residue that appears to be part of that subsite, then our model still holds.

6.3 Discussion

Analysis of protein structure in solution can be monitored by a variety of spectroscopic techniques, for example fluorescence, ORD, CD and nuclear magnetic resonance (see Freifelder, 1982 for an overview). Each technique has its advantages for a particular type of study. For this particular study we chose the method of circular dichroism to look for conformational changes in E.coli ICDH under particular situations.

The model proposed by Nimmo (1984) (Fig.1.5), suggests a conformational change may be involved in the mechanism of inactivation of ICDH by phosphorylation. In eukaryotic phosphorylation systems it has been generally assumed that phosphorylation stabilises different conformational states of the protein. Little direct evidence however has been established

relating to the magnitude or characteristics of these conformational changes. However, recently evidence relating to conformational changes on phosphorylation was achieved for glycogen phosphorylase (Sprang et al., 1988). These workers compared the refined crystal structures of dimeric glycogen phosphorylase b and a and revealed structural changes that represented the first step in the activation of the enzyme. On phosphorylation of serine-14, the N-terminus of each subunit assumes an ordered helical conformation and binds to the surface of the dimer. The consequent structural changes in the N- and C-terminal regions led to strengthened interactions between subunits and altered the binding sites for allosteric effectors and substrates (Sprang et al. 1988).

How can this type of situation be related to E.coli ICDH? Although phosphorylation of ICDH occurs it does not serve to alter allosteric properties of the enzyme but instead completely inactivates the enzyme activity. In the original model, the proposed conformational change was suggested to alter the enzyme structure in such a way as to 'close' the nucleotide binding site and hence prevent cofactor access and binding. The CD data obtained from the conformational analyses of ICDH indicated that no detectable change occurred on either cofactor binding or phosphorylation. This result seems in contrast to our original model. However, the results do not show that a conformational change has not occurred; they indicate only that no change detectable by circular dichroism occurred.

One interesting result was obtained from the CD analysis, an apparent inability of phosphorylated ICDH to bind the enzyme cofactor $NADP^+$. In the original model the phosphorylation of ICDH is proposed to produce a similar conformational change as $NADP^+$ binding and prevent binding by a charge-charge repulsion

mechanism. This can be modified in terms of these results to phosphorylation prevents binding by blocking the $NADP^+$ binding site, possibly involving a charge-charge repulsion mechanism. Work carried out by Garland and Nimmo (1984) using fluorescence spectroscopy indicated that phosphorylated ICDH was unable to bind its cofactor NADPH. Addition of NADPH to active ICDH resulted in an enhancement of the fluorescence of the coenzyme with no change in its emission or excitation spectra. The addition of NADPH to inactive ICDH had no effect on either the fluorescence yield or the excitation and emission spectra of the coenzyme (Garland & Nimmo, 1984).

It would appear then that the model proposed by Nimmo (1984) has correctly predicted the inability of phosphorylated ICDH to bind its cofactor, thus inactivating it. The lack of detection of a conformational change using CD does not rule out the possibility. Conclusive evidence however, on why phosphorylated ICDH is inactive will only be determined when the crystal structure of the enzyme is determined.

Figure 6.1 Circular dichroism spectrum of isocitrate dehydrogenase

Results are plotted as ellipticity (units per mean molar ellipticity) against wavelength (nm). Data obtained as described in Methods 2.12.

[X] = ellipticity

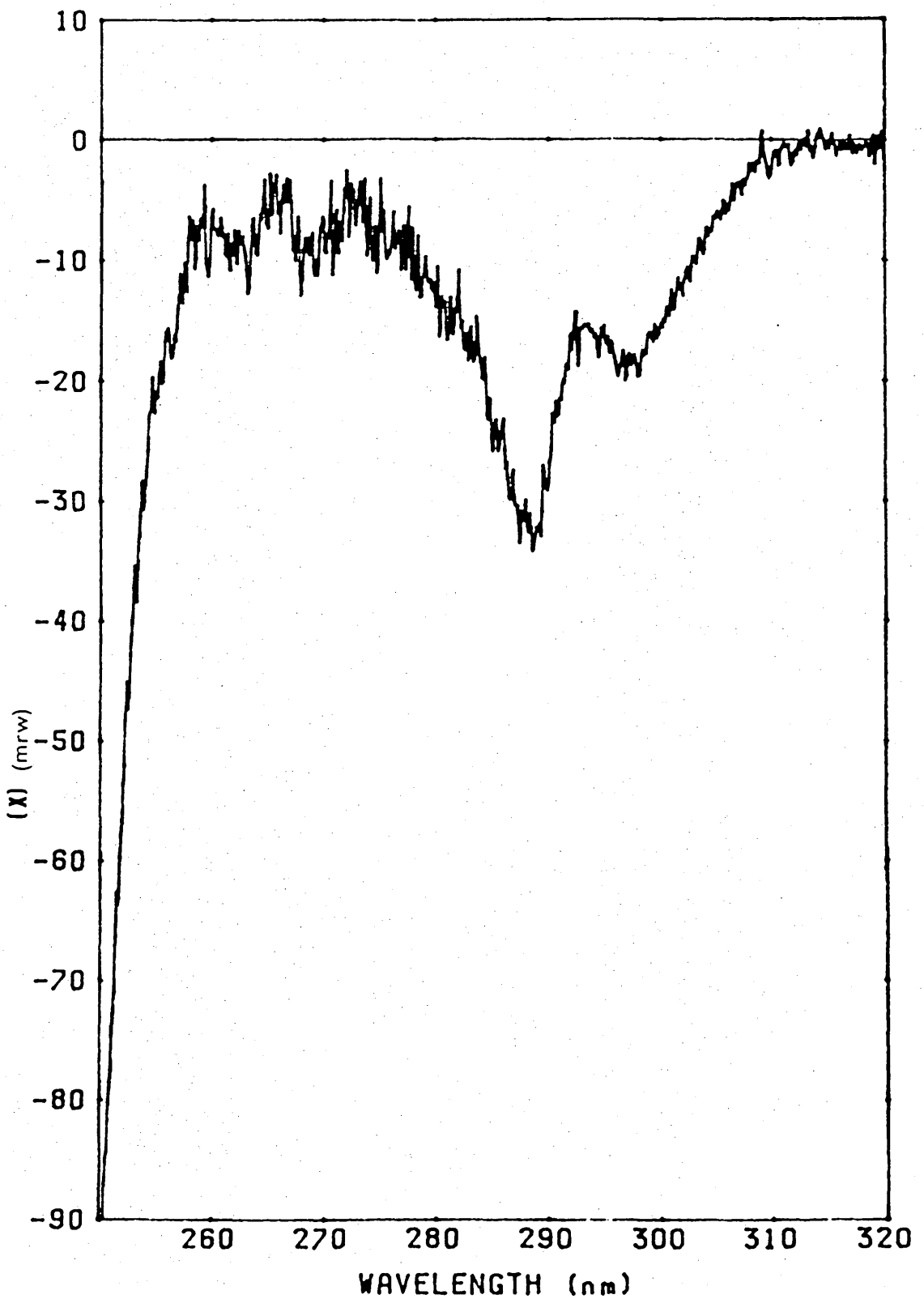

10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
(X) (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.2 Circular dichroism spectrum of isocitrate dehydrogenase in the presence of 150μM $NADP^+$

Results are plotted as for Figure 6.1.

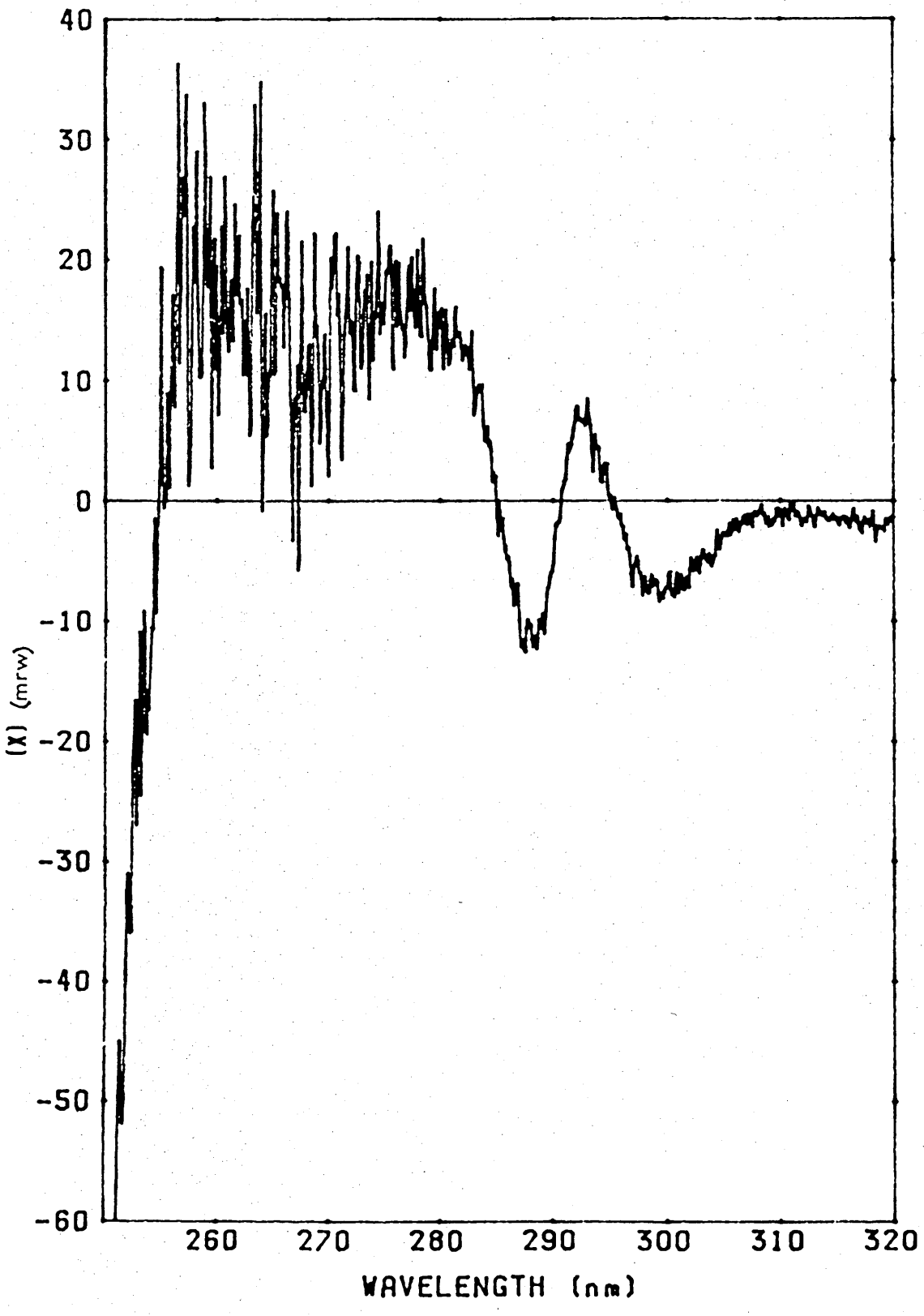

40
30
20
10
0
-10
-20
-30
-40
-50
-60
(X) (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.3 Circular dichroism difference spectrum of the isocitrate dehydrogenase + 150μM $NADP^+$ spectrum subtracted from the spectrum for isocitrate dehydrogenase.

Results are plotted as for Figure 6.1.

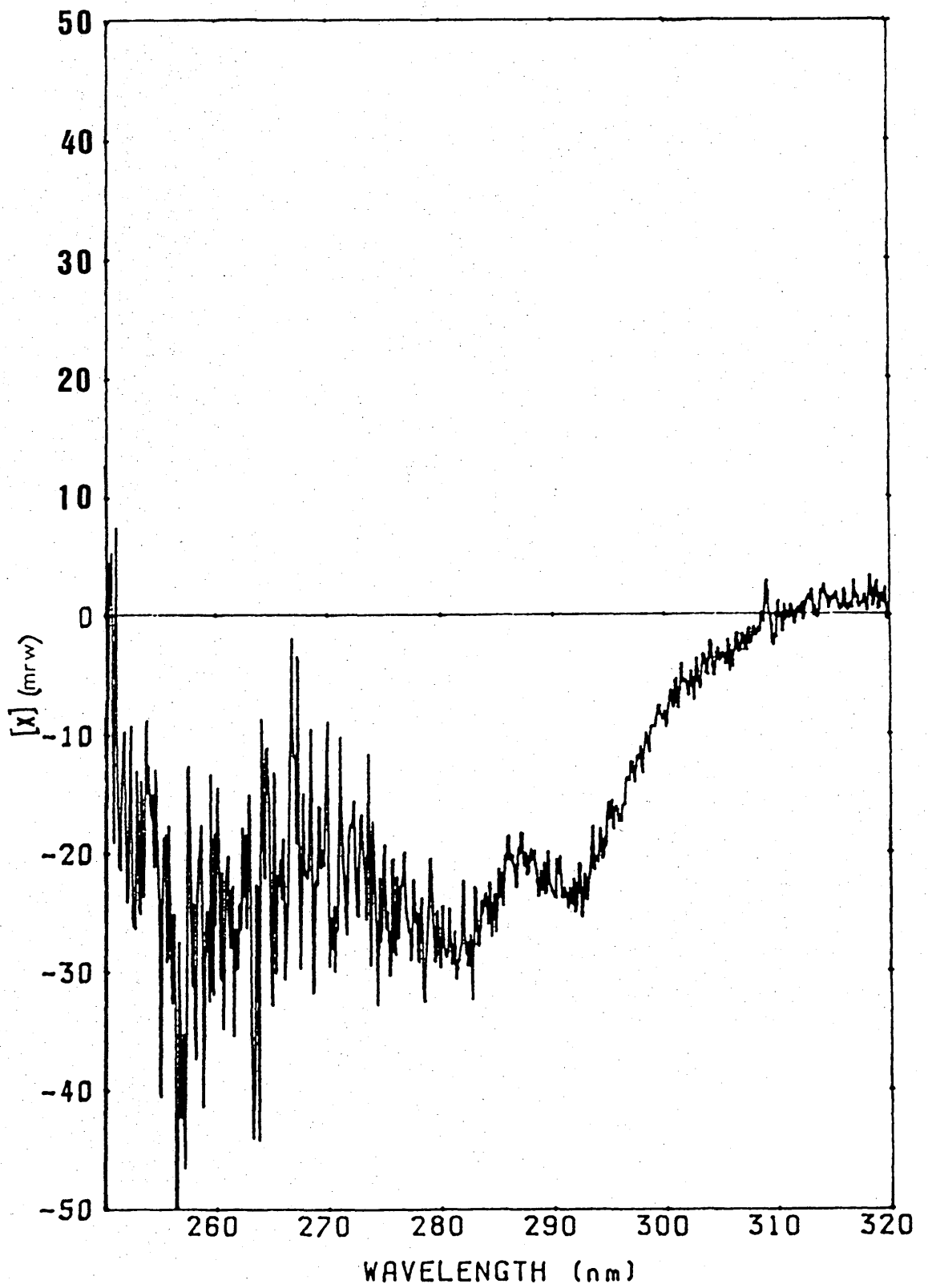

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.4 Circular dichroism spectrum of isocitrate dehydrogenase in the presence of 50μM NADPH

Results are plotted as for Figure 6.1.

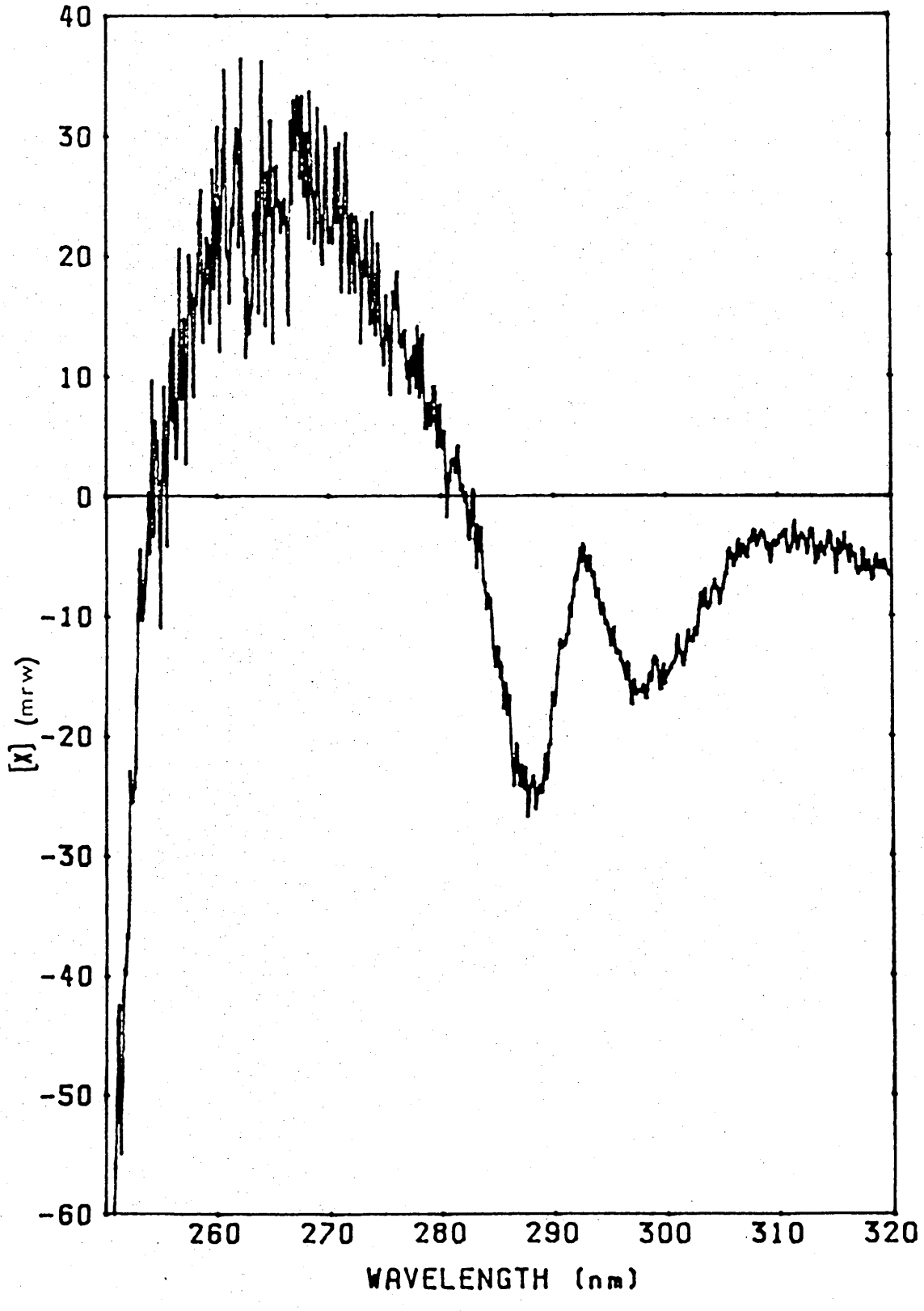

40
30
20
10
0
-10
-20
-30
-40
-50
-60
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.5 Circular dichroism difference spectrum of the isocitrate dehydrogenase + 50μM NADPH spectrum subtracted from the spectrum for isocitrate dehydrogenase.

Results are plotted as for Figure 6.1.

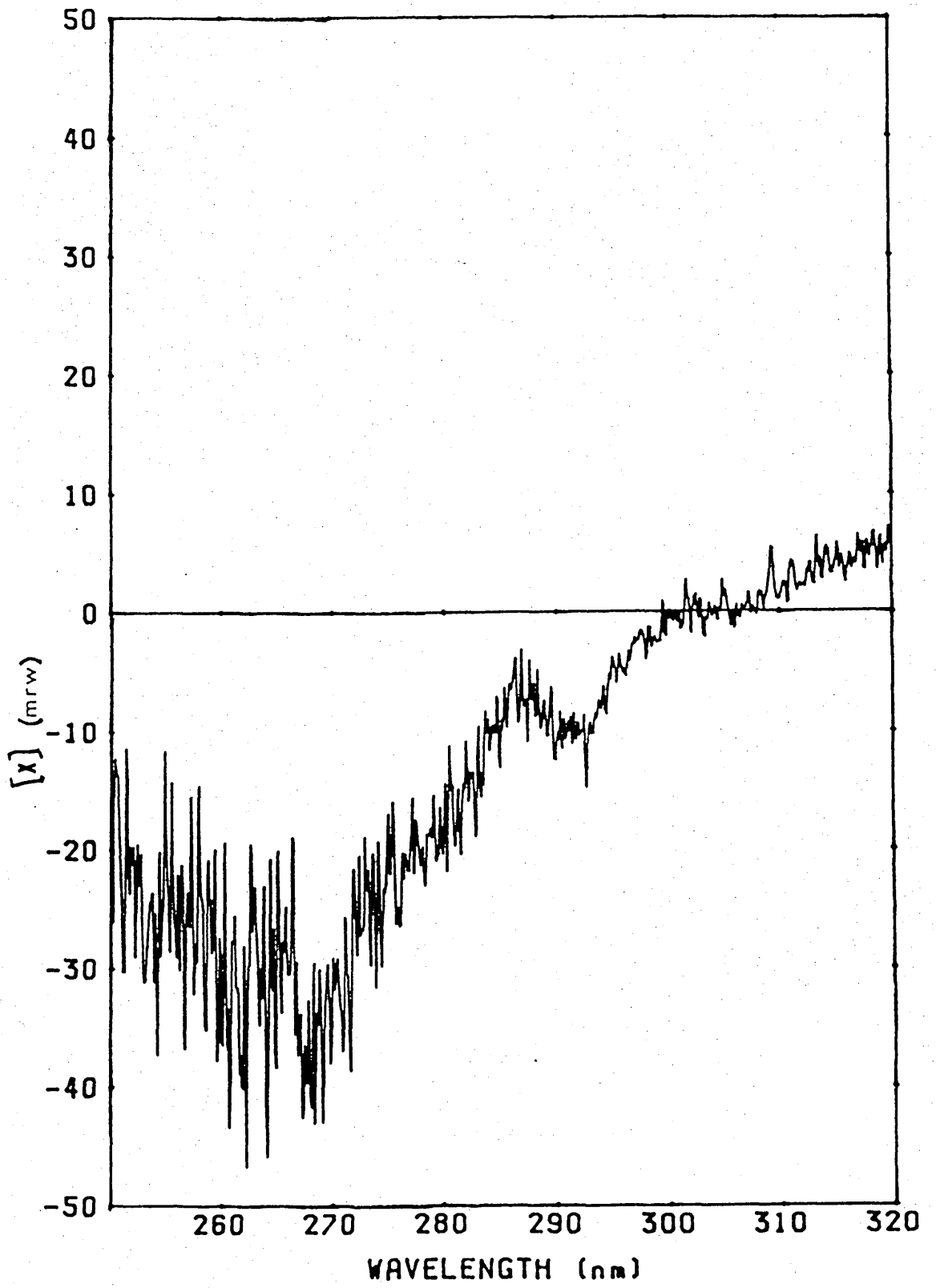

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.6 Circular dichroism spectrum of phosphorylated isocitrate dehydrogenase

Results are plotted as for Figure 6.1.

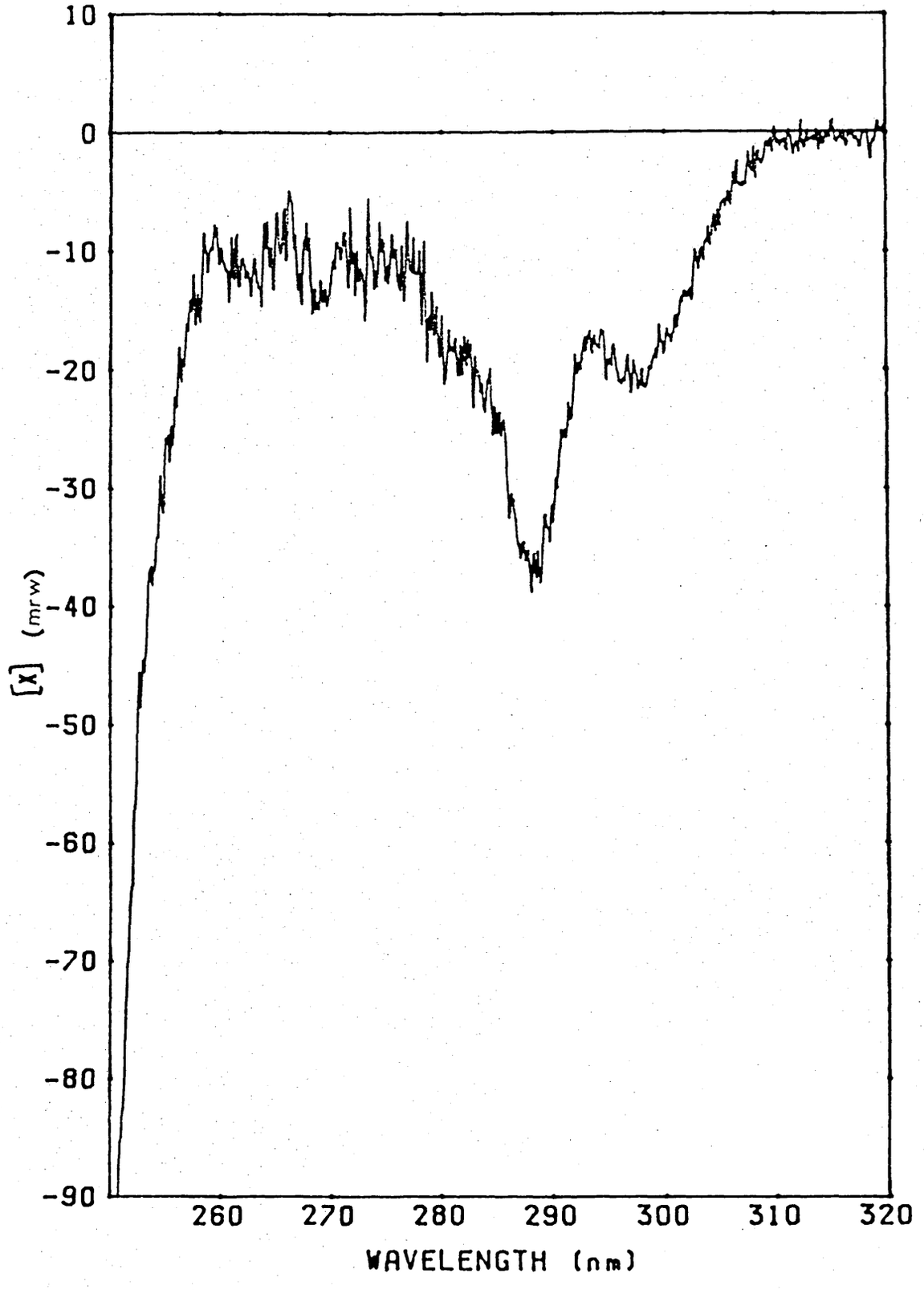

10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.7 Circular dichroism spectrum of phosphorylated isocitrate dehydrogenase in the presence of 150μM $NADP^+$

Results are plotted as for Figure 6.1.

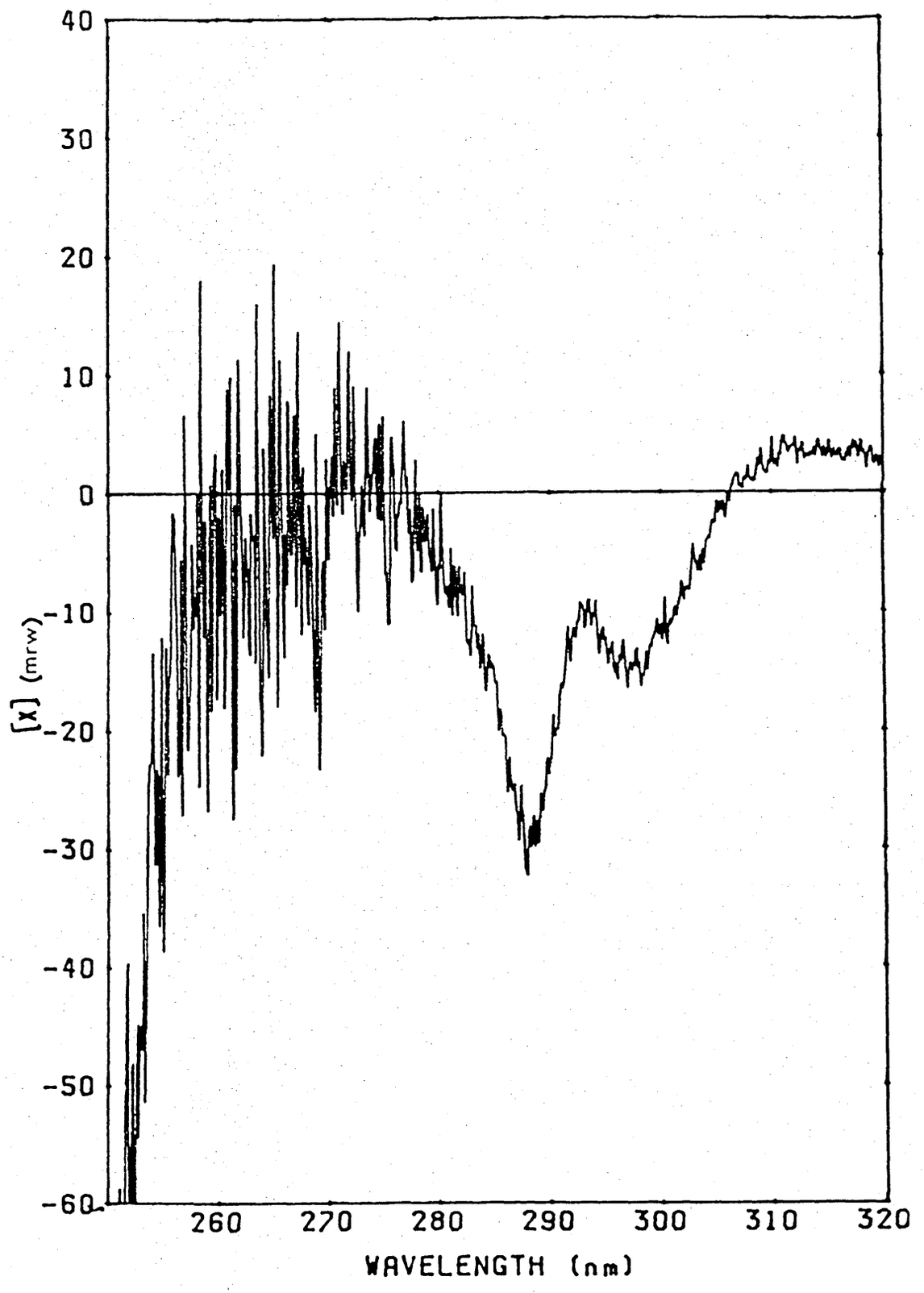

40
30
20
10
0
-10
-20
-30
-40
-50
-60
[x] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.8 Circular dichroism difference spectrum of the phosphorylated isocitrate dehydrogenase + 150μM $NADP^+$ spectrum subtracted from the spectrum for phosphorylated isocitrate dehydrogenase.

Results are plotted as for Figure 6.1.

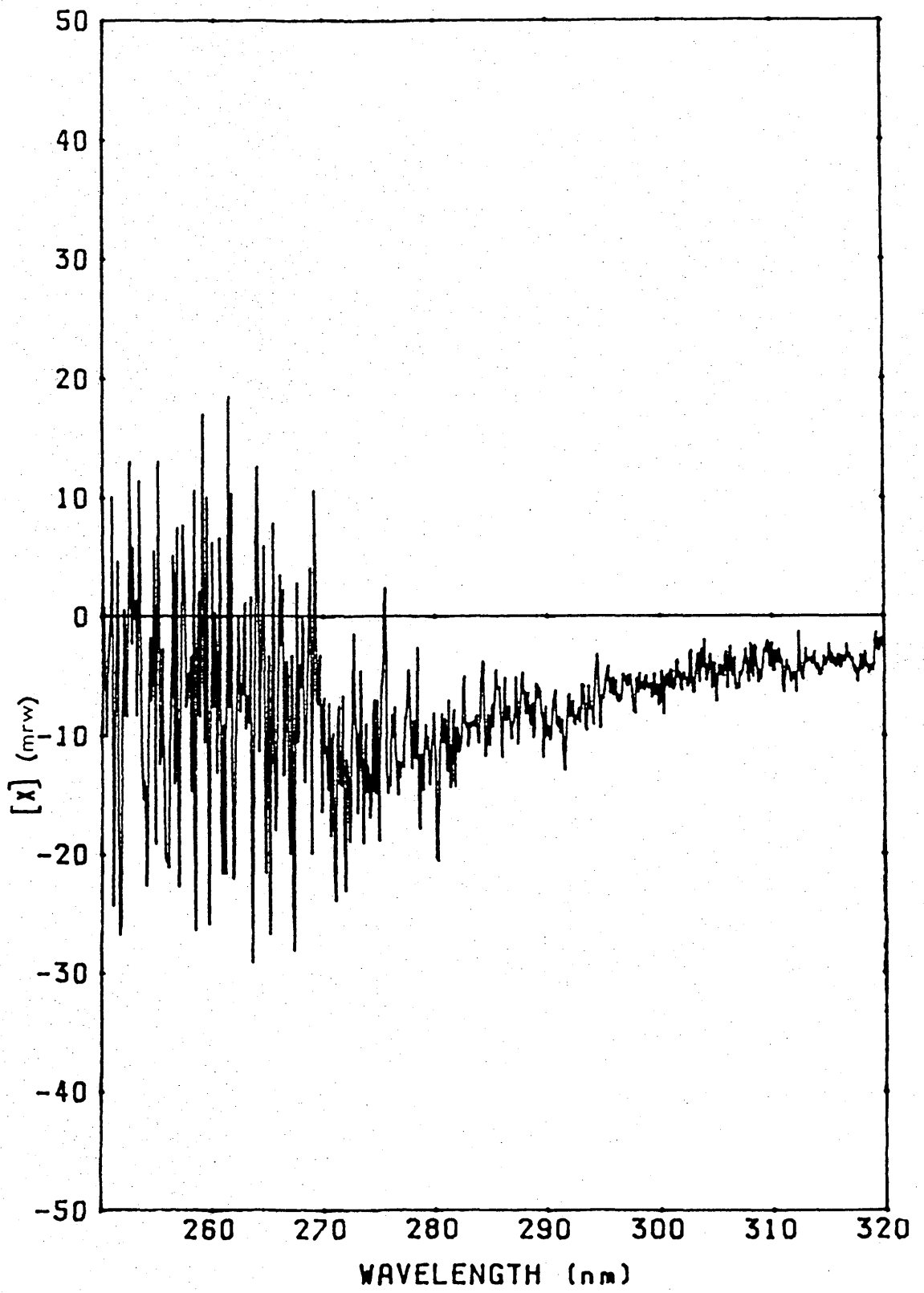

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.9 Circular dichroism spectrum of phosphorylated isocitrate dehydrogenase in the presence of 50μM NADPH.

Results are plotted as for Figure 6.1.

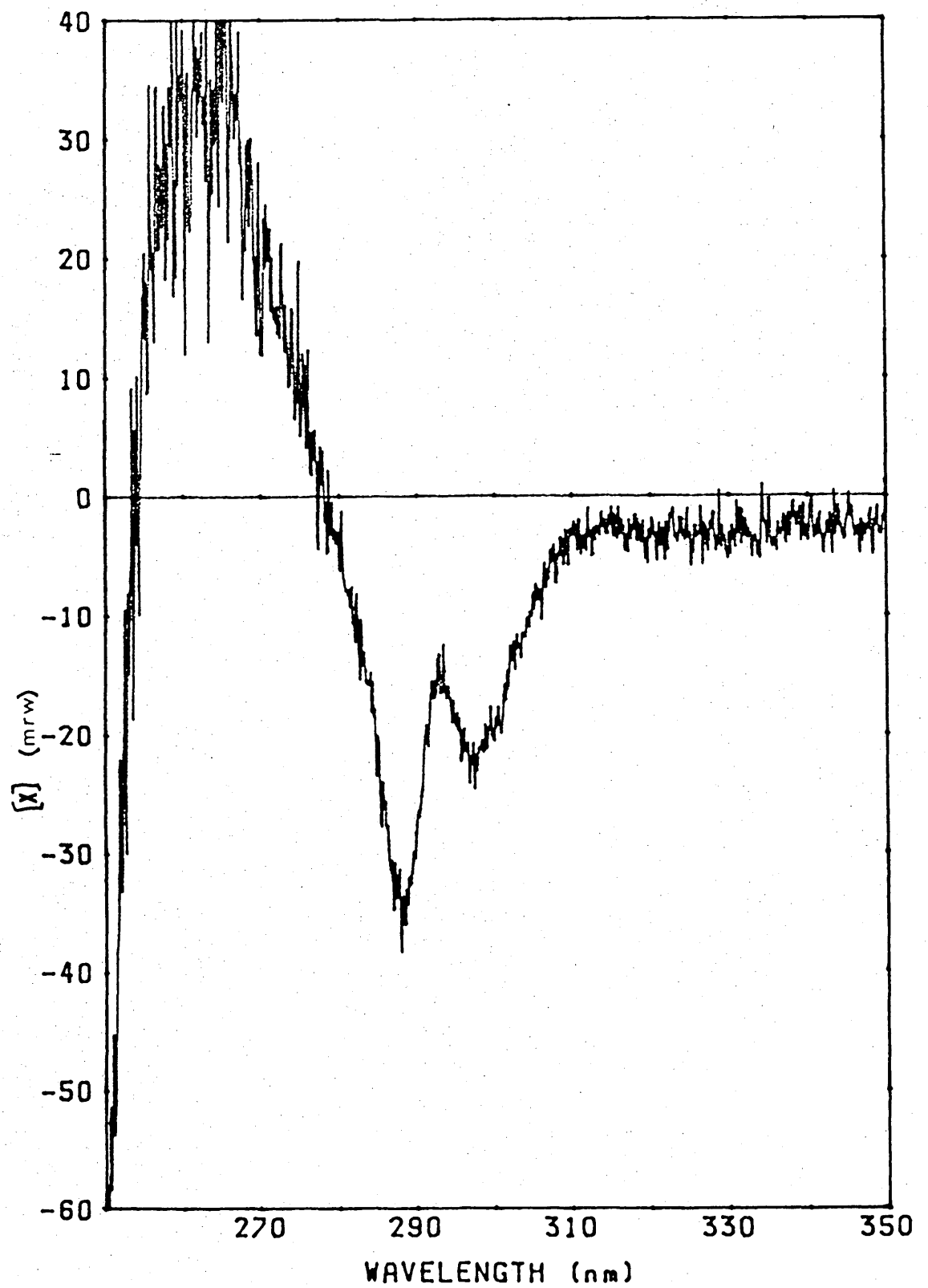

40
30
20
10
0
-10
-20
-30
-40
-50
-60
[x] (mrw)
270
290
310
330
350
WAVELENGTH (nm)

Figure 6.10 Circular dichroism difference spectrum of the phosphorylated isocitrate dehydrogenase + 50μM, NADPH spectrum subtracted from the spectrum for phosphorylated isocitrate dehydrogenase.

Results are plotted as for Figure 6.1.

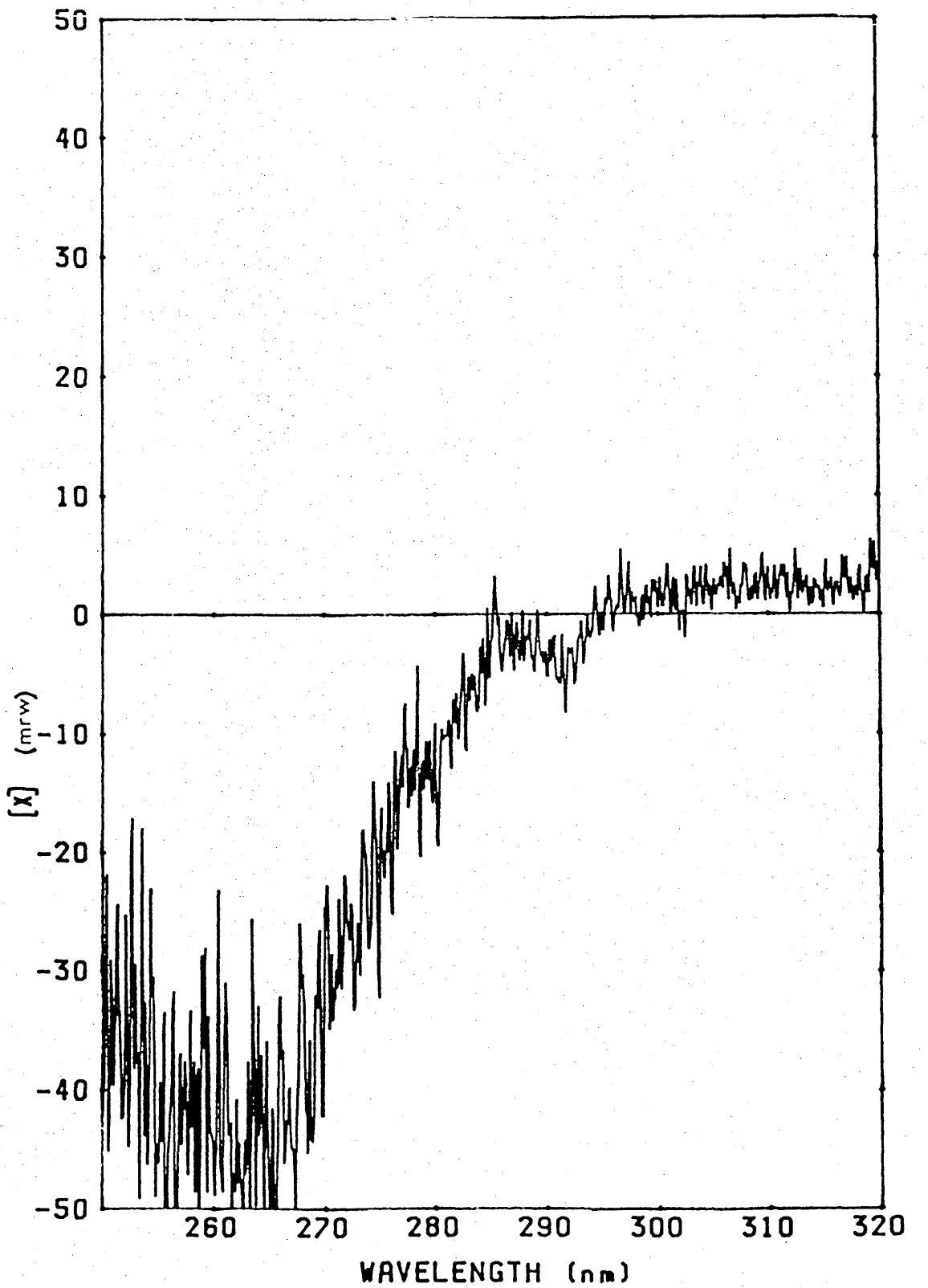

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.11 Circular dichroism difference spectrum of the phosphorylated isocitrate dehydrogenase spectrum subtracted from the spectrum for isocitrate dehydrogenase.

Results are plotted as for Figure 6.1.

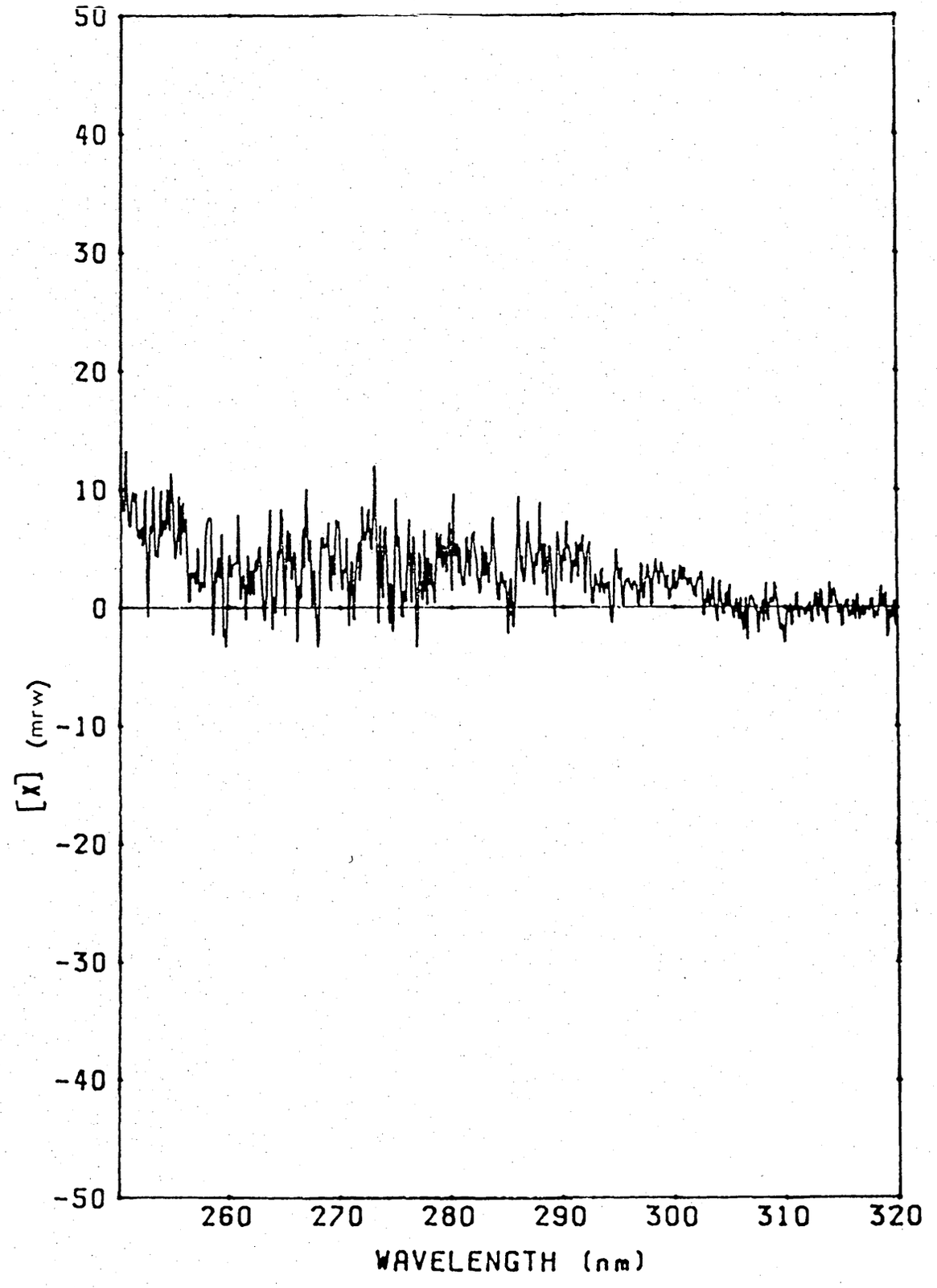

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.12 Circular dichroism difference spectrum of the phosphorylated isocitrate dehydrogenase + 150μM $NADP^+$ spectrum subtracted from the spectrum for isocitrate dehydrogenase + 150μM $NADP^+$.

Results are plotted as in Figure 6.1.

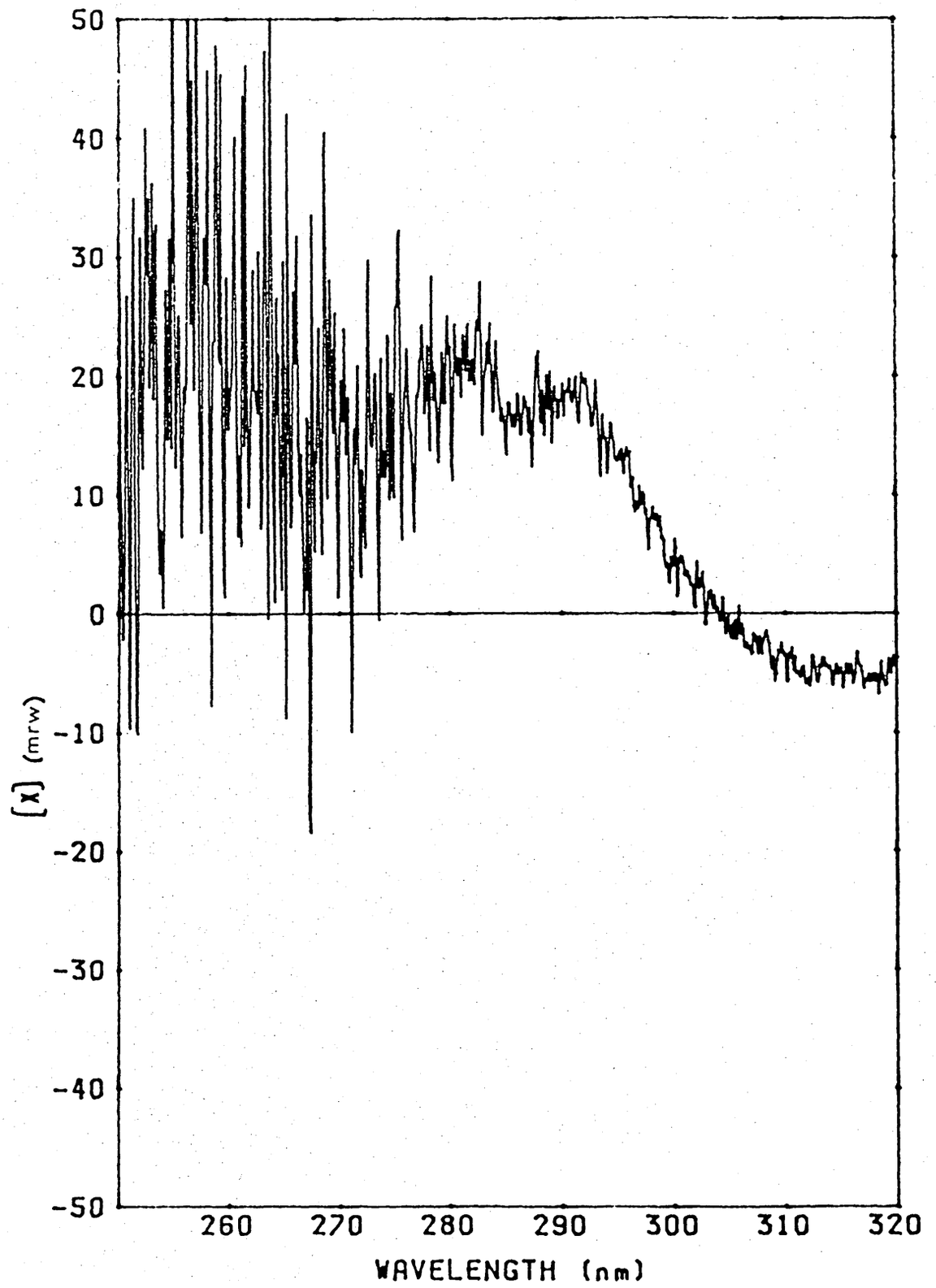

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.13 Circular dichroism difference spectrum of the phosphorylated isocitrate dehydrogenase + 50μM NADPH spectrum subtracted from the spectrum for isocitrate dehydrogenase + 50μM $NADP^+$.

Results are plotted as in Figure 6.1.

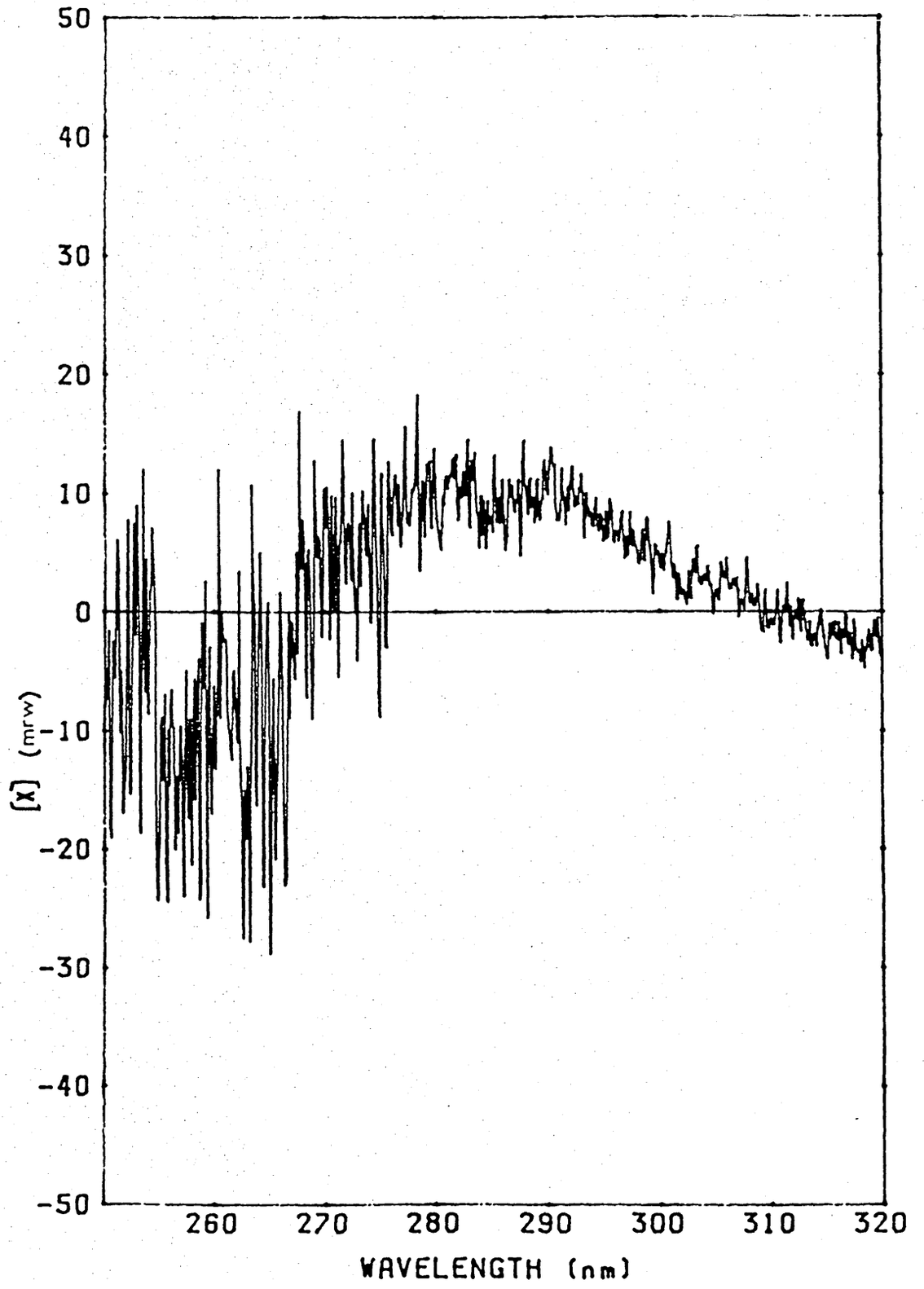

50
40
30
20
10
0
-10
-20
-30
-40
-50
[X] (mrw)
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.14 Circular dichroism spectrum of 150μM $NADP^+$

Results are plotted as voltage against wavelength (nm).

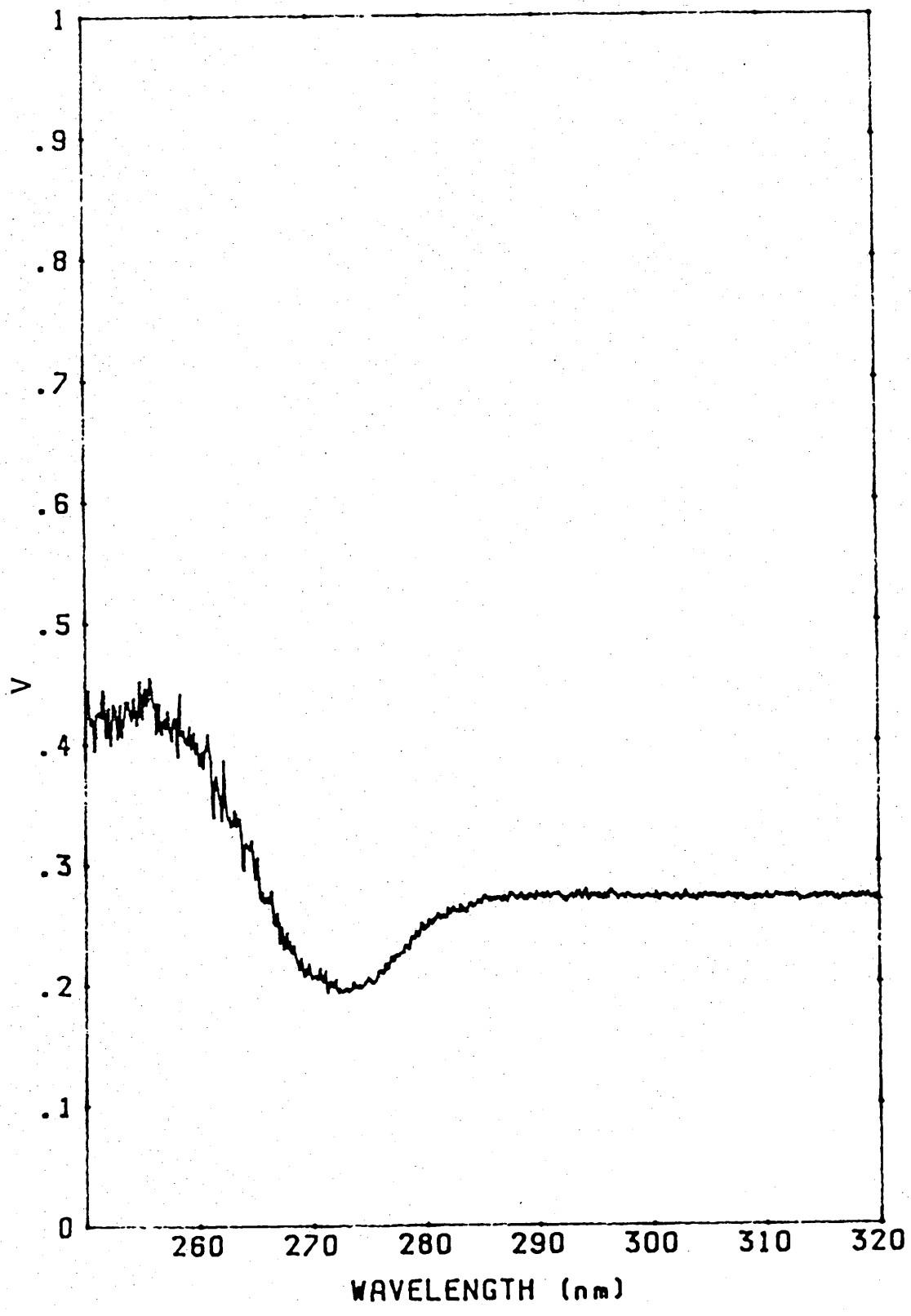
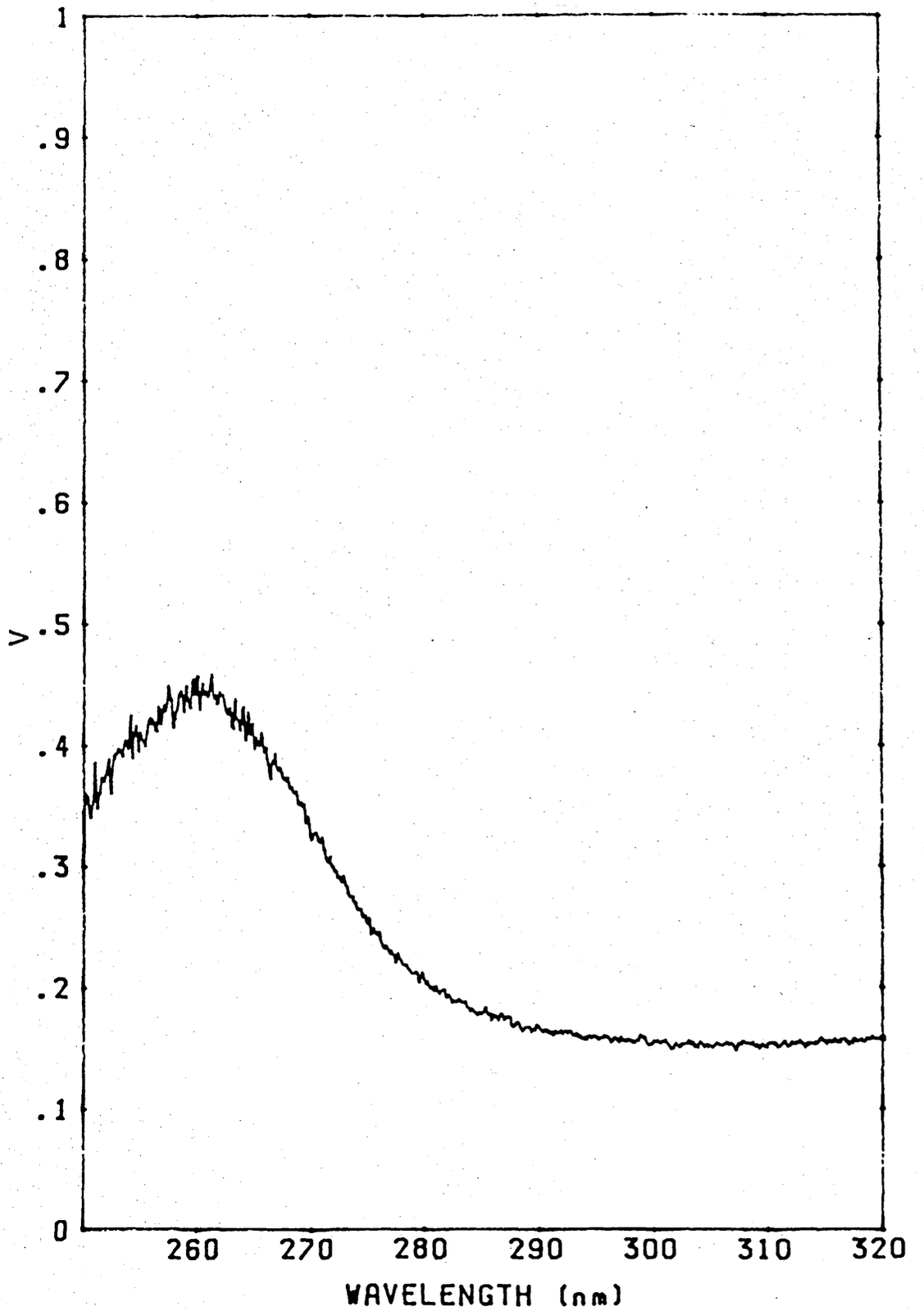

1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
V
260
270
280
290
300
310
320
WAVELENGTH (nm)

Figure 6.15 Circular dichroism spectrum of 50μM NADPH.

Results are plotted as voltage against wavelength (nm).


1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
V
260
270
280
290
300
310
320
WAVELENGTH (nm)

CHAPTER SEVEN

General Discussion

7.1 Role of phosphorylation of ICDH

The phosphorylation of ICDH in E.coli was the first example of the control of a bacterial enzyme by a phosphorylation/dephosphorylation mechanism. The present interest in the study of the phosphorylation of ICDH arose from the initial observations made by Holms and Bennett (1971) and Bennett and Holms (1975). These workers were primarily concerned with providing an explanation for the decrease in the level of ICDH activity when a culture of E.coli ML308, previously grown on limiting glucose adapted to acetate which had accumulated in the medium. Bennett and Holms concluded that covalent modification of ICDH was one possible explanation for the reversible inactivation of the enzyme. Since these early reports a great deal of information has been gained about the system. The work has been carried out by three groups, Koshland's (Berkeley), Reeves (Tempe) and here at Glasgow and has proved conclusively that the mechanism involved in the inactivation of ICDH is phosphorylation in both S.typhimurium and E.coli.

Bennett and Holms (1971) proposed that ICDH was inactivated in order to allow flux through the glyoxylate bypass. Work carried out by Koshland's group measuring fluxes through the system and by the Glasgow group studying the individual components of the system, both confirmed this initial hypothesis put forward by Bennett and Holms. Thus the role of phosphorylation of ICDH was to control usage of isocitrate under one particular condition, growth on acetate. This inactivation of ICDH allows a build-up in the levels of intracellular isocitrate enabling isocitrate lyase to operate and results in efficient use of acetate carbon via the glyoxylate bypass.

Although a lot of work has been carried out on the function of phosphorylation within the cell, and on the individual components of the system, little work has been done on why phosphorylated ICDH

is inactive. This provided a starting point for the work reported in this thesis.

7.2 The active site of isocitrate dehydrogenase

One of the main findings of this work was the identification of an arginine residue at the active site of ICDH. This residue could be protected either by NADP binding or phosphorylation. The data conformed to the hypothesis proposed by Nimmo (1984) that phosphorylation of ICDH occurred close to or at the $NADP^+$ binding site (Fig.1.5). However, we were unable to determine any sequence data to compare with the known protein and phosphorylation sequences.

The proposed location of the ICDH phosphorylation site raises the question concerning the evolution of such a control mechanism. Consider E.coli and its ability to grow on a variety of carbon sources. Here we make the assumption that the TCA cycle was the pathway first evolved by the cell. At a later stage the glyoxylate bypass was evolved to allow growth on acetate. This provided the cell with an alternative route for the available isocitrate during growth on acetate, and resulted in competition between ICDH and the glyoxylate bypass enzyme isocitrate lyase for their common substrate. To enable efficient use of acetate carbon via the glyoxylate bypass the cell would have to alter the activity of one of the two enzymes, either by decreasing the activity of ICDH or increasing the activity of ICL. In the case of E.coli it appears the former choice was adopted, it was easier for the cell to reduce the levels of ICDH activity rather than to create a more efficient lyase.

Consider then how the E.coli cell could regulate its level of ICDH activity. ICDH is not an allosteric protein and it can be said that it plays no role in the control of the TCA cycle except

during growth on acetate. One method of activity control would be for the ICDH to be controlled by an allosteric mechanism. To create an allosterically controlled ICDH would involve evolution of an allosteric binding site within the protein molecule which would necessitate considerable change to the protein structure. In the case of eukaryotes, proteins whose activities are controlled by phosphorylation are normally allosteric. The phosphorylation of such proteins usually has the effect of 'locking' the enzyme structure into one particular conformation and over-riding the allosteric control mechanism. In ICDH this is not possible since there is no allosteric control. However, one simple way of inactivating an enzyme via phosphorylation would be to modify its active site. In the case of ICDH the modification appears to prevent the binding of $NADP^+$.

The sequence of the ICDH protein is known. However, analysis of the primary structure reveals no protein region which bears any resemblance to known nucleotide binding domains (Thorsness & Koshland, 1987). This may indicate that ICDH, as a result of its control by phosphorylation, has evolved a slightly different nucleotide binding site to accommodate this function.

7.3 ICDH kinase/phosphatase

The mechanism of phosphorylation of ICDH differs significantly from that of eukaryotic enzymes. This is also reflected in the kinases which carry out the reactions. Eukaryotic kinases recognise and phosphorylate short linear sequences of amino acids. Such kinase recognition sequences are thought to occur on 'arm-like' structures located on the outside of the protein molecule. The phosphorylation of this structure is sufficient to lock the conformation into one particular shape. The ICDH kinase of E.coli, however, does not recognise as a potential substrate a

short linear sequence corresponding to the phosphorylation sequence. It seems only to recognise the whole protein as a substrate. This can be related to the location of the phosphorylation site within the protein itself. The active site of the enzyme will form an integral part of the protein. As a result the kinase must in turn 'surround' the protein to gain access to the active site. Thus substrate recognition involves the overall protein shape, and cannot be achieved by the phosphorylation site sequence alone located in the active site of the enzyme.

The gene for the ICDH kinase/phosphatase has been sequenced and it is unlike any determined for eukaryotic kinases. This suggests a different evolutionary origin for this enzyme. Similarly the kinase and phosphatase activities are located on the same bifunctional protein which is also contrary to eukaryotic protein kinases and their corresponding phosphatase activities (except maize pyruvate phosphate di-kinase interconverting enzyme) (Sugiyma, 1973). E.coli appears to have evolved a completely different phosphorylation system to that found in eukaryotes. The reasons for this difference may be in the availability of possible 'starter' proteins from which to evolve a kinase and phosphatase activity. Results from LaPorte's group suggest that the kinase and phosphatase activities share the same active site (Stueland et al., 1987). This would be consistent with the evolution of a reversible kinase activity further evolving to produce a kinase and phosphatase function. This would result in an ability to control both activities closely, a situation which occurs with the bifunctional kinase/phosphatase.

The origin of the ICDH kinase/phosphatase however still remains unknown. It is only with sequence comparison of the gene sequence to other known gene sequences that we might envisage an

answer, and at the same time discern why E.coli adopted a different evolutionary pathway to create a kinase quite distinct from that of eukaryotes.

7.4 Future Work

The work carried out in this thesis has provided a variety of directions for future work. The first relates to the arginine residue modified by phenylglyoxal. Recently a new arginine-modifying reagent, 4-(oxoacetyl) phenoxyacetic acid (OPA) has been developed (Duerksen-Hughes et al., 1987). This was reported to produce a stable modification of arginine residues. It would be interesting to repeat the phenylglyoxal labelling experiments with this reagent, which might yield a stable modification of ICDH. This would enable isolation of a labelled peptide for sequence analysis. However it is not clear in chemical terms that arginines modified by OPA would always be more stable than arginine residues modified by phenylglyoxal. Instability of the adduct might well be a problem with this reagent too.

Secondly we believe the arginine residue modified with phenylglyoxal is the arginine residue adjacent to the phosphorylatable serine residue in ICDH. We have the icd gene from E.coli ML308 and K12. The K12 gene has already been sequenced (Thorsness & Koshland, 1987) and the ML308 gene is currently being sequenced in Glasgow. Site-directed mutagenesis will be used as a tool to alter this arginine. This should help to clarify the functional role, if any, of this arginine residue. For example, an arginine to lysine change would retain the charge of the arginine residue, but should render the enzyme insensitive to phenylglyoxal inactivation.

A third direction relates to the conformation of ICDH. We used circular dichroism in the near U.V. region and were unable to detect any conformational changes, but seemed to detect an inability

of phospho-ICDH to bind $NADP^+$. This lack of detection of a conformational change on $NADP^+$ binding and phosphorylation does not necessarily mean that no change has occurred. Thus using circular dichroism in the far U.V. region which detects changes in amounts of secondary structure features such as alpha-helix and beta-sheet, might reveal a conformational change under these conditions.

Another interesting line of investigation concerns E.coli strain EB106. This is an icd mutant which is believed to contain a single point mutation in the icd gene. It synthesises a completely inactive ICDH protein (Apostolakos et al., 1982). We are interested in why this mutant ICDH is inactive. The protein is produced in the cell which suggests that it folds properly. However, it would be interesting to purify this protein and compare its conformation with those of active and inactive ICDH. Further, attempts to phosphorylate the protein, modify it with phenylglyoxal and study its ability to bind $NADP^+$ might be useful. Ultimately, sequencing of the mutant icd might throw further light on the structure/function relationships within ICDH.

References

Adams, M.J., Buehner, M., Chandrasekhar, K., Ford, G.C., Hackert, M.L., Liljas, A., Rossmann, M.G., Smiley, I.E., Allison, W.S., Everse, J., Kaplan, N.O. & Taylor, S.S. (1973) Proc. Natl. Acad. Sci. USA 70, 1968-1972.

Adler, A.J., Greenfield, N.J. & Fasman, G.D. (1973) in Methods in Enzymol. vol.XXVII 675-750.

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

Adler, S.P., Mangum, J.H., Magni, C. & Stadlman, E.R. (1974) Metabolic interconversions of enzymes (eds. E.H. Fischer, E.G. Krebs, H. Neurath and E.R. Stadlman) Springer-Verlag, Heidelberg, 221-233.

Apostolakos, D., Mentor, P.A., Rampach, B.J., Reeves, H.C. & Birge, E.A. (1982) Curr. Microb. 7, 45-47.

Ashworth, J.M. & Kornberg, H.L. (1966) Proc. Royal Soc. London (series B) 165, 179-188.

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

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

Blumenthal, K.M. & Smith, E.L. (1975) J. Biol. Chem. 250, 6555-6559.

Borders, C.L. & Riordan, J.F. (1975) Biochemistry 14, 4699-4704.

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

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

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

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

Britten, R. (1954) Science 119, 578.

Bylund, D.B. & Krebs, E.G. (1975) J. Biol. Chem. 250, 6355-6361.

Canovas, J.L. & Kornberg, H.L. (1965) Biochim. Biophys. Acta. 96, 169-172.

Canovas, J.L. & Kornberg, H.L. (1966) Proc. Royal Soc. London (series B) 165, 189-205.

Chaudhuri, S., Lambert, J.M., McColl, L.A. & Coggins, J.R. (1986) Biochem. J. 239, 699-704.

Cohen, P. (1983) Control of Enzyme Activity 2nd Ed., Outline Studies in Biology. Chapman & Hall, New York.

Cohen, P. (1988) Proc. Royal Soc. London (series B) 234, 115-144.

Cohen, S., Ushivo, H., Stoscheck, C. & Chinkero, M. (1982) J. Biol. Chem. 257, 1523-1531.

Cole, S.C.J., Yaghmaie, P.A., Butterworth, P.J. & Yon, R.J. (1986) Biochem. J. 233, 303-306.

Comis, A. & Easterbrook-Smith, S.B. (1985) Biochim, Biophys. Acta 842, 45-51.

Cori, G.T., Colowick, S.P. & Cori, C.F. (1938) J. Biol. Chem. 123, 381-389.

Cori, G.T. & Cori, C.F. (1945) J. Biol. Chem. 158, 321-332.

Cozzone, A.J. (1984) TIBS 9, 400-403.

Daile, P. & Carnegie, P.R. (1974) Biochem. Biophys. Res. Commun. 61, 852-858.

David, M., Rasched, I. & Sund, H. (1976) FEBS Lett. 62, 288-292.

Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404-427.

Deurksen-Hughes, P.J., Xu, X. & Wilkinson, K.D. (1987) Biochemistry 26, 6980-6987.

El-Mansi, E.M.T., MacKintosh, C., Duncan, K., Holms, W.H. & Nimmo, H.G. (1987) Biochem. J. 242, 661-665.

El-Mansi, E.M.T., Nimmo, H.G. & Holms, W.H. (1985) FEBS Lett. 183, 251-255.
Ernster, L. & Navazio, F. (1956) Experimental Cell Research 11, 483-486.
Fischer, E.H. & Krebs, E.G. (1955) J. Biol. Chem. 216, 121-132.
Freifelder, D. (1982) Physical Biochemistry (2nd Edition) W.H. Freeman & Co., New York.
Garland, D. & Nimmo, H.G. (1984) FEBS Lett. 165, 259-263.
Garnak, M. & Reeves, H.C. (1979a) Science 203, 1111-1112.
Garnak, M. & Reeves, H.C. (1979b) J. Biol. Chem. 254, 7915-7920.
Gilbert, H.F. & O'Leary, M.H. (1975) Biochem. Biophys. Res. Commun. 67, 198-202.
Gottschall, G., Giffham, F. & Antranikian, G. (1982) Biochem. Soc. Trans. 10, 324-326.
Graves, D.J. & Wang, J.H. (1972) Enzymes I 435-482 (ed. Boyer).
Gutte, B. & Merrifield, R.B. (1971) J. Biol. Chem. 246, 1922-1941.
Harvey, N.L., Fewson, C.A. & Holms, W.H. (1968) Lab. Pract. 17, 1134-1136.
Helmreich, E. & Cori, C.F. (1964) J. Biol. Chem. 239, 2440-2445.
Holms, W.H. & Bennett, P.M. (1971) J. Gen. Microbiol. 65, 57-68.
Holzer, H., Mecke, D., Wuff, K., Liess, K. & Heilmeyer, L. (1967) Adv. Enz. Reg. 5, 211-225.
Hoppe, J. (1985) TIBS 10, 246-249.
Huang, K-P. & Cabib, E. (1974) J. Biol. Chem. 249, 3815-3857.
Hummel, J.P. & Dreyer, W.J. (1962) Biochim. Biophys. Acta 63, 530-532.
Kantrowitz, E.R. & Lipscomb, W.N. (1976) J. Biol. Chem. 251, 2688-2695.
Keller, P.J. (1955) J. Biol. Chem. 214, 135-141.
Kemp, B.E., Benjamini, E. & Krebs, E.G. (1976) Proc. Natl. Acad. Sci. USA 73, 1038-1042.
Koland, J.G., O'Brien, T.A. & Gennis, R.B. (1982) Biochemistry 21, 2656-2660.
Kornberg, H.L. (1966) Essays in Biochemistry 2, 1-31.
Kornberg, H.L. & Madsen, N.B. (1957) Biochim. Biophys. Acta 24, 651-653.
Krebs, E.G. (1972) Curr. Top. Cell Reg. 5, 99-133.
Krebs, E.G. & Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923-959.
Krebs, E.G. & Fischer, E.H. (1956) Biochim. Biophys. Acta 20, 150-157.
Krebs, H.A. & Johnston, W.A. (1937) Enzymologia 4, 148-156.
Kuo, J.F. & Greengard, P. (1969) Proc. Natl. Acad. Sci. USA 64, 1349-1355.
Laemmli, U.K. (1970) Nature (London) 227, 680-685.
Langan, T.A. (1973) Adv. Cyc. Nucl. Res. 3, 99-153.
LaPorte, D.C. & Chung, T. (1985) J. Biol. Chem. 260, 15291-15297.
LaPorte, D.C. & Koshland, D.E. (1982) Nature (London) 300, 458-460.
LaPorte, D.C. & Koshland, D.E. (1983) Nature (London) 305, 286-290.
LaPorte, D.C., Walsh, K. & Koshland, D.E. Jr. (1984) J. Biol. Chem. 259, 14068-14075.
Lobb, R.R., Stokes, A.M., Hill, H.A.O. & Riordan, J. F. (1976) Eur. J. Biochem. 70, 517-522.
MacKintosh, C. & Nimmo, H.G. (1988) Biochem. J. 250, 25-31.
Malloy, P.J., Reeves, H.C. & Spiess, J. (1984) Curr. Microbiol. 11, 37-41.
Marr, J.J. & Weber, M.M. (1968) J. Biol. Chem. 243, 4973-4979.
Moyle, J. (1956) Biochem. J. 63, 552-558.
Nimmo, H.G. (1984) TIBS 9, 475-478.
Nimmo, H.G. (1986) Biochem. J. 234, 317-323.

Nimmo, G.A., Borthwick, A.C., Holms, W.H. & Nimmo H.G. (1984) Eur. J. Biochem. 141, 401-408.
Nimmo, H.G., Borthwick, A.C., El-Mansi, E.M.T., Holms, W.H., MacKintosh, C. & Nimmo, G.A. (1987) Biochem. Soc. Symp. 54, 93-101.
Nimmo, G.A. & Nimmo, H.G. (1984) Eur. J. Biochem. 141, 409-414.
Ochoa, S. (1948) J. Biol. Chem. 174, 133-157.
Patthy, L. & Smith, E.L. (1975) J. Biol. Chem. 250, 565-569.
Patthy, L. & Thesz, J. (1980) Eur. J. Biochem. 105, 387-393.
Patthy, L., Varadi, A., Thesz, J. & Kovacs, K. (1979) Eur. J. Biochem. 99, 309-313.
Perutz, M.F. (1978) Sci. Amer. 239, 68-88.
Peters, R.G., Jones, W.C. & Cromartie, T.H. (1981) Biochemistry 20, 2564-2571.
Pinna, L.A., Agostinis, P. & Ferrari, S. (1986) Adv. Protein Phosphatases III 327-368.
Plaut, G.W.E. (1963) The Enzymes (Boyer) 1st Ed., vol. VII, 105-126.
Powers, S.G. & Riordan, J.F. (1975) Proc. Natl. Acad. Sci. USA 72, 2616-2620.
Raglan, T.E., Kawasaki, T. & Lowenstein, J.M. (1966) J. Bac. 91, 236-244.
Reeves, H.C. & Ajl, S. (1960) J. Bac. 79, 341-345.
Reimann, E.M., Walsh, D.A. & Krebs, E.G. (1971) J. Biol. Chem. 246, 1986-1995.
Riordan, J.F. (1973) Biochemistry 12, 3915-3922.
Riordan, J.F. (1979) Molecular & Cellular Biochem. 26, 71-92.
Riordan, J.F. & Scandurra, R. (1975) Biochem. Biophys. Res. Commun. 66, 417-424.
Robinson, G.A., Butcher, R.W. & Sutherland, E.W. (1971) Cyclic AMp Academic Press, New York & London.
Rogers, T.B., Borresen, T. & Feeney, R.E. (1978) Biochemistry, 17, 1105-1109.
Ross, H.H. (1969) Anal. Chem. 41, 1260.
Shiio, J. & Ozaki, H. (1968) J. Biol. Chem. 64, 45-53.
Singh, T.J., Akatsuka, A. & Huang, K-P. (1982) J. Biol. Chem. 257, 13379-13384.
Smith, D.L., Chen, C.C., Bruegger, B.B., Holz, S.L., Halpern, R.M. & Smith, R.A. (1974) Biochemistry 13, 3780-3785.
Sprang, S.R., Achanya, K.R., Goldsmith, E.J., Stuart, D.I., Varvill, K., Fletterick R.J., Madsen, N.B. & Johnson, L.N. (1988) Nature (London) 336, 215-221.
Sprang, S.R. & Fletterick, R.J. (1979) J. Mol. Biol. 131, 523-551.
Springer, W.R. & Koshland, D.E. Jr. (1977) Proc. Natl. Acad. Sci. USA 74, 533-537.
Staros, J.V., Cohen, S. & Russo, M.W. (1985) Molecular Mechanisms of Transmembrane Signalling (Cohen, P. & Houslay, M.D. eds) vol.4, 253-277.
Stewart, J.M. & Young, J.D. (1984) Solid phase peptide synthesis (2nd Ed. Pierce Chemical Company).
Straud, R.M., Kossiakoff, A.A. & Chambers, J.L. (1977) Ann. Rev. Biophys. Bioenerg. 6, 177-193.
Stueland, C.S., Eck, K.R., Stieglbaur, K.T. & LaPorte, D.C. (1987) J. Biol. Chem. 262, 16095-16099.
Sugiyma, T. (1973) Biochemistry 12, 2862-2868.
Sutherland, E.W. Jr. & Woisilait, W.D. (1955) Nature (London) 175, 169-170.
Takahashi, K. (1968) J. Biol. Chem. 243, 6171-6179.
Thorsness, P.E. & Koshland, D.E. Jr. (1987) J. Biol. Chem. 262, 10422-10425.

Timasheff, S.N. (1970) The Enzymes (Boyer Ed., 3rd Ed.) vol. II 381-408.

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

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y-C., Tsulokawa, M., Mason, A., Seerburg. P.H., Grunfield, C., Rosen, O.M. & Ramachandran, J. (1985) Nature (London) 313, 756-761.

Vandenbunder, B., Dreyfus, M., Bertrand, O., Dognan, M.J., Sibilli, L. & Buc, H. (1981) Biochemistry 20, 2354-2360.

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

Walsh, K. & Koshland, D.E. Jr. (1984) J. Biol. Chem. 259, 9646-9654.

Walsh, K. & Koshland, D.E. Jr. (1985) J. Biol. Chem. 260, 8430-8437.

Walsh, D.A., Perkins, J.P. & Krebs, E.G. (1968) J. Biol. Chem. 243, 3763-3765.

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

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