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REFOLDING STUDIES ON 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES

Ellen Sarah Beaumont

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Division of Biochemistry, IBLS. October 1996

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Abstract

The 2-oxoacid dehydrogenase multienzyme complexes are high M_r assemblies occupying key positions in intermediary metabolism. Their function is to catalyse the irreversible oxidative decarboxylation of 2-oxoacids by the coordinated action of multiple copies of three separate enzymes termed E1 (2-oxoacid dehydrogenase), E2 (dihydrolipoamide acyltransferase) and E3 (dihydrolipoamide dehydrogenase). The 2-oxoacid dehydrogenase multienzyme complex family consists of the pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC) and branched chain 2-oxoacid dehydrogenase complex (BCDC). These complexes have been purified from a wide variety of organisms and in all complexes studied, the E2 component forms a symmetrical structural core to which the E1 and E3 subunits bind. The E1 and E2 components perform complex specific reaction steps, while the E3 component is responsible for the reoxidation of the lipoamide group in each of the complexes.

In eukaryotes, these nuclear encoded complexes are located in the mitochondrion, loosely associated with the inner face of the inner membrane. The *in vivo* translocation, folding and assembly of such large structures is an intriguing process which is at present not fully understood.

This thesis examines the GdnHCl-induced deactivation and subsequent reactivation on dilution from denaturant of OGDC and PDC from bovine heart and PDC from *E. coli*. Activity is found to be lost at relatively low GdnHCl concentrations in each complex (below 0.3 M). The *E. coli* PDC is shown to have spontaneous reactivation after higher concentrations of GdnHCl incubation than the bovine heart complexes. The deactivation and reactivation of the individual complex components has also been studied. The E2 component was found to reactivate after higher concentration GdnHCl incubations than the peripheral subunits. The deactivation and reactivation curves of complex intact and isolated components have been compared. For *E. coli* E3 this revealed a marked difference, with the intact E3

being considerably more stable than its isolated counterpart. This effect is thought to be due to the hydrophobic association of the E3 dimer with the E2 core, which has been shown to be reactivated after higher GdnHCl incubations.

The refolding environment of the E3 component has been studied in more detail in Chapter 5. E3 is a member of flavin-containing pyridine nucleotide-disulphide oxidoreductases. All members of this group studied to date are homodimers containing one flavin adenine dinucleotide (FAD) per subunit and a redox active disulphide. Purified E3 from bovine heart, yeast and *E. coli* have been unfolded using GdnHCl (as determined by CD spectra) and refolding has been attempted under a variety of conditions. The attempts to refold yeast and bovine E3 from a completely unfolded state have proved unsuccessful. The addition of the molecular chaperones groEL and groES (and ATP) to the refolding buffer of *E. coli* E3 gave a 15% recovery of activity, compared to no recovery when the protein was diluted into buffer alone. The effects of protein concentration and oxidation state of the refolding buffer have also been investigated. The single most important factor, however, has been found to be inclusion of FAD in the refolding buffer. A 5-fold molar excess of FAD (compared to E3 monomers) in the refolding buffer gave a 22% recovery of activity. It is thought that the FAD is creating a folding nucleation site on the E3 protein which encourages the protein down the correct folding pathway towards the native state.

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Finally, love and thanks to my family, Granny Smith, Lucy, Joe, Mum and Dad. Without your encouragement and financial and emotional support this would not have been possible.

Abbreviations

The abbreviations used in this thesis are set out in the Biochemical Journal "Instructions to Authors" with the following additions.

ATP	adenosine triphosphate
BCDC	branched chain 2-oxoacid dehydrogenase complex
BSA	bovine serum albumin
CD	circular dichroism
CS	citrate synthase
(k)Da	(kilo)Daltons
DCPIP	2,6-dichlorophenol-indophenol
DTT	dithiothreitol
E3	lipoamide dehydrogenase
EDTA	ethylene diamine tetra acetate
FPLC	fast protein liquid chromatography
GdnHCl	guanidinium chloride
h	hour
Hsp	heat shock protein
IPTG	isopropyl β-D-thiogalactopyranoside
min	minute
mM	millimolar
MOPS	3-[N-Morpholino] propane-sulphonic acid
M_r	relative molecular mass
MPa	mega pascals
MSUD	maple syrup urine disease
NAD^+	nicotineadenosine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide

OAA	oxaloacetate
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBC	primary biliary cirrhosis
PDC	pyruvate dehydrogenase complex
PDI	protein disulphide isomerase
PEG	poly ethylene glycol
PMSF	phenymethanesulphonyl fluoride
PPI	peptidyl prolyl *cis-trans* isomerase
SDS	sodiumdodecyl sulphate
TCA	trichloroacetic acid/tricarboxylic acid cycle
TEMED	N,N,N^{1},N^{1}-tetramethylethylene diamine
ThDP	thiamine diphosphate
Tris	Tris(hydroxymethyl)aminomethane
UV	ultra violet
v	volume
w	weight

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CHAPTER 1

INTRODUCTION

1.1 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES

1.1.1 Three principal systems

The 2-oxoacid dehydrogenase complexes represent classical examples of multienzyme complexes. The pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC) and branched chain 2-oxoacid dehydrogenase complex (BCDC) are all members of the thiamine pyrophosphate-requiring 2-oxoacid dehydrogenase complex family. As our understanding of macromolecular assembly and organisation of protein structure and function stands to benefit directly from a deeper knowledge of these complexes and because of the key positions in metabolism they occupy (see Figure 1.1), they have been studied in detail from numerous sources since the initial purification and characterisation of PDC from *Escherichia coli* (Koike *et al.*, 1960, 1963; Reed and Willms, 1965; Linn *et al.*, 1972; Danson *et al.*, 1979; Henderson and Perham, 1980; Stanley and Perham, 1980; Kresze and Ronft, 1981; Jaenicke and Perham, 1982). In eukaryotes, the complexes are present in the mitochondria and have high M_r values (2.5×10^6 to 9.0×10^6) (Linn *et al.*, 1972). They can be seen as particles of 30 to 40 nm in the electron microscope and are significantly larger than ribosomes (Oliver and Reed, 1982).

1.1.2 Reaction sequence of the 2-oxoacid dehydrogenase complexes

The 2-oxoacid dehydrogenase complexes catalyse the decarboxylation and dehydrogenation of 2-oxoacids in a sequential and co-ordinated multistep process involving the activities of multiple copies of three separate enzymes to generate CO_2 and the corresponding acyl CoA and NADH (Reed, 1974; Yeaman, 1989). The overall reaction catalysed by the 2-oxoacid dehydrogenases can be summarised as follows:

$$RCO\text{-}COOH + CoASH + NAD^+ \longleftrightarrow RCOSCoA + CO_2 + NADH + H^+$$

These complexes were first postulated to be one of the classic examples of a "swinging arm" mechanism (Reed, 1974; Hammes, 1981), whereby rotation of the substrate is permitted between the three active sites in the constituent enzymes whose participation is required in the overall catalysis. More recently it has been found that the swinging arm alone is not sufficient to allow adequate rotation of the substrate, and that the whole lipoyl domain has a highly flexible conformation. Figure 1.2 schematically represents the reaction mechanism of the 2-oxoacid dehydrogenase complexes where each component has a specific role in the catalytic process. The substrate-specific E1 enzyme (2-oxoacid dehydrogenase), which requires thiamine pyrophosphate (ThDP) as an essential cofactor, catalyses the decarboxylation of the appropriate 2-oxoacid with the formation of a ThDP intermediate and a molecule of CO_2. E1 is also responsible for the reductive acylation of the lipoic acid moiety which is covalently attached to dihydrolipoamide acyltransferase (E2) as is discussed later. E2 transfers the acyl group to the CoA acceptor leaving the dithiolane ring of the lipoic acid in the reduced state. E2 also forms the structural core of the complexes and is arranged in octahedral (24 subunits) or icosahedral (60 subunits) symmetry depending on the specific complex, and in the case of PDC on the source of the complex (see section 1.2.2). Dihydrolipoamide dehydrogenase (E3) then reoxidises the lipoamide group, transferring the reducing equivalents onto NAD^+ via its tightly bound FAD cofactor (Brown and Perham, 1976).

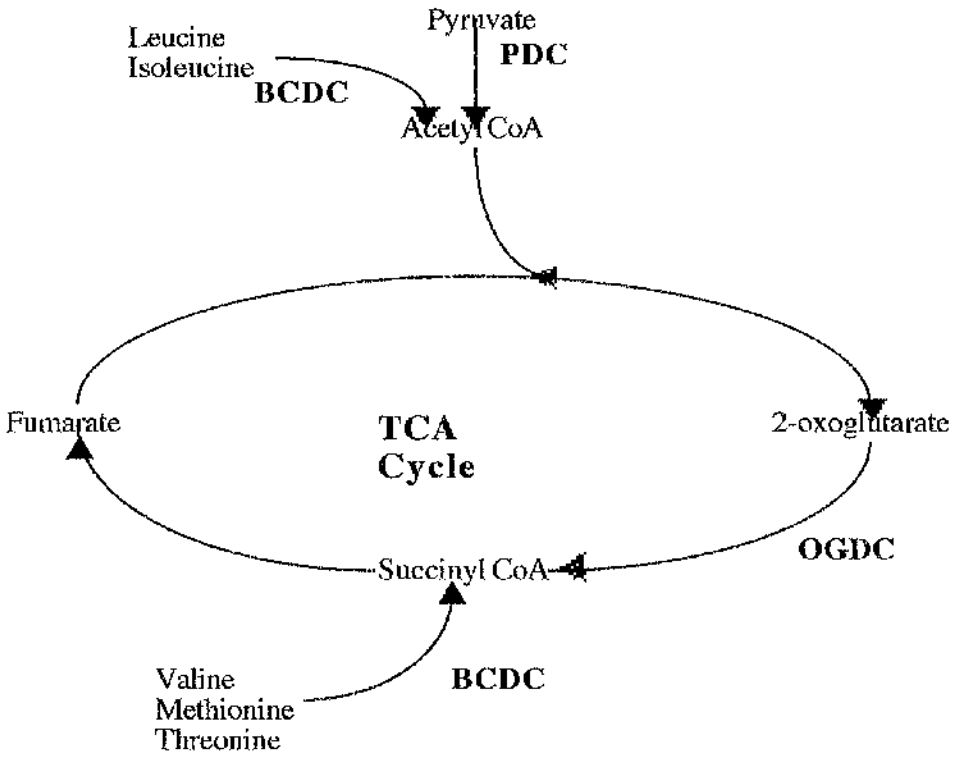


Figure 1.1 An outline of the tricarboxylic acid cycle illustrating the pivotal positions of the 2-oxoacid dehydrogenase complexes in central metabolism

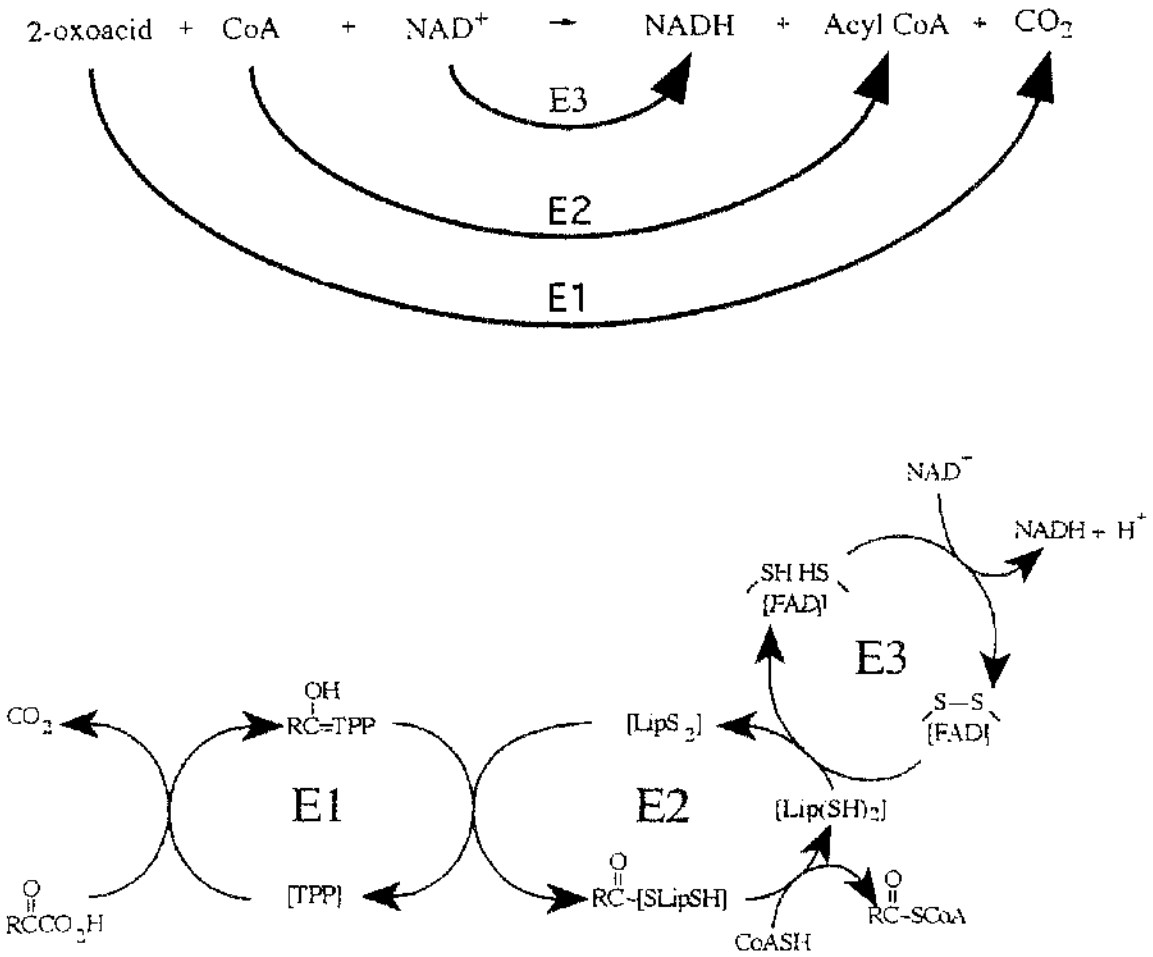


Figure 1.2 Schematic representation of the reaction mechanism of the 2-oxoacid dehydrogenase complexes highlighting the specific reactions catalysed by the component enzymes

1.2 COMPONENT ENZYMES OF 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES

1.2.1 2-oxoacid dehydrogenase (E1)

The 2-oxoacid dehydrogenase (E1) component catalyses the only irreversible reaction in the multistep activity of the 2-oxoacid dehydrogenases. This is the first reaction in the complex: the thiamin diphosphate-dependent oxidative decarboxylation of the 2-oxoacid is followed by the reduction of the lipoyl group covalently bound to a specific lysine residue of the E2 lipoyl domain. To enable this reaction to proceed, E1 non-covalently binds thiamin diphosphate (ThDP), a cofactor widely employed by enzymes catalysing reactions involving the rupture of the carbon-carbon bonds adjacent to a 2-oxo (keto) group. The decarboxylation step is the rate-limiting step in the overall reaction catalysed by the complexes (Danson *et al.*, 1978; Akiyama and Hammes, 1980; Cate *et al.*, 1980). The reaction is thought to proceed via a covalent adduct of ThDP with the formation of a ThDP-bound intermediate and one molecule of CO_2 (Reed, 1974).

There are two different forms of the E1 component. The first is a homodimer (α_2) with a subunit M_r of approx. 100 000 and is found in octahedral OGDC and PDC. The second is a heterotetramer ($\alpha_2\beta_2$) with subunit M_r values of approximately 41 000 and 36 000 respectively and is found in the octahedral BCDC and icosahedral PDC. In the heterotetrameric form, the E1α subunit appears to comprise two large domains with M_r values of 31 000 and 10 000 (Koike *et al.*, 1992). The genes encoding the E1α and E1β chains of PDC and BCDC and the unsplit E1 chain of OGDC may share a common ancestor (Matuda *et al.*, 1991). However, sequences available for E1 of PDC and OGDC show little homology even when extracted from the same source (Darlison *et al.*, 1984). As an example, the E1 chains from *E. coli* PDC and OGDC show a pronounced lack of sequence similarity (Guest *et al.*, 1989). A 30-amino acid residue motif has, however,

been identified in all known ThDP-dependent enzymes, irrespective of their lack of sequence homology, which is predicted to be involved in ThDP binding (Hawkins *et al.*, 1989).

1.2.2 Dihydrolipoamide acyltransferase (E2)

The dihydrolipoamide acyltransferase (E2) component of the complexes is the key to their structure and function. It has three major roles. Firstly, it forms a structural core to which the E1 and E3 components bind. Secondly, it is an acyltransferase, catalysing the formation of the acyl-CoA product. Thirdly, it provides the attachment site for the lipoic acid cofactor which interacts with the different active sites in the complexes. All E2 enzymes possess highly segmented structures containing several functional domains (Bleile *et al.*, 1979; Perham 1991). A schematic representation of the domain structure of E2 from various sources is shown in Figure 1.3. The C-terminus of the E2 polypeptide forms a compact inner domain which contains the E2 binding sites allowing the E2 polypeptides to interact with each other to form the core of the complex. This domain also contains the catalytic activity of E2 (Packman *et al.*, 1988). Adjacent to this domain, there is a distinct region of polypeptide consisting of approximately 50 amino acids which is responsible for the binding of E3 to the central core. At the N-terminus of the E2 polypeptide are the lipoate domains. The lipoyl moieties are attached via an amide linkage to the ε-amino group of a lysine residue within a lipoate domain. This linkage provides a flexible arm about 14 Å in length permitting the lipoyl moiety to rotate amongst the active sites of E1, E2 and E3 in what is called a "swinging arm" active site coupling mechanism (Koike *et al.*, 1963). The flexibility of the whole domain, as well as the "swinging arm", is intrinsic to the coupling process (Miles *et al.*, 1988; Machado *et al.*, 1993). The role of the lipoyl domains is that of guiding the cofactor lipoamide through the various active centres. In order to promote reductive

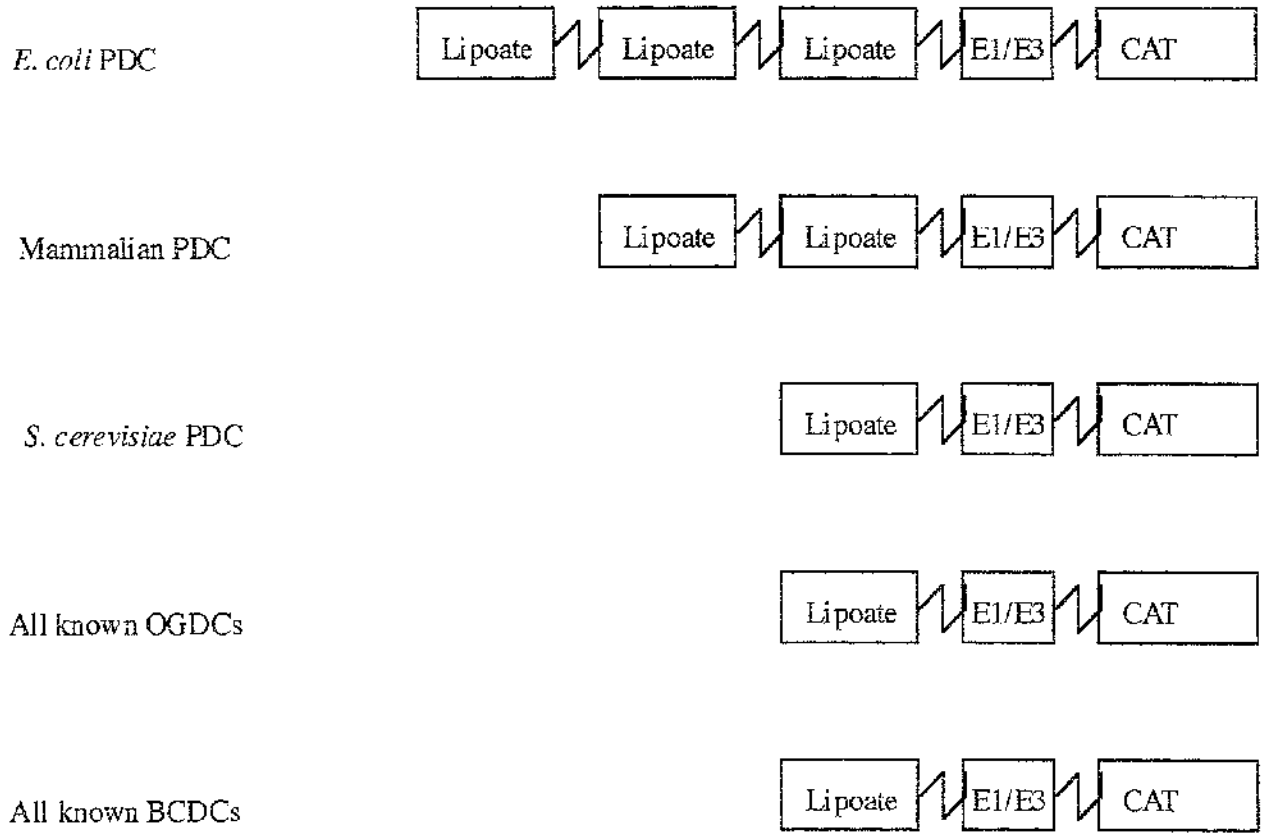


Figure 1.3 Comparison of the domain structures of E2 components from various sources

The lipoic acid containing domain (lipoate), peripheral subunit binding domains (E1/E3) and the inner catalytic domain (CAT) are connected via linker regions which are rich in alanine and proline residues. (This figure is adapted from that of Thekkumara *et al.*,1989).

acylation of the dithiolane ring by the E1 component, the lipoyl group must be attached to a specific lipoyl domain. This indicates that the decarboxylase subunit binds and recognises the domain itself and not only the lipoyl group (Packman *et al.*, 1988).

The complexes vary in the number of lipoyl domains which are present in the E2 polypeptide. E2 from PDC of *E. coli* is remarkable in that it contains three highly-conserved lipoyl domains (Stephens *et al.*, 1983). Surprisingly, deletion of two of the three lipoate domains by site-specific mutagenesis still allows assembly of a functional complex with full catalytic activity (Guest *et al.*, 1985). The catalytic activity can be abolished when all three domains are deleted or rendered unlipoylatable by lipoyl-Lys→Gln substitutions (Allen *et al.*, 1989). Complexes containing mixtures of wild-type and mutant lipoyl domains possess high levels of activity, although some impairment has been reported when the only wild-type domain is the one closest to the peripheral subunit binding domain (Allen *et al.*, 1989). Presumably this is a reflection of the reduced conformational flexibility of this construct. A reduction in complex activity is also observed by increasing the number of lipoyl domains to between four and nine per E2 chain. Under-lipoylation of the domains participating in catalysis and steric hindrance from unlipoylated domains are thought to be involved in the partial activity loss (Machado *et al.*, 1992). As the additional domains are not required for the catalytic functioning of the complex, the interesting question remains as to the purpose of this duplication. However, while deletion of two of the three *E. coli* lipoyl domains still allows a complex with full catalytic activity to assemble, there is evidence that when these bacteria are cultured with wild-type *E. coli*, the wild-type has a greater growth rate (Guest *et al.*, 1985). This is thought to be due to the increased flexibility of the outer lipoyl domain allowing the surrounding E1 components to be serviced more effectively. The domains are joined together by conformationally flexible linker regions, rich in alanine, proline and acidic residues, which facilitate active site coupling. The importance of these linker regions to a high catalytic efficiency is inferred from the impairment that

accompanies the deletion or shortening of these sequences, and by the enhancement which occurs on incorporating a polyproline linker (Miles *et al.*, 1988; Turner *et al.*, 1993).

It has been found that the domain sequences are sufficiently well-conserved in evolution for analogous segments to be clearly identified in E2 components of all of the 2-oxoacid dehydrogenase complexes studied so far. As well as the variations in the number of lipoate domains per E2 chain, however, differences are also apparent in the lengths and compositions of the interdomain linker sequences (Guest *et al.*, 1989; Perham and Packman, 1989). A search for amino acid sequences similar to the C-terminal domain of the E2 subunit of *E. coli* PDC has found nine sequences with extensive similarity, eight of which are E2 subunits from a variety of species (Russell and Guest, 1991). The other was for a mitochondrial ribosomal protein, MRP3, from *Neurospora crassa,* now also thought to be an E2 component. Russell and Guest (1991) have compared 13 E2 sequences, including those from human, bovine, rat, yeast and *E. coli* sources. This study has revealed that 34% of the aligned residues are identical or show a high degree of conservation and has also helped identify residues which are potentially important for structure, catalytic activity and substrate-specificity of the acyltransferases.

The E2 components of the complexes are arranged with point group symmetry. OGDC and BCDC have 24 copies of E2 arranged with octahedral (cubic) symmetry as does PDC from Gram-negative bacteria. In contrast, PDC from mammals, birds, yeast and Gram-positive bacteria have 60 copies of E2 arranged in icosahedral symmetry (Perham *et al.*, 1987). The structure and molecular architecture of the 2-oxoacid dehydrogenase multienzyme complexes is discussed in more detail in a later section.

1.2.3 Dihydrolipoamide dehydrogenase (E3)

Dihydrolipoamide dehydrogenase (E3) is responsible for the NAD^+ dependent re-oxidation of the dihydrolipoamide groups present on the E2 components, thus allowing the acyltransferase component to re-enter the catalytic cycle (Yeaman, 1989). E3 also functions as an integral component of the glycine decarboxylase complex (GDC) which, like the 2-oxoacid dehydrogenase complexes, is located in the mitochondrion in eukaryotic cells. E3 has also been discovered in the absence of the aforementioned complexes. In the bloodstream form of *Trypanosoma brucei* there is a single mitochondrion and an absence of the multienzyme complexes mentioned above. E3 has been found located outside the mitochondrion, associated with the plasma membrane (Danson *et al.*, 1987; Jackman *et al.*, 1990). Serrano (1992) has reported the presence of E3 activity in two cyanobacteria, the unicellular *Synechoccus* PCC 6301 and the filamentous *Anabaena cylindrica*. The 2-oxo acid dehydrogenase complexes are believed to be absent in filamentous cyanobacteria (Smith, 1973; Bothe and Nolteernsting, 1975). Since a low GDC activity was detected in cell free extracts of *Anabaena cylindrica* ,the function of cyanobacterial E3 remains unclear. It has been suggested that E3 may be involved in a glycine cleavage system (Serrano, 1992). The overall reaction catalysed by E3 is:

$$\text{Dihydrolipoamide} + NAD^+ \longleftrightarrow \text{Lipoamide} + NADH + H^+$$

During the reaction, electrons are transferred from lipoamide first via the disulphide/dithiol and then via its cofactor FAD to NAD^+ as the final electron acceptor (Ghisla and Massey, 1989). All E3 enzymes isolated to date have been found to be homodimers with a subunit M_r of approximately 50 000. Each subunit contains a

molecule of non-covalently bound FAD. Four distinct domains can be identified within each monomer. From the N-terminus these are; an FAD binding domain, an NAD binding domain, a central domain and an interface domain. The structural similarities between the FAD and NAD binding domains suggest that they may have evolved from a common ancestor.

E3 has been isolated from a wide variety of sources and is generally believed to be the identical gene product in all three members of the 2-oxoacid dehydrogenase complexes as well as in GDC (for a review, see Carothers *et al.*, 1989). However, there is some conflicting evidence suggesting the existence of isoforms of E3. In rat liver mitochondria two immunologically distinct forms of E3 have been identified. It has been suggested that from this source the E3 associated with GDC is different from that associated with the 2-oxoacid dehydrogenase complexes (Carothers *et al.*, 1987).

The sequencing of E3 enzymes from a wide variety of sources has provided much information allowing comparisons to be made between species. It has been shown that E3 is a highly conserved enzyme across species. For example, the sequence of human liver E3 exhibits 96% homology with that of E3 from porcine heart (Otulakowski and Robinson, 1987) and 44% identity with *E. coli* E3 (Pons *et al.*, 1988). Three dimensional structures have also been determined for E3 components from several sources including yeast (Takenaka *et al.*, 1988), *Pseudomonas putida* (Mattevi *et al.*, 1992a) and *Pseudomonas fluorescens* (Mattevi *et al.*, 1992b). It has also been reported that E3 exhibits both structural and functional similarities with several members of the pyridine nucleotide disulphide oxidoreductases, in particular with glutathione reductase (GR) (Takanaka *et al.*, 1988). GR is responsible for catalysing the NADPH dependent reduction of glutathione disulphide. Therefore E3 and GR act in opposite directions by passing reducing equivalents to NAD^+ or from NADPH respectively. Both enzymes contain a redox-active disulphide bridge that undergoes oxidation-reduction during catalysis causing them to cycle between oxidised and 2 electron reduced forms

(Williams *et al.*, 1989). As well as GR and E3, other members of the family of homodimeric FAD-dependent disulphide oxidoreductases include mercuric reductase, thioredoxin reductase and trypanothione reductase. E3 is the only member of this family that belongs to a multienzyme complex, although an increasing number of non-complex bound functions of E3 are being reported, (Williams, 1992 and references therein) as discussed earlier in this section.

Each subunit of E3 binds non-covalently a molecule of flavin adenine dinucleotide (FAD). Attachment of this cofactor is thought to occur during the folding of mature polypeptides within the mitochondrion and it has previously been suggested that this may represent a key step in the folding of E3 and the subsequent assembly of the mature complex (Ycaman, 1986). The involvement of FAD in the folding of E3 is investigated further in Chapter 5.

1.2.4 Component X

In addition to its three constituent enzymes, mammalian PDC has been found to contain a tightly-associated 50 000-M_r polypeptide termed component X (De Marcucci and Lindsay, 1985). It was initially suggested that component X was a proteolytic fragment of E2, but this suggestion was ruled out using refined immunological techniques employing subunit-specific antisera and the finding that anti-X serum elicits no cross-reaction with the other components including the intrinsic kinase of PDC (De Marcucci and Lindsay, 1985). Component X has been found to contain an additional lipoate domain and it was first thought that there were about 6 copies of the protein per complex (Jilka *et al.*, 1986; Hodgson *et al.*, 1986). More recent evidence suggests that there are 12 copies of X in bovine PDC, although it is not yet known if they are bound to the complex individually or as 6 dimers (Sanderson *et al.*, 1996). A similar protein has been found in PDC from yeast (Behal *et al.*, 1989). There is no evidence to date for the

existence of a component X in PDC from prokaryotes, nor in OGDC or BCDC, but this possibility cannot be ruled out at present.

The role of component X in PDC remains unclear. Limited proteolysis of component X in mammalian PDC leads to a lower affinity of E3 for the core assembly, so one possibility is that it is involved with the binding of the E3 component and facilitating its participation in electron transfer (Gopalakrishnan *et al.*, 1989; Neagle *et al.*, 1991; Lawson *et al.*, 1991). Mutagenesis of the yeast gene for component X has indicated that it is not required for the formation of the E2 core, that it is essential for E3 binding and that the lipoyl domain can be deleted without effect (Lawson *et al.*, 1991). Its lipoyl domain, though capable of becoming reductively acetylated (Jilka *et al.*, 1986; Hodgson *et al.*, 1986), apparently serves no catalytic function.

1.3 COMPLEX CONTROL

PDC is well designed for fine regulation of its activity. Phosphorylation of the E1 subunit causes inactivation of the complex by a dramatic reduction of the V_{max}. Interconversion of the active and inactive forms of E1 is a dynamic process that leads rapidly to the establishment of steady states, in which the fraction of phosphorylated enzyme can be varied over a wide range. BCDC is controlled in a similar fashion, although the kinases and phosphatases are specific for each complex. The kinases are tightly bound to, and copurify with, their respective complexes, whereas the phosphatases are loosely associated and are purified as distinct, soluble enzymes. The pyruvate dehydrogenase kinase has been purified to apparent homogeneity from extracts of bovine kidney mitochondria and its structure elucidated (Stepp *et al.*, 1983). It is a hetero-dimer consisting of subunits termed α and β, which have molecular masses of 48 000 and 45 000 respectively. Limited proteolytic studies first indicated that the α-subunit contained the kinase active site and that the β-subunit may serve a regulatory

role (Stepp *et al.*, 1983). Using purification, N-terminal sequencing and cDNA cloning and sequencing, rat heart has been shown to contain two PDC kinases, termed p45 and p48. Northern blotting has shown that mRNA for p45 is abundant in most rat tissues studied whereas p48 is abundant only in heart (Popov *et al.*, 1993; Popov *et al.*, 1994). Limited immunological data suggest that the bovine kidney kinase α-subunit may correspond to rat heart p45 (Priestman *et al.*, 1994). Kinase activity appears to be specific for pyruvate dehydrogenase as little activity is exhibited toward a number of other substrates including BCDC, rabbit skeletal muscle phosphorylase *b*, glycogen synthase *a*, histones or casein (Reed *et al.*, 1985). The kinase has been found to phosphorylate three serine residues on the α-subunit of E1 (Yeaman, *et al.*, 1978; Sugden *et al.*, 1979). Phosphorylation occurs markedly faster at site 1 than at the other two sites, and phosphorylation at site 1 correlates closely with inactivation of E1. The activity of the kinase is stimulated by acetyl-CoA and NADH, products of the complex, and this stimulation is antagonised by the substrates CoA and NAD^+. Inhibition is also caused by pyruvate and by ThDP, which presumably binds directly to the active site of the E1 substrate and thereby alters the conformation about phosphorylation site 1 so that the serine hydroxyl group is less accessible to the kinase (Reed and Yeaman, 1987).

Pyruvate dehydrogenase phosphatase has been isolated from bovine heart and kidney mitochondria (Teague *et al.*, 1982; Pratt *et al.*, 1982). It has been found to have a molecular mass of approximately 150 000 and consists of two subunit types, the catalytic activity residing in a subunit of 50 000 and the larger subunit containing a molecule of FAD (Teague *et al.*, 1982). The phosphatase is highly specific for PDC, with minimal activity against phosphorylated BCDC (Damuni *et al.*, 1984). Also, it has been found that the phosphatase has an absolute requirement for Mg^{2+} and is stimulated by micromolar concentrations of Ca^{2+} which act by promoting binding of the phosphatase to the E2 core of the complex. NADH inhibits phosphatase activity, and this inhibition is reversed by NAD^+ (Pettit *et al.*, 1975). The amino acid sequences of

pyruvate dehydrogenase phosphatases from several sources have recently been deduced. Sequence alignment of sequences from rat (Tamura *et al.*, 1989), rabbit and human (Mann *et al.*, 1992), *Saccharomyces cerevisiae* (Maeda *et al.*, 1993) and the protozoan parasite *Leishmania chagasi* (Burns *et al.*, 1983) has revealed five distinct regions of significant sequence identity. Site-directed mutagenesis is being carried out with the pyruvate dehydrogenase phosphatase catalytic subunit to ascertain whether the highly conserved residues are involved in Mg^{2+} binding, substrate binding and/or catalysis (Reed *et al.*, 1996).

1.3.1 Acute control of the complexes

As the 2-oxoacid dehydrogenase complexes occupy key positions in intermediary metabolism it is essential that their activities be precisely regulated. All members of the family are subject to product inhibition which is reversed competitively by the substrates. The activities of both OGDC and PDC are also subject to control by changes in the intramitochondrial concentrations of free Ca^{2+} (Denton and McCormack, 1985). The effect of Ca^{2+} ions on OGDC is via direct binding to the complex, causing allosteric activation by reducing the K_m for the substrate, 2-oxoglutarate (Lawlis and Roche, 1981). This effect is antagonised by ATP which increased the K_m for 2-oxoglutarate. This mechanism is thought to underlie the activation of OGDC by Ca^{2+}-mobilising hormones such as vasopressin and the α-adrenergic action in liver (Denton and McCormack, 1985). PDC can also be activated by such hormones, however, this activation is not via direct allosteric activation but via stimulation of PDC phosphatase as discussed in the previous section.

1.4 STRUCTURE OF THE 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES

The structure of these complexes is such that a number of E2 polypeptides form a core around which are arranged multiple copies of E1 and E3 components which bind non-covalently (Reed, 1974). As mentioned in section 1.2.2, the arrangement of the E2 core is complex and species-specific as there are two distinct core structures, the cube and the pentagonal dodecahedron. OGDC and BCDC have 24 subunits of E2 arranged with octahedral symmetry as does PDC from Gram negative bacteria such as *E. coli* as illustrated in Figure 1.4 (Oliver and Reed, 1982). The second E2 core arrangement is composed of 60 subunits arranged with icosahedral symmetry and is found in PDC from Gram positive sources, yeast such as *S. cerevisiae*, and also from mammalian, avian and fungal sources (Perham, 1991). In E2 cores with octahedral symmetry, twelve E1 dimers bind along the twelve edges of the core and six E3 dimers bind along the six faces. In E2 cores with icosahedral symmetry, 20-30 E1 tetramers bind to the thirty edges and again six E3 dimers bind to six of the twelve faces of the pentagonal dodecahedron (Reed *et al.*, 1975; Barrera *et al.*, 1972). The number of E3 dimers bound to each complex, however, has recently been reexamined due to the evidence that component X facilitates the binding of E3 dimers to the complex in PDC (Lawson *et al.*, 1991; De Marcucci *et al.*, 1995). As evidence suggests that there are 12 copies of component X in bovine PDC, up to 12 E3 dimers could be bound to the core complex (Sanderson *et al.*, 1996).

1.4.1 Mitochondrial targeting and assembly

PDC, OGDC and BCDC in eukaryotes are located in the mitochondrion, probably in loose association with the inner face of the inner membrane. The individual

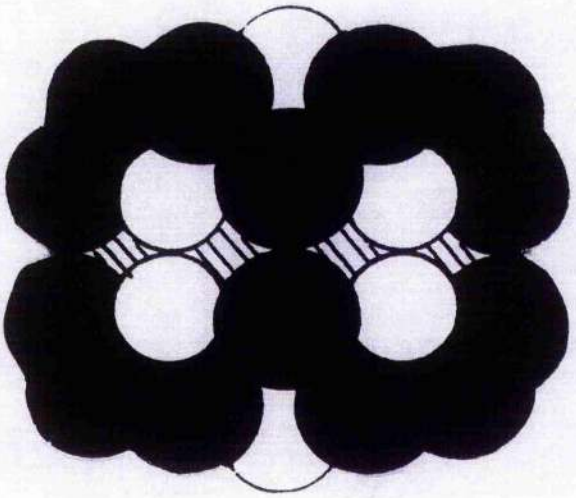


Figure 1.4 Model of the pyruvate dehydrogenase complex from *E. coli*

The transacetylase core (E2) is shown hatched, the pyruvate dehydrogenase component (E1) is shown in black and the dihydrolipoyl dehydrogenase component (E3) is shown in white. This figure is reproduced from Stryer (3rd edition, p381).

polypeptides of the complexes are nuclear coded, since all the translation products of the mitochondrial genome have been identified as components of the major respiratory chain complexes of the inner membrane involved in oxidative phosphorylation (Chomyn *et al.*, 1985). The translocation and assembly of such large complexes is necessarily a multistage process involving a sequence of molecular events, the details of which are gradually being elucidated.

As with the majority of mitochondrial precursors, the initial cytosolic translation products of the components would each be expected to contain amino-terminal extensions which are proteolytically cleaved during or shortly after entry into the organelle. This has been found to be the case for each of the complexes, PDC (Hunter and Lindsay, 1986), OGDC (De Marcucci *et al.*, 1988) and BCDC (Clarkson and Lindsay, 1991). The E2 components of the complexes, however, have been found to possess extended presequences, approx. 55-75 amino acids in length (Lindsay, 1989). There is some evidence that elongated signal sequences contain additional information to that which is present in shorter (25-35 amino acid) amino-terminal sequences, perhaps to specify more precisely the delivery destination i.e. the inner membrane, outer membrane or intermembrane space (Hurt *et al.*, 1985). Chaperone proteins may well be involved in maintaining the individual polypeptides in a loosely-folded state which is required to facilitate their movement across organelle membranes and may also be involved in their folding once inside the mitochondrion, which is discussed further in Chapter 5. *In vitro* evidence that bovine PDC can regain full catalytic activity when its purified native components are mixed in the absence of chaperone proteins, suggests that their assistance is not required in the assembly of the folded components within the mitochondrion. As the E2 and X subunits form the core to which the other components of the complex bind, the construction of the correct core conformation probably holds the key to the assembly of the complexes. Three general schemes of assembly of the central core can be envisioned. Firstly a sequential mechanism, in which individual

monomeric subunits are combined to generate progressively larger E2-X multimers, with one subunit being added at each step. Secondly a random mechanism, in which different oligomeric structures, composed of varying numbers of individual E2 subunits, combine spontaneously to form the final structure. Thirdly an ordered mechanism in which a single defined oligomeric E2 intermediate assembles into progressively larger E2-X structures. Behal *et al.* (1994) have found evidence supporting the third suggested mechanism using sedimentation velocity analysis on reassembling E2-X complexes. The detection of a stable 8 S oligomeric structure strongly suggests that there is a key assembly intermediate between the monomeric 1.5-3 S species and the mature 32 S E2-X subcomplex in bovine PDC. This assembly intermediate is widely believed to be a trimer (Behal *et al.*, 1994; Wynn *et al.*, 1994). Component X is not thought to be influential in the E2 assembly process. Lawson *et al.* (1991) demonstrated that component X is not required for E2 assembly in yeast PDC. Bovine PDC E2 has also shown reversible dissociation in the absence of component X (De Marcucci *et al.*, 1995).

1.5 PHYSIOLOGICAL DISORDERS

The importance of the 2-oxoacid dehydrogenase complexes and their control is demonstrated by the occurrence and severity of a number of related disorders.

Primary biliary cirrhosis (PBC) is a chronic liver disease characterised by progressive inflammatory destruction of intrahepatic bile ducts which leads to fibrosis, liver cell damage and ultimately liver failure (Kaplan, 1987; Sherlock and Dooley, 1993). PBC-specific auto-antibodies can be detected by indirect immunofluorescence (Walker *et al.*, 1965). A serological marker for the diagnosis of this autoimmune process is the presence of antimitochondrial autoantibodies directed against a family of antigens termed M2. Several constituents of the M2 antigens are components of PDC, OGDC and BCDC. The major autoantigen is believed to be the PDC E2 component (Palmer *et al.*,

1993) and the lipoic acid moiety is thought to play an important role in antibody recognition (Fussey *et al.*, 1990).

Various forms of metabolic acidosis can be traced to regulatory abnormalities of the 2-oxoacid dehydrogenase complexes, such as Maple Syrup Urine Disease (MSUD). This autosomal recessive inborn error of metabolism results in an accumulation of branched-chain amino acids in the urine of sufferers and was first characterised by its high prevalence in the Mennonite populations of Pennsylvania (DiGeorge, 1982). Several phenotypes have been described, ranging from the classical form, characterised by severe mental and physical impairment and early death (Menkes *et al.*, 1954; Dancis *et al.*, 1959), to a number of milder conditions with later onset (Dancis *et al.*, 1967; Schulman *et al.*, 1970; Scriver *et al.*, 1971). Molecular analysis of BCDC in cell lines derived from patients with MSUD has revealed that the mutations causing this disease are heterogeneous and include missense, deletion, insertion and splicing abnormalities of any subunit of BCDC (reviewed by Indo and Matsuda, 1996).

Blass *et al.* (1971) documented a second inborn error of metabolism involving deficiencies of PDC causing a broad spectrum of conditions characterised by low complex activity (below 30% of normal). These deficiencies are associated with primary lactic acidosis and neurological disabilities of varying severity including Leigh's disease, an autosomal recessive condition affecting normal brain function (Johnston *et al.*, 1984). Until relatively recently, little was known of the genetic mutations associated with these conditions. Since complex activity depends on the correct translation of E1, E2 and E3 (and in PDC E1α, E1β and X) as well as the regulatory enzymes, mutations in many gene loci are possible. Using assays of individual component activities, immunological screening with component-specific antisera and assessment at the mRNA level employing cDNA clones encoding individual components, several types of specific

genetic mutations have been identified. The most common form of PDC deficiency is due to mutations located in the E1 α gene of chromosome X (Robinson *et al.*, 1989). Initial information suggested that males were more severely affected by such mutations, the frequency and range of severity being greater in females. However, due to the unpredictable patterns of inactivation of the 2 X-linked E1 α alleles in females, there is a variable degree of penetrance, especially within the brain (Brown *et al.*, 1989). While mutations found in males appear to be associated with predicted preservation of minimal E1α function, heterozygous females can survive with certain mutations that would not be predicted to have any residual function. In such females, cells in which the normal allele is expressed should function normally, while neighbouring cells expressing the abnormal allele would not be expected to survive (Dahl, 1995). It is still unclear which stage of development may be the most vulnerable to the deficiency caused by these mutations. During normal male reproduction, the problem of lack of expression of the E1α gene on chromosome X in haploid spermatozoa is resolved by testis-specific expression of the E1α gene on chromosome 4 (Iannello *et al.*, 1993). It is possible that ova containing a defective E1 α allele which is not able to produce any PDC activity may not survive in the ovary, accounting for the apparently frequent disappearance of these alleles (Kerr *et al.*, 1996). The notable rarity of E3-linked deficiencies reflects the severity of the associated condition. Impairment of E3 function causes a reduction in all three 2-oxoacid dehydrogenase complex activities resulting in the accumulation of lactic, 2-oxo- and branched-chain amino acids and their derivatives in the plasma and urine of sufferers (Liu *et al.*, 1993).

It has been suggested that deficiencies in OGDC activity are linked to Alzheimer's disease. Lowered interconversion between 2-oxoglutarate and glutamate, caused by impaired 2-oxoglutarate oxidation, is believed to result in the accumulation of glutamate, a potent neurotoxin when energy metabolism is suppressed (Beal, 1992).

Sheu *et al.* (1994) have studied cultured skin fibroblasts from familial Alzheimer patients. Their studies have revealed defective OGDC activity and abnormalities of the E2 component. Other mitochondrial proteins including PDC were found to have normal activity levels. Mutations in the gene encoding E2 may cause the defect in OGDC activity.

1.6 THE PROTEIN FOLDING PROBLEM

The first half of the genetic pathway whereby information from DNA is transferred into a three-dimensional functional protein has been well characterised. Until relatively recently, however, little research has been undertaken to elucidate the steps undergone by the nascent polypeptide during and after emerging from the ribosome to reach its final native structure. Pioneering work by Christian Anfinsen beginning in the 1960s on bovine pancreatic ribonuclease led to the acceptance of the thermodynamic hypothesis which states that the amino acid sequence of a given protein determines its tertiary structure (Anfinsen, 1973). The extrapolation from early experimental results of this type was that a protein *in vivo* could fold and assemble spontaneously in a process intrinsic to its primary structure, independent of other factors. Levinthal (1968) has estimated that it would take in the order of 10^{50} years for a polypeptide of 100 amino acid residues to reach a particular conformation by random processes and although the figure is probably a little lower when taking steric hindrance into account, it is obvious that an ordered folding pathway of some description is in operation. The way in which amino acid sequence dictates tertiary structure, however, is not a simple code like the mRNA triplet code. It can be shown that amino acid substitutions are allowed at many residue positions without drastic changes in the folding pattern. Furthermore strikingly similar structures have been determined for evolutionary distant proteins with only a few

amino acids in common. Determining the folding pathway may be the principal means of understanding the complexity of the folding process itself.

A general folding pathway for monomeric proteins may be described in simple terms by the equation

$$U \overset{\text{fast}}{\longleftrightarrow} I \overset{\text{slow}}{\longleftrightarrow} N$$

where U, I and N represent the unfolded, intermediate and native states, respectively. The initial fast steps in protein folding involve the rapid formation of secondary structure, which occurs on the millisecond time scale, and the formation of an intermediate structure represented by I in the above equation (Ptitsyn *et al.*, 1990; Christensen and Pain, 1991). This intermediate state, termed a molten globule or compact folding intermediate, differs from the native by the absence of close packing throughout the molecule and by a substantial increase of fluctuations in side chains as well as of larger segments of the molecule. The molten globule form exists in rapid equilibrium with the unfolded state. The rate determining step in protein folding is the transition from I to the native state, a process which involves changes in tertiary structure. Not all proteins have been found to fit with this framework model. For example the folding of barstar, the inhibitor of barnase in the bacterium *Bacillus amyloliquefaciens*, can be explained better by the hydrophobic collapse model (Agashe *et al.*, 1995). This model gives overriding precedence to a non-specific collapse of the polypeptide chain which facilitates subsequent formation of specific secondary and tertiary structures.

The folding process is not always completely successful as incorrect intra- or inter-polypeptide interactions can take place. This can lead to the formation of inactive aggregates and has been observed *in vitro* where the yield of refolded protein varies greatly depending on the chosen conditions and *in vivo* in the case of formation of

inclusion bodies during protein overexpression (LaVallie *et al.*, 1992) or during heat shock and other stress conditions within the cell (Parsell *et al.*, 1993). Aggregation of this type is thought to be caused by the exposure of hydrophobic residues on the molten globule. A growing list of human diseases are being found also to involve altered protein folding. Cystic fibrosis, for example, has been found to be caused by mutations of the gene encoding an ATP-dependent Cl^- channel called the cystic fibrosis transmembrane conductance regulator (CFTR). In 70% of cases the mutation is a deletion of residue 508, located in the amino-terminal nucleotide-binding domain of this five domain protein. As a consequence, Cl^- conductance is decreased in diseased epithelial cells. Thermodynamic studies *in vitro* have shown that this mutation does not have a large effect upon the stability of the native state, which is functional under physiological conditions, but dramatically destabilises a 67 amino acid peptide model of a folding intermediate (Thomas *et al.*, 1992). These findings strongly suggest that the defect is in the folding pathway itself (Thomas *et al.*, 1995).

1.6.1 Comparing *in vitro* and *in vivo* protein folding

The traditional approach to studying the folding process, with a view to understanding the pathway involved, is to investigate the purified protein in an *in vitro* system. Chaotropic agents are used to denature the protein and these agents are then removed to initiate refolding. Commonly used chaotropes include urea and guanidinium chloride (GdnHCl) which unfold the protein by interfering with hydrophobic interactions. In many studies, these agents have helped provide information on the structure of intermediates on the refolding pathway using techniques such as circular dichroism and fluorescence spectroscopy, ^{1}H-exchange NMR, gel exclusion chromatography, urea gradient gel electrophoresis and analytical ultracentrifugation (e.g. Holladay *et al.*, 1974; Christensen and Pain, 1991; Zhi *et al.*, 1992; Behal *et al.*, 1994).

Protein folding *in vivo*, however, happens cotranslationally, that is, elements of secondary structure, or even whole domains, can fold before the synthesis of the polypeptide is complete. Indeed, it has been found in many cases that protein refolding occurs more quickly than its synthesis in the cell. Failure of a protein to refold may be caused in some cases by the sequential nature of the process. For example, when the synthesis of a polypeptide consisting of 100 amino acids is complete, around 40 residues remain within the ribosome. Unfolding the native state of such a protein cannot therefore recreate the conditions experienced by the nascent chain, and may effect the folding process. Following co- and post-translational events within the cell is a relatively recent field and poses many practical problems, despite which some interesting progress is being made. Allen *et al.* (1995) have investigated the co-translational *N*-linked glycosylation of tissue-type plasminogen activator (t-PA) in cell culture. This study uses a pulse-chase approach in conjunction with immunoprecipitation and reducing and non-reducing SDS-PAGE to demonstrate that folding and disulphide bond formation in t-PA determines its extent of core *N*-linked glycosylation.

In vitro, aggregation can be suppressed by varying the conditions of the refolding environment. The temperature, pH and protein concentration of an optimum refolding reaction can be far removed from the situation encountered by the same protein in the cell. Indeed in the highly viscous intracellular environment where protein concentration can be 200 mg/ml it is unclear whether spontaneous folding is possible at all.

Refolding *in vitro* varies in time from seconds to hours and is often dependent on the chosen experimental conditions. Indeed some proteins, particularly multi-subunit ones such as glutamate dehydrogenase, have eluded all attempts to refold after unfolding of the polypeptide chains to date. There are many factors which govern the differences observed in the efficiencies of nascent folding and refolding respectively. As the length of a polypeptide chain increases, there is a tendency to form domains within the chain which can unfold and refold independently. The rate limiting step in the formation of

active protein is often the correct 'pairing' of these domains. During the biosynthesis of proteins, it is likely that co-translational folding may help to prevent incorrect pairing by allowing domains to fold sequentially (Jaenicke, 1981; Tsou, 1988). Also, pauses in translation due to rare codons may give time for structural correction and adjustment during biosynthesis (Purvis *et al.*, 1987). Part of the discrepancy between *in vivo* and *in vitro* (re)folding can be accounted for by the fact that the cell contains additional 'tools' for efficient folding, such as protein disulphide isomerase, peptidyl prolyl *cis-trans* isomerase and chaperone proteins, which are discussed in more detail in the following sections.

Despite the discrepancies between the time-scale of (re)folding, the product of the *in vitro* or *in vivo* pathway is a protein with identical conformation, biological activity and relative molecular mass for a given substrate (Jaenicke, 1987). This would suggest that the *in vitro* method of studying protein folding is indeed a valid way of providing valuable information about the folding process. There are benefits and drawbacks of studying both the *in vivo* and *in vitro* systems, but it is clear that the whole picture will only be obtained by combining results from both types of experiments.

1.7 CATALYSIS OF PROTEIN FOLDING

The rate-determining steps of protein folding *in vitro*, and thus presumably *in vivo*, involving the isomerisation of covalent bonds can be catalysed by protein disulphide isomerase and peptidyl prolyl *cis-trans* isomerase. These enzymes do not determine the folding pathway but accelerate the slowest steps of the folding process.

1.7.1 Protein Disulphide Isomerase

Protein disulphide isomerase (PDI) is an abundant soluble protein which catalyses thiol : disulphide interchange reactions in protein substrates. This leads to a net protein disulphide formation, isomerisation or reduction, depending on the initial substrates and imposed thiol : disulphide redox potential. PDI is found within the lumen of the endoplasmic reticulum (E.R.) and it has been found that the level of secretory protein synthesis of different cell types correlates well with the abundance of PDI (Freedman, 1989). *In vitro* research has shown that the rate of protein folding can be increased by PDI. The human chorionic gonadotropin beta subunit (hCG-beta), for example, based on the gel migration of folding intermediates and the order of formation of six disulphide bonds, has been shown to have indistinguishable *in vivo* and *in vitro* folding pathways. The same rate-limiting step was found in both environments. However, the half time for this step in the cell is 4 min whereas *in vitro* the half time is over 80 min. PDI has been shown to significantly decrease the *in vitro* half time without changing the order of disulphide bond formation (Huth *et al.*, 1993).

The mammalian enzyme is a dimer with identical M_r 57 000 subunits, each containing duplications of domains which show a strong homology to thioredoxin reductase (Edman *et al.*, 1985). Like thioredoxin, the active site of PDI has two conserved cysteine residues in the sequence WCGHCK which participate in the thiol : disulphide exchange reactions. The enzyme is identical to the β-subunit of prolyl-4-hydroxylase (Pihlajaniemi *et al.*, 1987), an enzyme which causes extensive modification of proline residues in nascent collagen molecules. As yet it is unknown whether the active site cysteine residues involved in the thiol : disulphide exchange reaction are also involved in the hydroxylation reaction. PDI also shows homology to a surprisingly large number of other proteins suggesting that it may have multiple roles concerned with protein modification and assembly within the E.R.. In addition, PDI is also a component

of the microsomal triglyceride transfer protein complex which facilitates the incorporation of triglycerides into nascent very low density lipoproteins within the E.R. (Wetterau *et al.*, 1990).

Interestingly it has been proposed that PDI itself is a chaperone protein, one which also contains PDI activity (Wang and Tsou, 1993) John *et al.* (1993) have shown that the β-subunit of prolyl-4-hydroxylase, which is identical to PDI, is essential for the correct folding and assembly of the $\alpha_2\beta_2$ functional complex of prolyl-4-hydroxylase, as it prevents the misfolding and aggregation of the α–subunit. As the PDI also forms part of the final functional complex, this chaperone-like function is quite different from those already documented. However, the enzyme has also been shown to bind peptides which have no obvious sequence similarities to the α–subunit, a property similar to chaperone proteins which is discussed in more detail later. The binding by PDI of a number of denatured polypeptides and their release only in the presence of ATP (Nigam *et al.*, 1994) outlines the intriguing dual action of PDI *in vitro* which has still to be confirmed *in vivo*.

1.7.2 Peptidyl prolyl cis-trans isomerase

Peptidyl prolyl *cis-trans* isomerase (PPI) has been shown to catalyse a rate-limiting step in *in vitro* protein folding, the cis-trans isomerisation of proline peptide bonds. The efficiency of catalysis of rotation around the X-pro peptide bond by PPI is variable and dependent on the target protein. The slow steps of refolding can be accelerated rapidly in the case of immunoglobulin light chains (Lang *et al.*, 1987) and ribonuclease T1 (Fischer *et al.*, 1989) while more moderate catalysis is observed with porcine ribonuclease (Lang *et al.*, 1987) and type III collagen (Bächinger, 1987). Other proteins, such as bovine ribonuclease A, show no acceleration of refolding in the presence of PPI. Lang *et al.* (1987) have proposed that the variability observed in the

efficiency of catalysis by PPI may be due to the fact that in some cases the Xaa-Pro bond is simply inaccessible owing to earlier folding events on the folding pathway creating pockets of native tertiary structure.

PPIs are highly abundant ubiquitous proteins found in the endoplasmic reticulum of all tissues and organisms from bacteria to mammals. The three-dimensional structure of two different representatives of the PPI family has been elucidated and this evidence supports a 'catalysis by distortion' mechanism (Jaenicke, 1995). As has been suggested for PDI, PPI may also have a chaperone-like activity, for example in preventing the aggregation of carbonic anhydrase (Freskgard *et al.*, 1992). More direct evidence is required to fully implicate PPIs as catalysts of protein folding.

1.8 MOLECULAR CHAPERONE PROTEINS

The term molecular chaperone was first reported by Laskey *et al.* (1978) to describe the function of nucleoplasmin, an acidic nuclear protein that mediates the *in vitro* assembly of nucleosomes from separated histones and DNA but is not a component of the nucleosome. The term was later extended to describe the postulated roles of a wider range of different proteins in mediating protein folding and assembly. Molecular chaperones are now described as a functional class of unrelated families of protein that mediate the correct non-covalent assembly of other polypeptide-containing structures, but are not normally components of these assembled structures when the latter are carrying out their normal biological functions. All of the molecular chaperones studied to date act not by providing steric information essential for assembly, but by inhibiting incorrect interactions which would produce non-functional structures and, in some cases, large insoluble aggregates. Structural homology is seen between members of the same chaperone class but not between classes. The major chaperone proteins are briefly discussed in the following section with particular emphasis on the prokaryotic

chaperonins groEL and groES and their role in the sequential mechanism of chaperone proteins.

1.8.1 The Nucleoplasmins

Nucleoplasmin is a nuclear protein, most abundant in amphibian oocytes where it can constitute up to 10% of total nuclear protein (Mills *et al.*, 1980). At physiological ionic strength, nucleosome cores are unable to form in the absence of nucleoplasmin, resulting from the formation of a precipitate between the basic histones and acidic DNA. In the presence of a molar excess of nucleoplasmin the strong positive charge of the histones is shielded by the binding of the chaperone. In this way initial non-specific DNA-histone interactions are prevented allowing the formation of the most energetically stable conformation, the nucleosome (Laskey *et al.*, 1978). Nucleoplasmin is believed to be Mg-ATP independent, which distinguishes it from other known chaperone proteins such as the Hsp 70 and chaperonin 60 classes discussed later. As it has been demonstrated that under non-physiological conditions, nucleosomes can be reconstituted from separated DNA and histones in the absence of nucleoplasmin, by prolonged dialysis from high salt concentration (2M NaCl) to lower salt concentrations (Felsenfeld, 1978), it is clear that the chaperone imparts no steric information during its action.

Since the discovery of nucleoplasmin, several homologues have been identified, including the nucleolar protein XLNO-38 which is thought to mediate the assembly of ribosomes. A family of nucleoplasmin-like proteins may exist which carry out various chaperoning functions within the cell (Dingwall and Laskey, 1990).

1.8.2 The hsp70 family

The hsp70 proteins were initially discovered as proteins whose synthesis was increased on heat-shock. They are now recognised as a large, highly conserved group, not all of which are heat inducible, found throughout eukaryotic cells. *E. coli* contains a heat-inducible hsp70 called dnaK. *DnaK* genes in other bacteria have not as yet been extensively studied, but most bacteria contain only one (Lund, 1995). In the yeast *Saccharomyces cerevisiae*, however, the situation is far more complex with at least six hsp70 homologues in the cytosol (SSA1-SSA4, SSB1 and SSB2), the mitochondria (SSC1p) and in the endoplasmic reticulum (Kar2), (Craig *et al.*, 1993). Studies in other eukaryotic cells show that hsp70 proteins can migrate to the nucleus, are present in chloroplasts and their presence in other organelles has also been postulated.

Genetic studies show that these proteins are important and sometimes essential for cell function. Although *E. coli* can survive the loss of its *dnaK* gene, such cells are clearly distressed as they only grow at low temperatures and tend to form filaments. Overexpression of DnaK has been observed to stimulate the membrane translocation of a hybrid protein which is usually unable to cross the inner membrane (Phillips and Silhavy, 1990). Strong associations have been observed in mammalian cell lines between hsp70 and ribosomes by immunoprecipitation experiments, suggesting that the hsp70 is binding to proteins as they emerge from the ribosome to prevent their aggregation or misfolding (Beckmann *et al.*, 1990). The ability of hsp70 to bind to unfolded proteins *in vitro* has led to the suggestion that one of their roles *in vivo* is the protection of partially denatured proteins (Pelham, 1986), although they may also target some denatured proteins to the degradative pathways. It has also been demonstrated that hsp70s play a role in reactivation of damaged proteins. This was shown with the enzyme luciferase, which is rapidly inactivated in *E. coli* that is heated to 43°C but recovers activity on return of the cells to 37°C. This reactivation is substantially reduced in *dnaK*

mutant cells, although this is obviously not the only component involved as a reduction in recovery of luciferase activity is also observed in cells with mutant *dnaJ*, *groEL* and *groES* genes (Schroeder *et al.*, 1993). The *dnaJ* gene was first identified in *E. coli* because mutations in this gene block DNA replication of phage λ. The *dnaJ* gene is located in an operon immediately downstream of *dnaK* and considerable amino acid conservation has been observed between DnaJ proteins from a number or sources, particularly in a 70 amino acid section termed the J region (Silver and Way, 1993). It has been suggested that certain DnaJ proteins interact with only certain Hsp70s, implying that DnaJ, and not solely the peptide-binding pocket of Hsp70, determine substrate specificity (Brodsky *et al.*, 1993). Alternatively, Hsp70 could be targeted to a particular substrate via cocompartmentalization of certain DnaJs and Hsp70s (e.g. Caplan *et al.*, 1992; Hattori *et al.*, 1993). Many studies have shown that proteins need to be in an unfolded state to cross membranes (Eilers and Schatz 1988), and it seems likely that hsp70 homologues on both sides of the membrane maintain protein in this state to enable membrane translocation to occur.

Hsp70 proteins have been shown to be involved in protein translocation across membranes, protein translation and protection and recovery from heat shock. Other roles, such as uncoating of clathrin cages, initiation of DNA replication and sensing the temperature of the cell, have also been suggested. Interaction with proteins with DnaJ motifs appears to be an important part of at least some of these processes, all of which seem likely to stem from the interaction of hsp70 with the unfolded or partially unfolded protein. Hsp70 proteins , certainly in the case of BiP, the hsp70 homologue found in the endoplasmic reticulum, are believed to exist in an oligomeric form which can be mobilised into an active monomeric form on the binding of substrate polypeptides (Blond-Elguindi *et al.*, 1993). This binding takes place with the C-terminal region of the hsp70 protein and is weakened in the presence of hydrolysable ATP (Flynn *et al.*, 1989; Langer *et al.*, 1992a). Nucleotide binding to the N-terminus causes a conformational

change in the hsp70 protein, as judged by protease susceptibility (Liberek *et al.*, 1991a), which is presumably transmitted to the C-terminal domain, causing the polypeptide to be released. Hsp70 chaperones are believed to interact with other proteins *in vivo*, including DnaJ and GrpE, which help to regulate the chaperones ATPase activity and thus polypeptide release (Liberek *et al.*, 1991b).

1.8.3 The Hsp90 family

Another abundant and ubiquitous class of heat-shock proteins which have some properties typical of molecular chaperones *in vivo* are in the 80-90 kDa range, and are generally referred to as the hsp90 proteins. Like hsp70s, members of the hsp90 class of chaperones are highly conserved in bacteria, yeast and mammals with approximately 40% identity between the various eukaryotic hsp90s and the *E. coli* homologue HtpG, which is actually a rather diminutive member of this family at 62.5 kDa (Bardwell and Craig, 1988). In eukaryotic cells, hsp90s comprise 1-2% of cytoplasmic protein under normal conditions, a figure which rises under conditions of stress (Hendrick and Hartl, 1993). In *S. cerevisiae* two homologues of hsp90 exist. The first, Hsc82, is constitutively expressed while the second, Hsp82, has a low basal level of expression which can be induced 10 to 15 fold on heat-shock (Borkovich *et al.*, 1989). Inactivation of both of these genes is lethal at any temperature, although cells can survive single mutations at temperatures up to 37.5°C. Studies on HtpG have shown that it helps the cell grow more efficiently at high temperature, but it is not essential for growth.

Hsp90s have been found to associate with a variety of other proteins including cellular protein kinases and steroid hormone receptors (Gething and Sambrook, 1992) where the common role of the hsp90 protein in these cases seems to be in stabilising the target protein in a non-native conformation. Hsp90-bound steroid receptors retain a high affinity for the steroid hormone but are unable to bind DNA and therefore unable to

activate transcription. The displacement of hsp90 from the receptor occurs on steroid binding which then enables the steroid/steroid receptor complex to bind DNA and activate transcription (Sanchez *et al.*, 1987). It has also been shown that hsp90s can aid the prevention of aggregation of chemically denatured proteins such as citrate synthase and the F_{ab} fragment of a monoclonal antibody providing further evidence of the chaperoning properties of this class of proteins (Wiech *et al.*, 1992).

Nadeau *et al.* (1993) have shown that members of the hsp90s have ATPase activity which can sometimes be stimulated by the binding of substrate proteins. The mechanistic role of this stimulation is not yet clear. Hsp90s also undergo slow autophosphorylation on serine and threonine residues which has been found to have no effect on the ATPase activity but has been suggested that this may regulate the ability of hsp90 to bind its protein targets. It appears that hsp90s have a multitude of functions conferred by the ability to bind reversibly to proteins with exposed hydrophobic surfaces. The interaction of hsp90s with such proteins and with other classes of chaperone proteins may well be crucial in the regulation of a wide range of intracellular processes.

1.8.4 The sHSP family

Small heat shock proteins (sHSP) are a conserved group of heat-shock inducible proteins with molecular masses between 15 and 30 kDa. The sHSPs have been shown to be homologues of α-crystallin, a predominant eye lens protein which is also present in other tissues, not only at the amino acid sequence level (deJong *et al.*, 1988) but also on a structural level (Merck *et al.*, 1993). The sHSPs share functional chaperone properties with the α-crystallins in that they prevent misfolding of a variety of thermally and chemically denatured proteins (Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993). This reactivation, certainly in the case of both citrate synthase and α-glucosidase, in the

presence of either sHSPs or α-crystallin seems to be independent of ATP (Jacob *et al.*, 1993). It remains to be seen whether the sHSPs have specific functions within the cell, independent of other chaperone proteins, or whether they act in concert with other regulatory proteins *in vivo*.

1.8.5 The hsp60 family

The hsp60 proteins were the first proteins to be recognised as having chaperone function (Hemmingsen *et al.*, 1988). Like the hsp70 proteins, they are encoded by highly conserved genes and are found in all organisms and many cellular locations. This family of proteins, also called the chaperonins, includes two types of protein, generally called chaperonin 60 and chaperonin 10, or GroEL and GroES in *E. coli*, the organism on which a large proportion of chaperonin research has been carried out (Sabil and Wood, 1993). GroEL is a heat-shock protein which can be increased from about 1% to 10% of the total soluble cytoplasmic protein following cellular stress (Hemmingsen *et al.*, 1988) but is essential for cellular growth at all temperatures (Fayet *et al.*, 1989). The *groE* gene, which encodes both groEL and its partner chaperonin groES, was initially discovered because mutations in it prevented the growth of several bacteriophages. Assembly of the bacteriophage λ and T4 head structures and T5 tail structures does not proceed in the absence of groEL (Georgopoulos *et al.*, 1972; Georgopoulos *et al.*, 1973; Zweig and Cummings, 1973). Hsp60 was first identified in mitochondria in *Tetrahymena thermophilia* as a groEL related stress protein during heat shock conditions (McMullen and Hallberg, 1987). Early studies such as these indicated a role in the assembly of other proteins and it now seem clear that this is a general function for these proteins within the cell. Highly related hsp60 homologues have not been detected in the eukaryotic cytosol or the endoplasmic reticulum. However, in the cytosol of many organisms including thermophilic archaebacteria, plants and mammals, proteins with

weak hsp60 homology have been identified which appear to have chaperonin-like functions (Lewis *et al.*, 1992; Trent *et al.*, 1991; Yaffe *et al.*, 1992; Mummert *et al.*, 1993). This group of proteins, typified originally by the mouse protein called TCP1, assemble into a hetero-oligomeric protein complex called TRiC (TCP1 ring complex) within the cell and are generally referred to as the TRiC family. The TRiC family may be regarded as a sub-class of chaperonins which are postulated to carry out groEL-like functions within the eukaryotic cytoplasm. As yet no equivalent of the co-chaperonin, groES, has been identified for TRiC. It may be that a co-chaperonin is not required, since studies comparing the refolding of tubulin by TRiC and the *E. coli* chaperonins demonstrated that TRiC was able to fold tubulin efficiently while groEL was only able to do so in the presence of groES (Burston and Clarke, 1995).

1.8.5.1 Structure and function of groEL and groES

GroEL is an oligomeric protein consisting of 14 identical subunits arranged in 2 stacked 7-membered rings with a central cavity, known as the 'double doughnut' (Hohn *et al.*, 1979; Hendrix 1979). Each subunit has a molecular mass of 57.3 kDa, giving a total molecular mass of over 800 kDa (Hemmingsen *et al.*, 1988). Electron microscope analysis of negatively stained oligomeric groEL has indicated that the overall cylindrical shape has a height and diameter of approximately 160 nm and 130 nm respectively (Ishii *et al.*, 1992; Langer *et al.*, 1992b). Each subunit consists of three distinct domains. The largest of these, the equatorial domain, contains the N-terminal and C-terminal residues which from crystallographic data appear to project into the central channel (Braig *et al.*, 1994). The equatorial domain is highly α-helical and serves as the structural foundation providing most of the contacts with adjacent subunits in the ring and in addition contains the ATP binding site. The apical domain forms the opening of the central channel and possesses a degree of local flexibility enabling a wide range of unfolded polypeptides to

be accommodated. The intermediate domain, as its name suggests, forms the link between the equatorial and apical domains. Flexibility has also been observed *en-bloc* with the intermediate domain acting as a hinge between the two other domains. GroEL possesses a weak ATPase activity ($k_{cat} \approx 0.06$ sec^{-1} per subunit) (Braig *et al.*, 1993) which is dependent on the presence of Mg^{2+} and K^{+} ions for maximal activity (Viitanen *et al.*, 1990).The crystal structure of groEL complexed to ATP-γ-S has been resolved to 2.4 Å, providing additional structural information (Boisvert *et al.*, 1996). Each subunit of ATP is observed to bind to a novel pocket, formed from amino acids which are highly conserved among chaperonins. Further, conformational shifts in the equatorial domain have been identified on ATP binding.

The *gro* E operon also encodes groES which has a subunit M_r of 10 368 (Hemmingsen *et al.*, 1988). GroES exists in a homo-oligomeric form composed of seven subunits arranged in a ring-like structure 8 nm in diameter with a 2 nm electron dense central channel (Chandrasekhar *et al.*, 1986). GroES has been defined as a (co-) chaperonin as have its homologues in mitochondria (Hsp10) and plastids (cpn10) which have a similar structural arrangement (Hemmingsen *et al.*, 1988). The crystal structure of groES has been resolved to 2.8 Å (Hunt *et al.*, 1996) confirming the domed shape of the heptamer, the inside surface of which is hydrophilic and highly charged. Each subunit of groES contains a mobile loop segment which is involved in groEL binding. In the crystal structure this loop is disordered in six of the subunits. The single well-ordered copy is observed to extend from the bottom outer rim of the groES dome, suggesting that the cavity within the dome is continuous with the polypeptide binding chamber of groEL in the chaperonin complex (Hunt *et al.*, 1996). Crystal structures of other members of the cpn10 family have also revealed a flexible region extending from the lower rim of the dome (e.g. cpn10 from *Mycobacterium leprae*, Mande *et al.*, 1996a).

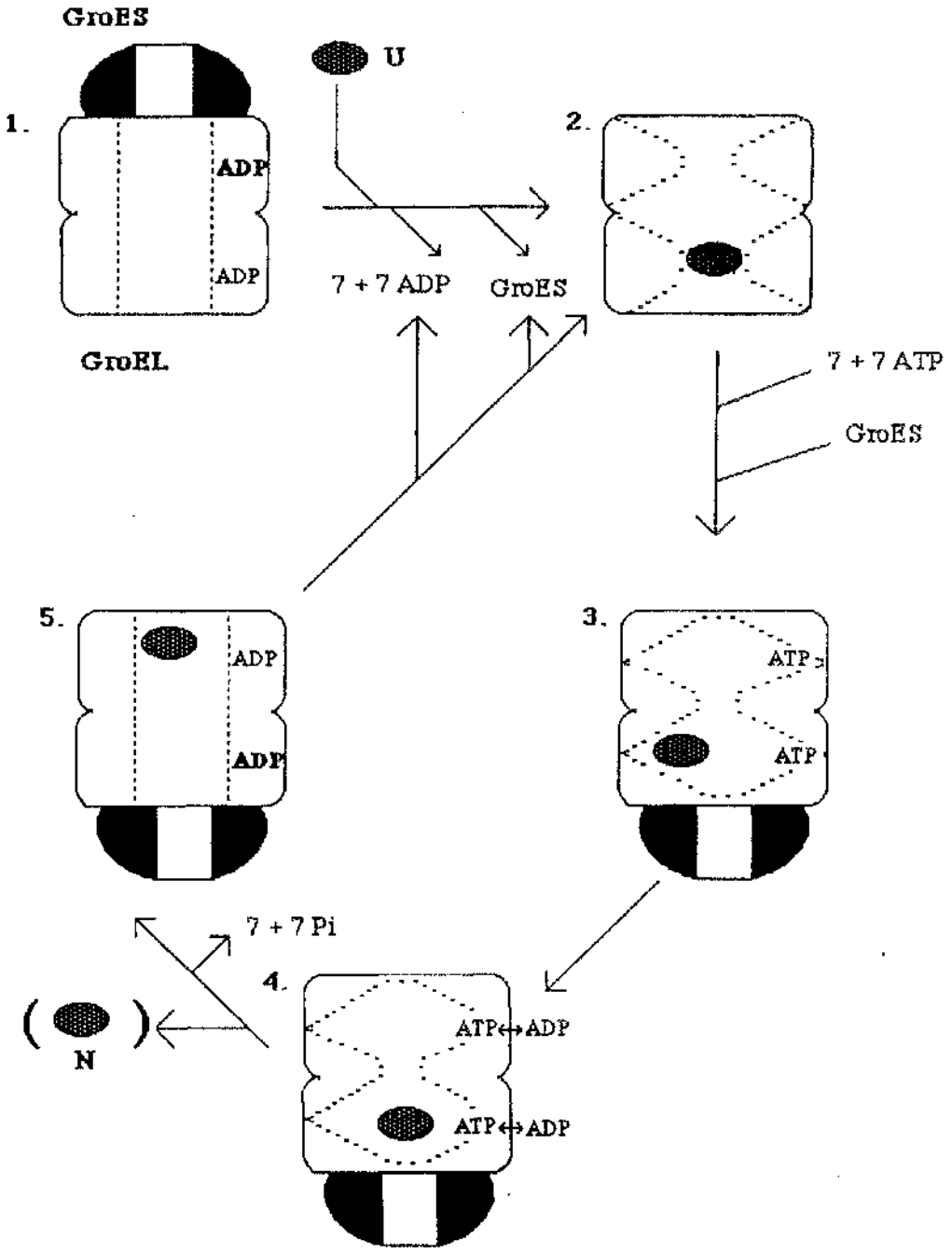
1.8.5.2 Method of groEL and groES action and their role in the sequential mechanism of chaperone proteins

Studies indicate that groEL binds one molecule of substrate protein within the central cavity in a conformation resembling the molten globule (Martin *et al.*, 1991; Langer *et al.*, 1992; Braig *et al.*, 1993; Chen *et al.*, 1994; Hayer-Hartl *et al.*, 1994). Protein folding is achieved through cycles of ATP dependent protein release and rebinding (Martin *et al.*, 1991; Weissman *et al.*, 1994). This process is thought to be regulated by groES which binds nucleotide-dependently to one end of the groEL cylinder, leaving the cavity of one toroid available for the association of substrate protein (Martin *et al.*, 1991; Langer *et al.*, 1992). GroES binding increases the cooperativity of the groEL ATPase activity and after ATP hydrolysis, stabilises the seven interacting groEL subunits in the ADP-bound state (Martin *et al.*, 1993). GroES dissociates after ATP hydrolysis in the uninhibited groEL toroid and its reassociation with the substrate bound groEL complex results in ATP-dependent protein release. This sequence of events is illustrated in Figure 1.5.

The main stress proteins of *E. coli* function in an ordered protein-folding reaction. First DnaK (hsp70) recognises the nascent peptide chain and cooperates with DnaJ in stabilising an intermediate conformational state lacking ordered tertiary structure. The protein is then transferred to groEL (hsp60) via GrpE and ATP hydrolysis. GroEL then interacts with groES as described above in order to produce the native protein. In this way, groEL and groES are the final stage of the protein folding mechanism (Langer *et al.*, 1992a). Homologues of each of the chaperones involved in this *E. coli* sequence exist in mammalian cells where a similar pathway is thought to operate.

Figure 1.5 Model for the groEL-groES assisted folding of polypeptides

1. The groEL-groES complex is favoured under physiological conditions. The groEL ring associated with groES is stabilised in a high affinity ADP-binding state (shown in bold type), the other ring has a lower affinity for ADP. **2.** Upon binding of the unfolded polypeptide (U), ADP and consequently groES dissociate. **3.** ATP binds, weakening the interaction between the polypeptide and groEL, and allows groES to rebind. Proteins with a low affinity with groEL may be released at this stage. **4.** ATP hydrolysis releases the bound polypeptide into the central cavity, allowing it to fold. Proteins attaining (near) native structure after this cycle are released (N) **5.** Generation of the ADP state increases the binding affinity of groEL for groES. Polypeptides which have still not reached the (near) native state rebind and the reaction cycle continues (from step **2.**) Between steps **5.** and **2.** the groEL protein is rotated 180°, with the substrate polypeptide remaining bound in the same ring. This model was taken from Martin *et al.*, (1993).


GroES
U
1.
ADP
ADP
GroEL
7 + 7 ADP
GroES
2.
7 + 7 ATP
GroES
5.
ADP
ADP
3.
ATP
ATP
7 + 7 Pi
N
4.
ATP↔ADP
ATP↔ADP

1.9 PROTEIN (RE)FOLDING STUDIES

Since the refolding experiments on bovine pancreatic ribonuclease (Anfinsen, 1961), the unfolding by various methods and refolding of a vast number of proteins, both monomeric and oligomeric, have been studied. A wide range of physical parameters and solvent compounds have been used as denaturing agents including GdnHCl, urea, SDS, LiCl, high temperatures, extremes of pH and high hydrostatic pressure (Tanford, 1968; Tanford, 1970). The extent to which the native structure is perturbed differs widely with the protein being studied, but to a certain extent, denaturants show additive effects. As a result of the structure-function relationship of proteins, monitoring biological function represents the most sensitive method of following the refolding process. Secondary and tertiary structural probes, and in oligomeric proteins, the state of association are also important approaches used to characterise the folding process.

It has been adequately demonstrated that the majority of denatured proteins, both monomeric and oligomeric, can be renatured to a degree under appropriate conditions (Ghèlis and Yon, 1982). In general, a low protein concentration is employed during renaturation to minimise undesirable side reactions such as aggregation and sulfhydryl oxidation. It has also been found that the addition of ligands, such as substrates of enzymes, and even low concentrations of detergents can increase the degree of renaturation of a protein (Tandon and Horwitz, 1987; Mendoza *et al.*, 1991).

Much interest has been shown in the refolding of nuclear encoded mitochondrial proteins as it is known that such proteins cross the mitochondrial membranes in an unfolded conformation and so folding and, for oligomeric proteins, subsequent association, occurs after translocation. The deactivation and reactivation of citrate synthase (CS) has been studied by several laboratories (Srere, 1966; Wu and Yang, 1970; Greenblatt and Sarkissian, 1972; West *et al.*, 1990; Zhi *et al.*, 1992). West *et al.*

(1990) showed that deactivation of CS in GdnHCl at concentrations less than 1.5M caused substantial activity loss. On dilution this loss of activity is completely reversed. The loss of activity in GdnHCl occurs at much lower concentrations than changes in circular dichroism, fluorescence and exposure of sulphydryl groups, which all occur between 1.8M and 3M GdnHCl. This suggests that GdnHCl at low concentration causes a change in CS that is more subtle than global unfolding which occurs at higher concentrations. Zhi *et al.* (1992) have studied the reactivation of GdnHCl-denatured CS under a variety of conditions. The addition of bovine serum albumin (BSA), oxaloacetate (OAA) and glycerol to the renaturation buffer were found to promote reactivation. The effect of these substrates was found to be additive with respect to the yield of folded CS. The precise mechanism by which BSA, OAA and glycerol promote refolding is uncertain. BSA is generally thought to stabilise structures that approach the native conformation. The action of glycerol may be similar to that of BSA, as substances like polyethylene glycol, which mimics the water exclusion and viscosity effects of glycerol, do not enhance renaturation. OAA may bind to a partially formed active site, which then acts as a folding nucleation point.

The refolding of denatured CS by the groE system has also been investigated (Buchner *et al.*, 1991; Zhi *et al.*, 1992). Folding assistance is demonstrated by groE in both these reports, although the conclusions about the essential presence of groES are different in the two studies. It is worth noting that while reports of *in vitro* renaturation of proteins with the assistance of only groEL are interesting mechanistically, their importance is probably limited owing to the presence of both components of the groE system in cellular protein folding.

To date, the groE assisted refolding of relatively few chosen proteins appears to have been studied in detail. Some, such as firefly luciferase, have been chosen because of the availability of extremely sensitive assay techniques, in this case a luminescence-based enzyme assay. Firefly luciferase is also a useful substrate because its spontaneous

refolding upon dilution from denaturant is inefficient (Nimmersgern and Hartl, 1993). Other substrates, such as rhodanese were perhaps initially selected because of the relative simplicity of a monomeric system (Mendoza *et al.*, 1991). Additionally, some substrates have been studied which are capable of folding reversibly under *in vitro* conditions in the absence of additional components, such as enolase (Kubo *et al.*, 1993), lactate dehydrogenase (Badcoe *et al.*, 1991) and dihydrofolate reductase (Viitanen *et al.*, 1991). The addition of groEL to these systems which are capable of spontaneous refolding has been found to dramatically reduce the yield of refolded protein, showing that groEL is recognising an intermediate state of the folding protein. To further the understanding of groE-mediated folding, it is important that in future a wider range of substrate proteins are employed as it is clear that a large number of proteins *in vivo* associate with the groE system during folding.

The dissociation, unfolding and unassisted renaturation of bovine heart PDC has recently been reported (West *et al.*, 1995). The results of this study show that the integrity of this complex is perturbed by relatively low levels of GdnHCl which lead to dissociation of the E1 and E3 components from the E2/X complex core. It was found that this dissociation leads to loss of the overall activity of the complex without causing any major loss of structure of the individual components of the complex. It was further reported that the individual components lose secondary and tertiary structure, as monitored by far-UV CD and protein fluorescence respectively, at higher levels of GdnHCl incubation than those at which activity is lost. Also, it was found that the ability to regain activity on subsequent dilution of the GdnHCl to 0.03M correlated with the integrity of the secondary and tertiary structure. In this respect, the components of bovine PDC behave in a manner typical of a number of other imported mitochondrial enzymes, such as glutamate dehydrogenase (West and Price, 1988), fumarase (Kelly and Price, 1991), citrate synthase (West *et al.*, 1990) and NAD^+-dependent isocitrate synthase (Kelly *et al.*, 1993).

This thesis details the purification and characterisation of 2-oxoacid dehydrogenase multienzyme complexes from various sources. The deactivation and reactivation of the complexes and their component subunits has been studied using a variety of techniques to monitor structural and activity changes during these processes. E3 from bovine, yeast and bacterial sources has been purified to enable the effect of various *in vitro* refolding environments to be investigated, including the addition of the bacterial chaperone proteins groEL and groES.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and biochemicals

Acrylamide, dithiothreitol (DTT), 2-mercaptoethanol, polyethylene glycol (PEG), sodium dodecyl sulphate (SDS) and N,N,N^{1}N^1-tetramethylethylene diamine were obtained from BDH Chemicals Ltd., Poole, Dorset, England. N,N^1-methylenebisacrylamide and Triton X-100 were obtained from Fisons, Loughborough, England. Guanidine hydrochloride (GdnHCl) and isopropylthio-β-D-galactoside were obtained from Gibco BRL, Paisley, Scotland. Acetyl coenzymeA (acetyl-1-^{14}C) was obtained from Dupont, Belgium. Bactotryptone and yeast extract were obtained from Difco, Detroit, Michigan, U.S.A. All other chemicals were of analytical grade and were obtained from Sigma Chemical Co., Poole, Dorset, England.

2.1.2 Enzymes and proteins

Bovine serum albumin (BSA), yeast PDC and molecular mass markers for gel filtration chromatography were obtained from Sigma Chemical Co., Poole, Dorset. Molecular mass marker proteins for M_r determination by SDS-PAGE were purchased from Pharmacia Ltd., Milton Keynes.

2.1.3 Chromatography media

Sephacryl S-200 was obtained from Pharmacia, Milton Keynes, Buckinghamshire. DEAE-cellulose was obtained from Whatman Biochemicals, Maidstone, Kent.

2.1.4 Pre-packed media

Pre-packed Mono Q, Resource Q, alkyl-Superose and Superose 6 columns were obtained from Pharmacia and used on a Pharmacia FPLC system.

2.1.5 Bacterial strains

The bacterial strain *E. coli* JRG2872 carrying the plasmid pG5501 was used to overproduce *E. coli* PDC (Russell *et al.*, 1992). This IPTG-inducible system overexpresses PDC on a tac promoter.

For overexpression of groEL, the bacterial strain was *E. coli* DH1 carrying the plasmid pGT3270. This plasmid contains the *groE* genes on a 2.1 kb *Eco* R1-*Hind* III DNA fragment (McLennan *et al.*, 1993), a gift from Dr. N. F. McLennan, University of Edinburgh, Scotland.

Bacterial stocks were prepared by mixing 5 ml of L-broth culture taken after overnight growth, with an equal volume of sterile glycerol and stored frozen at -70°C.

2.1.6 Media

Luria broth has been used to grow *Escherichia coli* cells and consists of:-

Bactotryptone 10g/l

Yeast extract 5g/l

NaCl 10g/l

Sterilisation was achieved by autoclaving at 15psi.

2.2 GENERAL LABORATORY PROCEDURES

2.2.1 Measurement of pH

pH measurements were carried out using a CD 620 digital pH meter (Cambridge, U.K.) calibrated at room temperature.

2.2.2 Dialysis

Dialysis was carried out using tubing obtained from Medicell International Ltd., London, which was of 6.3 or 14.3 mm diameter depending on the volume to be dialysed. The tubing was pre-treated by boiling for 10 min in 2% (w/v) $NaHCO_3$, 1 mM EDTA pH 8. Pre-treated tubing was stored in 100% (v/v) ethanol and rinsed thoroughly in distilled water before use.

2.2.3 French pressure cell

Cells were broken by 2 passages through an automatic French pressure cell at 95 MPa (14000 psi internal pressure). The cell was precooled on ice before use (cat. no. 4-3398A, American Instruments Company, Silver Spring, Maryland, U.S.A.).

2.2.4 Protein estimation

Protein concentrations were determined by the method of Bradford (1976) with BSA as standard.

2.3 PREPARATION OF PDC AND OGDC FROM BOVINE HEART

PDC and OGDC were prepared by initially taking 600g of either fresh beef heart or beef heart stored at -80°C within 3 h of slaughter, from which fat and connective tissue had been removed. Cubes of tissue were blended and extracted in 50mM MOPS buffer, pH 7.0, containing 2.7 mM-EDTA, 3% (v/v) Triton X-100, 0.1 mM-DTT, 0.1 mM-PMSF, 0.1 mM-benzamidine/HCl and 0.2% (v/v) anti-foam. After spinning at 15000g for 20 min, the collected supernatant was adjusted to pH 6.45 with 10% (v/v) acetic acid. At this stage the extracts contained assayable amounts of OGDC activity. The presence of lactate dehydrogenase made PDC activity difficult to estimate this early in the purification. Both complexes were precipitated by the addition of 0.12 vol. of a 35% (w/v) PEG solution. After stirring on ice for 30 min, the solution was spun at 30000g for 15 min. Pellets were re suspended in 50 mM-MOPS, pH 6.8, containing 1% (v/v) Triton X-100, 2.7 mM-EDTA, 0.1 mM-DTT, 0.1 mM-PMSF and 0.1 mM-benzamidine by homogenisation with a loose-fitting Teflon-glass homogenizer. This extract was adjusted to 13 mM-$MgCl_2$ and 50mM-sodium phosphate, pH 7.0. The pH was then again lowered to 6.45 with 10% (v/v) acetic acid before the addition of 0.12 vol. 35% (w/v) PEG for a second precipitation. The 2-oxoacid dehydrogenase complexes were separated by differential precipitation with PEG. Usually between 0.04-0.06 vol. 35% (w/v) PEG was required to precipitate 90% of the OGDC activity. OGDC was pelleted by spinning at 40000g for 10 min prior to overnight re suspension in a minimal volume of 50 mM-MOPS buffer, pH 7.0, containing 1% (v/v) Triton X-100, 2.7 mM EDTA, 0.1 mM-DTT, 0.1 mM-PMSF and 0.1-mM benzamidine. The PDC containing supernatant fraction was concentrated by centrifugation for 2.5 h at 150000g in a Beckman Ti70 rotor. The pellets were re suspended in the same buffer as the OGDC. Both complexes were stored at a final concentration of 5-10 mg/ml at 4°C or in 50% (v/v) glycerol at -20°C.

2.4 PREPARATION OF E3 FROM BOVINE HEART OGDC

The method for mammalian E3 purification was developed by Ms. Saiqa Khan, Division of Biochemistry and Molecular Biology, University of Glasgow. NaCl was added to the purified complex to 2M giving a protein concentration of ~3 mg/ml. This solution was incubated on ice for 15 min to allow adequate time for component dissociation. A further incubation at 65°C for 10 min then followed, which induced denaturation and aggregation of the less stable complex components. Centrifugation was then carried out at 15000g for 10 min to pellet the insoluble aggregates. The E3 remained in the supernatant and was dialysed to remove salt before using experimentally.

2.5 PREPARATION OF PDC FROM *Escherichia coli*

E. coli strain JRG 2872, kindly donated by Prof. J. R. Guest, University of Sheffield, overexpresses PDC from a tac promoter (Russell, *et al.*, 1992). Cultures (250 ml) in Luria broth containing glucose (0.2% w/v) and ampicillin (50μg/ml) were grown at 37°C with vigorous shaking to mid-exponential phase (A_{650}=0.25-0.50) and induced by the addition of IPTG to 60μM. After 6-8 h, cells were harvested, washed in buffer 1 [20 mM-potassium phosphate, pH 7.8, containing 2 mM-Na_2EDTA, 1 mM-benzamidine/HCl and 1 mM-PMSF] and disrupted using a French pressure cell in the same buffer, as detailed in section 2.2.3. The crude extracts were clarified by centrifugation at 150000g for 30 min. PDC was sedimented by further centrifugation at 150000g for 4h. The PDC containing pellet was re suspended in buffer 1 and further purified by FPLC ion-exchange chromatography using a Resource Q column with a 0-700 mM-NaCl gradient in buffer 1. Fractions containing PDC were pooled and

concentrated by sedimentation as before. Purified complexes were stored in buffer 1 plus glycerol (50% v/v) at -20°C.

2.6 PREPARATION OF E3 FROM BACTERIAL PDC

Bacterial E3 was purified from two stages of the whole complex preparation. The supernatant from the final high speed spin was found to contain large amounts of E3 (28-40% of total yield). This was purified using an adaptation of the method described in section 2.4. NaCl was added to the supernatant to a final concentration of 2M and then incubated at 65°C for 20-30 min. Again the sample was spun for 10 min at 15000g and the salt removed by dialysis into the appropriate buffer before using experimentally. Secondly, a small amount (3-7% of total yield) of E3 was located in a peak from the Resource Q salt gradient, the final step in the bacterial PDC purification process. Heat treatment was carried out on the pooled fractions from this peak to further purify if required.

2.7 PREPARATION OF GroEL

The groEL purification protocol described below was based on adaptations, obtained from Dr. G. J. Thomson, to the procedure described previously (Hendrix, 1979; Chandresekhar, 1986). Solutions throughout the preparation were kept at 4°C. GroEL could not be assayed as the ATPase activity is too weak, so a Phastgel system was used to identify groEL containing fractions. The bacterial strain used was *E. coli* DH1 carrying the plasmid pGT3270 containing the groE genes on a 2.1 kb *Eco RI-HindIII* DNA fragment, kindly donated by Dr. N. F. McLennan, ICMB, University of Edinburgh, Scotland, UK (McLennan *et al.*, 1993). Bacterial stocks were stored at -70°C in 50% (v/v) glycerol. The *E. coli* were grown on Luria broth containing glucose (0.1%

w/v) until the cell suspension had reached saturation (OD_{600} of approximately 5) before harvesting the cells by centrifugation (4500g for 15 min). Cells could be used immediately or stored in pellet form at -20°C until use, the latter option aiding the next step in the purification process. Around 20g of wet weight cells were obtained from a typical 4l culture. Cells were re suspended in extraction buffer (50mM Tris-HCl buffer, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine) and broken using a French pressure cell as detailed previously. Cell wall debris was then removed by centrifugation at 15000g for 40 min. Following cell breakage an equal volume of streptomycin sulphate (0.05 mg/ml) was added to the supernatant which was stirred on ice for 1 h. The extract was then centrifuged for 45 min at 25000g. At this stage the solution was gradually brought to 55% (w/v) saturation by addition of solid $(NH_4)_2SO_4$ and, after stirring for 1 h on ice, the precipitated protein was collected by centrifugation at 30000g for 30 min. Precipitated protein was resuspended in 4 ml of S-200 buffer (50mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 10% (w/v) glycerol, 5 mM 2-mercaptoethanol, 1M KCl and 0.5% (w/v) Triton X-100) and loaded onto a Sephacryl S-200 column (120 cm x 3 cm) equilibrated in the same buffer. GroEL containing fractions, eluting in the void volume of the column, were pooled and dialysed against Mono Q 10/10 buffer (20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA). The dialysed solution was applied to the anion exchange column and eluted with a 25 ml 0-1M linear NaCl gradient in the above buffer. Fractions containing groEL were pooled and dialysed against Alkyl-Superose buffer (50mM potassium phosphate pH 7.0, containing 1.5M $(NH_4)_2SO_4$). This was then loaded onto an Alkyl-Superose HR 5/5 hydrophobic interaction chromatography column equilibrated in the above buffer. GroEL was eluted with a linear 1.5-0M $(NH_4)_2SO_4$ gradient.

2.8 STORAGE OF PURIFIED PROTEIN

Purified mammalian OGDC, bacterial PDC and groEL were dialysed against 100 volumes of 50mM Tris-HCl, pH 7.6, containing 50% (v/v) glycerol and stored at -20°C.

2.9 DENATURATION CONDITIONS

The unfolding of the complexes and purified components was studied in 50mM-potassium phosphate pH 7.6. Solutions of protein (final concentration 0.5-1.0 mg/ml) were incubated in the presence of varying concentrations of GdnHCl for 15 min at 4°C before measurements of catalytic activity, CD or fluorescence were taken. There was no further significant change on incubation for a further 45 min.

2.10 PREPARATION OF DIHYDROLIPOAMIDE

Dihydrolipoamide was prepared from the oxidised form of DL-6,8-thioctic acid amide (DL-lipoamide). 60 mg of DL-lipoamide was dissolved in 1.2 ml of 50% (v/v) ethanol in 1M potassium phosphate buffer pH 8, to which 2.4 mg of freshly prepared 5% (w/v) $NaBH_4$ was added for 10 min. This was then neutralised with 1.2 ml of 3M HCl. The dihydrolipoamide was extracted into the upper solvent layer by adding 3 x 3 ml volumes of toluene. Evaporation of the toluene under N_2 left crystalline dihydrolipoamide which was stored at -20°C until use (Kochi and Kikuchi, 1976).

2.11 ENZYME ASSAYS

2.11.1 2-oxoacid dehydrogenase complexes

The whole complex activities of PDC and OGDC were assayed spectrophotometrically at 340nm, as the rate of formation of NADH, at 30°C (Danson *et al.*, 1978). Approximately 2-5μg of complex were assayed in the presence of 670μl **Solution A** (50mM potassium phosphate, pH 7.6, containing 3 mM NAD^+, 2 mM $MgCl_2$ and 0.2 mM ThDP), 14μl **Solution B** (0.13M cysteine-HCl, 0.13 mM Li_2CoASH) and 14μl **Solution C** (100 mM pyruvic acid/2-oxoglutarate for PDC and OGDC respectively). The above assay solution was preincubated at 30°C and the reaction initiated by the addition of enzyme.

2.11.2 2-oxoacid dehydrogenase (E1)

The activity of the E1 component, pyruvate dehydrogenase in PDC and 2-oxoglutarate dehydrogenase in OGDC, was measured by following the reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm. The protein was first incubated at 30°C in solution A (as described in the previous section) and DCPIP. The reaction was then initiated by the addition of the appropriate solution C (as described in the previous section) (Khailova *et al.*, 1976).

2.11.3 Dihydrolipoamide acetyltransferase (E2)

The activity *E. coli* E2 was measured isotopically via the conversion of [1-^{14}C] acetyl-CoA to the radiolabelled S-acetyl dihydrolipoamide intermediate which is extracted into toluene, leaving the unreacted [1-^{14}C] acetyl-CoA in the aqueous phase (Butterworth *et al.*, 1974). The reaction mixture contained 0.1 ml of 50 mM potassium phosphate, pH 7.4, containing 5 mM cysteine and various amounts of GdnHCl, 10 µl of 10 mM dihydrolipoamide in ethanol, 20 µl of 2.5 mM of mixed cold acetyl-CoA and [1-^{14}C] acetyl-CoA, giving a specific activity of 3 x 10^5 cpm/µmol. 5-10 µg of PDC and water made the total volume of the reaction mixture 0.2 ml. The reaction was started by the addition of the acetyl-CoA mixture and incubated at 25°C for 2 min. At this stage the reaction was stopped by adding 0.5 ml of toluene and the mixture shaken for 10 s on a Vortex mixer to extract the radioactive S-acetyldihydrolipoamide. An aliquot (0.1 ml) of the toluene layer was then withdrawn and radioactivity measured in a Beckman liquid scintillation spectrometer. Controls were also carried out from which enzyme (or dihydrolipoamide) was omitted. This background reading, typically around 40 cpm, was subtracted from the experimental readings.

2.11.4 Dihydrolipoamide dehydrogenase (E3)

Assays were performed using the method of Jackman *et al.* (1990) at 30°C. The assay buffer used was 50 mM-potassium phosphate, pH 7.6, containing 3 mM-NAD^+, 1 mM $MgCl_2$, 0.2mM ThDP and 24μg of dihydrolipoamide (for preparation see section 2.10). The reaction was initiated by the addition of enzyme and the activity followed by monitoring NADH formation at 340nm.

2.12 ATPase ACTIVITY OF GroEL

The ATPase activity of groEL was measured spectrophotometrically by coupling the hydrolysis of ATP to the oxidation of NADH by the coupling enzymes pyruvate kinase and lactate dehydrogenase. Assays were performed in 50 mM triethanolamine buffer pH 8.0 containing 10 mM magnesium acetate. Concentrations of substrates and coupling enzymes in the buffer were; 0.5 mM ATP, 1.0 mM phospho*enol* pyruvate, 0.15 mM NADH, 2.5 μg/ml pyruvate kinase and 8.3 μg/ml lactate dehydrogenase. The oxidation of NADH was monitored at 340 nm (Price *et al.*, 1993).

2.13 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis in the presence of SDS was performed using the method of Laemmli (1970), with a 3% stacking gel and a 10% or 12% running gel. The ratio of acrylamide : bisacrylamide was 30 : 0.8 for each gel. Polymerisation was induced by the addition of 0.03% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. After electrophoresis, protein on the gels was visualised by staining with Coomassie blue for at least 90 min at room temperature whilst rotating at slow speed on a shaker. The Coomassie reagent was 0.1% (w/v) Coomasie Brilliant Blue G250 in 50% (v/v) methanol and 10% (v/v) glacial acetic acid. Destaining was carried out in 10% (v/v) methanol and 10% (v/v) glacial acetic acid, the destain being replaced at intervals until the background was fully destained and bands clearly visible.

2.14 CIRCULAR DICHROISM

Circular dichroism (CD) is a spectroscopic property which is sensitive to molecular conformation, and thus is widely used in the study of protein structure. The

near-ultraviolet (UV) CD bands of proteins (340-250 nm) are principally due to tryptophan, tyrosine, phenylalanine and cystinyl groups, and they reflect the tertiary and quaternary structure of the protein. The far-UV CD bands of proteins (260-178 nm) derive from the amide chromophore and reflect the secondary structure of the protein (α-helix, β-sheet etc.). For most proteins the native state has a much more intense CD spectra over most of the far- and near-UV wavelength. The large loss of intensity which occurs in both of the wavelength regions upon treatment of the protein with a denaturant, such as GdnHCl, combined with fluorescence measurements, allow the stability of tertiary and secondary structure to be compared.

CD spectra were recorded at 20°C on a JASCO J-600 spectropolarimeter. All spectra were recorded by Dr. Sharon Kelly, Department of Biological and Molecular Sciences, University of Stirling.

2.15 FLUORESCENCE

Fluorescence spectroscopy is a sensitive technique which can be employed continuously or discontinuously to obtain information on protein tertiary structure around fluorogenic amino acids. Fluorescence emission is observed on the return to the ground state of an excited electron. Nonfluorescent compounds lose the energy of the excited molecule as heat, while fluorescent compounds emit part of this energy as light. Due to the energy loss, the fluorescence emission is shifted to a longer wavelength compared to that of excitation. In the fluorescence experiments shown in Chapter 5, the excitation wavelength is 290 nm and emission has been monitored either by scanning from 310 nm to 400 nm, or at 340nm each second over 5 min to follow the tertiary structural changes during protein refolding.

Fluorescence changes were recorded at 25°C on a Perkin Elmer LS50 spectrofluorimeter with slit width setting at 5.0.

2.16 LIGHT SCATTERING MEASUREMENTS

Light scattering is a useful tool in following the association and dissociation of subunits of multimeric proteins. It has the advantage over other methods that measurements can be made very rapidly. The protein solution is illuminated with a beam of light with a wavelength far from any absorption band in the solvent or the protein. The amount of light scattered in directions other than that of the incident beam is proportional to the M_T of the protein in solution. Subunit association or aggregation can therefore be detected by an increase in intensity of scattered light over time.

Light scattering experiments were carried out at 25°C using a Perkin Elmer LS50 spectrofluorimeter. The illuminating wavelength was 350 nm. Detection of scattered light (350 nm) was at 90° to the incident beam. Slit width setting was 2.5.

CHAPTER 3

PURIFICATION AND INVESTIGATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

3.1 Introduction

The effect of structural changes on the activity of enzymes has been widely studied. Changes in primary structure aside, chaotropes such as urea and GdnHCl have been instrumental in enabling detailed information to be collected on the unfolding and subsequent renaturation processes. Comparison of the unfolding and inactivation of several enzymes in the presence of GdnHCl and urea have suggested that the active sites are usually situated in a limited region of the enzyme molecule that is more susceptible to denaturants than the protein as a whole (Tsou, 1986). It has been noted that different chaotropes do not necessarily produce the same end product. The dimeric pig heart citrate synthase, for example, is dissociated into monomers on treatment with GdnHCl but remains dimeric when incubated with a comparable concentration of urea (Wu and Yang, 1970). Subtle differences exist, therefore, in the mechanism of action these reagents in disrupting non-covalent interactions both within and between polypeptide chains. Previous work (West *et al.*, 1990) has suggested that GdnHCl denatures multienzyme complexes in three steps. Initially, subunit dissociation occurs at low GdnHCl concentrations. This is followed by local perturbation of the active site of individual components of the complex at intermediate GdnHCl concentrations. Beyond this secondary and tertiary structural changes occur progressively with increasing denaturant concentrations. Throughout this investigation GdnHCl has been employed, primarily to enable this work to be usefully compared to that of West *et al.* (1995).

This chapter deals with the purification and characterisation of OGDC and PDC from bovine heart and PDC from *E. coli* strain JRG 2872. Purification was followed by assay of intact complex activity, monitoring NADH formation spectrophotometrically at 340nm, and by SDS-PAGE. Initial characterisation of the complexes was carried out using GdnHCl-induced unfolding and subsequent refolding which was again monitored

using the intact complex. Also, circular dichroism has been employed to monitor secondary structural changes resulting from GdnHCl-induced denaturation.

3.2 OGDC and PDC purification from bovine heart

OGDC and PDC were purified from bovine heart as described in the Materials and Methods section. This protocol is based on that of Stanley and Perham (1980), with adaptations, outlined by De Marcucci *et al.* (1985). The same preparation can be used to purify both complexes although the amount of PEG used in the final cut is an important factor in the purity of the complex. Throughout the procedure estimates of protein concentration were made using the Bradford assay. Whole complex activity was also monitored. A typical set of data for the OGDC purification procedure is shown in Table 3.1. Purification was also followed by SDS-PAGE. Figure 3.1 shows samples taken from an OGDC preparation at the same stages as are shown in Table 3.1.

In this way both complexes were obtained with specific activities of 3-5 units/mg when assayed at 25°C. The extent of cross-contamination was estimated to be less than 1% on the basis of activity measurements.

3.3 Loss of activities of PDC and OGDC from bovine heart

The loss of activity of the complexes when incubated with and assayed in the presence of increasing GdnHCl concentrations is illustrated in Figure 3.2A. From this graph it can be seen that both complexes have lost all activity after incubation in 0.3M GdnHCl. OGDC activity is reduced to 50% of its original value at 0.11M GdnHCl whereas for PDC this value is 0.07M.

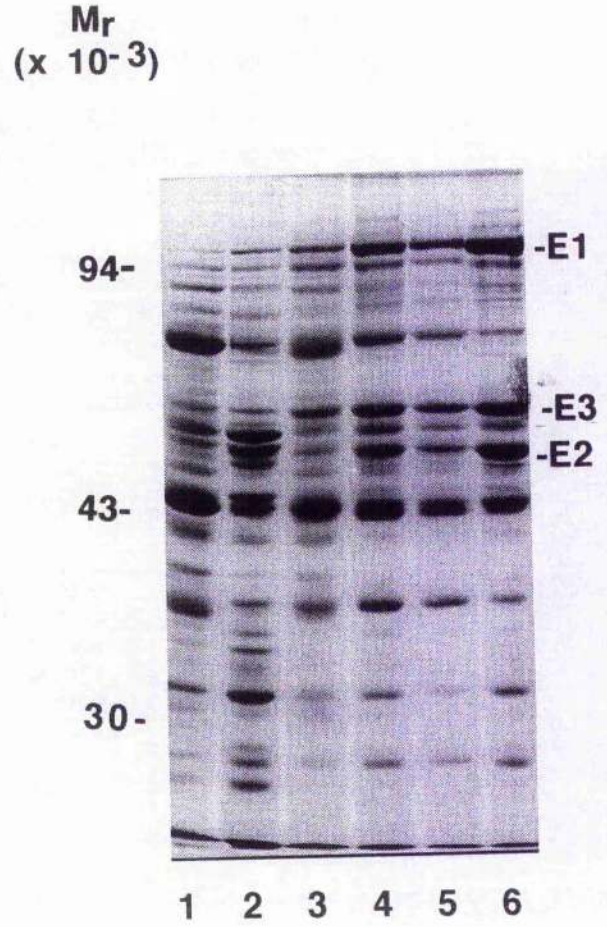
	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	% Recovery	Purification factor
Homogenate	1650	13.3	21900	2.69	4440	0.203	100	1.00
After 1st PEG cut	580	23.5	13600	6.14	3560	0.262	80.2	1.29
After muslin filtering	400	8.1	3240	8.40	3360	1.04	75.7	5.12
After 2nd PEG cut	200	6.5	1300	13.0	2600	2.00	58.6	9.85
Before final PEG cut	188	4.5	846	10.3	1940	2.29	43.7	11.28
Resuspended pellet	18.5	22.0	407	83.8	1550	3.81	34.9	18.8

Table 3.1 Purification table for bovine heart OGDC

Samples from each step of the purification were analysed for protein concentration and whole complex activity as described in Materials and Methods. The table shows the average data from two separate preparations. A Unit is defined as the amount of enzyme which converts 1 μmol of substrate to product in 1 min.

Figure 3.1 SDS-PAGE analysis of samples taken during trypic digestion of purified bovine heart OGDC

OGDC was resolved on 10% SDS-PAGE and stained with Coomassie blue. Lane M, low M_r proteins (10 μg); lanes 1 to 6, 15 μg samples taken at the same stages of the preparation as are shown in Table 3.1.


Mr
(x 10-3)
94-
43-
30-
-E1
-E3
-E2
1
2
3
4
5
6

3.4 Reactivation of beef heart OGDC and PDC by dilution of GdnHCl

The results of experiments in which either OGDC or PDC were incubated in GdnHCl for 15 min at 4°C, and then diluted into buffer for assay so as to lower the residual GdnHCl concentration to below 0.03M, are shown in Figure 3.2B. It is clear that the ability of PDC to regain complex activity decreases dramatically when the initial GdnHCl concentration is above 0.7M and is lost completely after pre-exposure to concentrations above 1.8M. OGDC can be seen to be more sensitive to GdnHCl incubation as its ability to regain complex activity decreases rapidly when it is incubated above 0.3M. For OGDC 50% of activity is regained after dilution from 0.5M GdnHCl incubation, a concentration at which PDC regains all of its activity.

3.5 PDC purification from *E. coli*

PDC was purified from *E. coli* strain JRG 2872 using the procedure reported by Russell *et al.* (1992), with minor adaptations which are outlined in Materials and Methods. Throughout the procedure estimates of protein concentration were made using the Bradford assay. Whole complex activity was also monitored as described previously. A typical set of data for the purification procedure is shown in Table 3.2. Purification was also followed using SDS-PAGE. Figure 3.3 shows samples taken from an PDC preparation at the same stages as are shown in Table 3.2.

3.6 Loss of activity and subsequent reactivation of *E. coli* PDC

The loss of intact complex activity was monitored after incubation with GdnHCl for 15 min at 4°C. The results are shown in Figure 3.4. Comparing Figure 3.4 with Figure 3.2 it can be seen that the reversible nature of inhibition of bovine PDC by

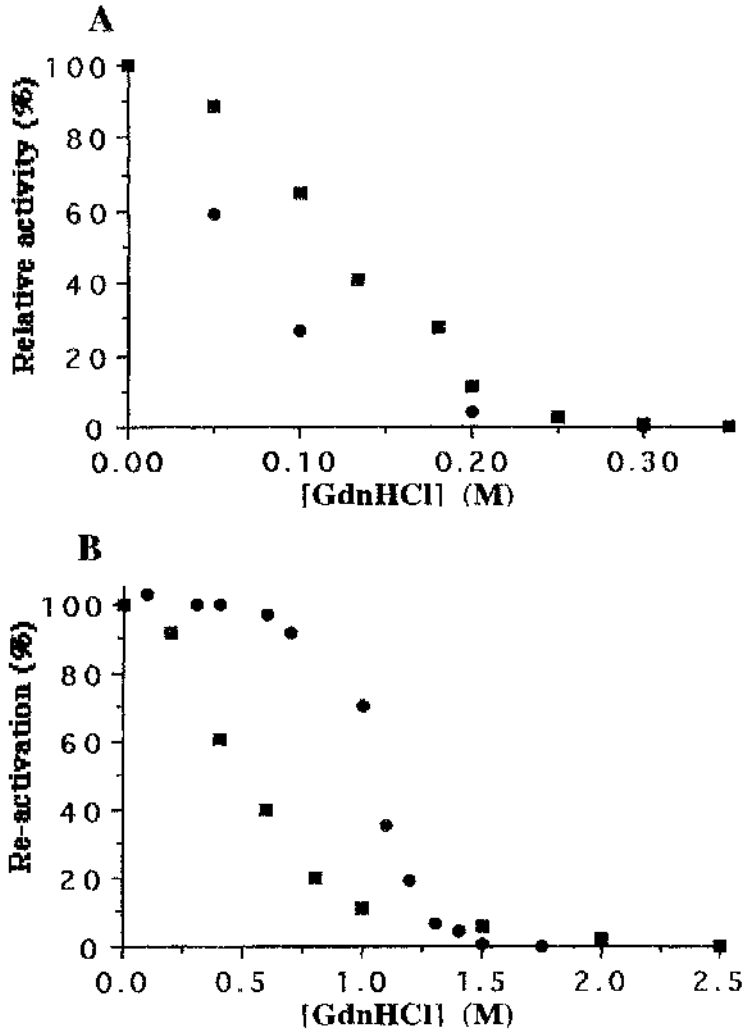


Figure 3.2 Inactivation and reactivation of bovine heart PDC and OGDC

Enzyme complexes were incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (shown in **A** where assay mixture contained the same [GdnHCl] as incubation) or reactivation (shown in **B** where [GdnHCl] is reduced to below 0.03M by dilution) was assayed. The concentrations of PDC (■) and OGDC (●) were both 1 mg/ml. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations.

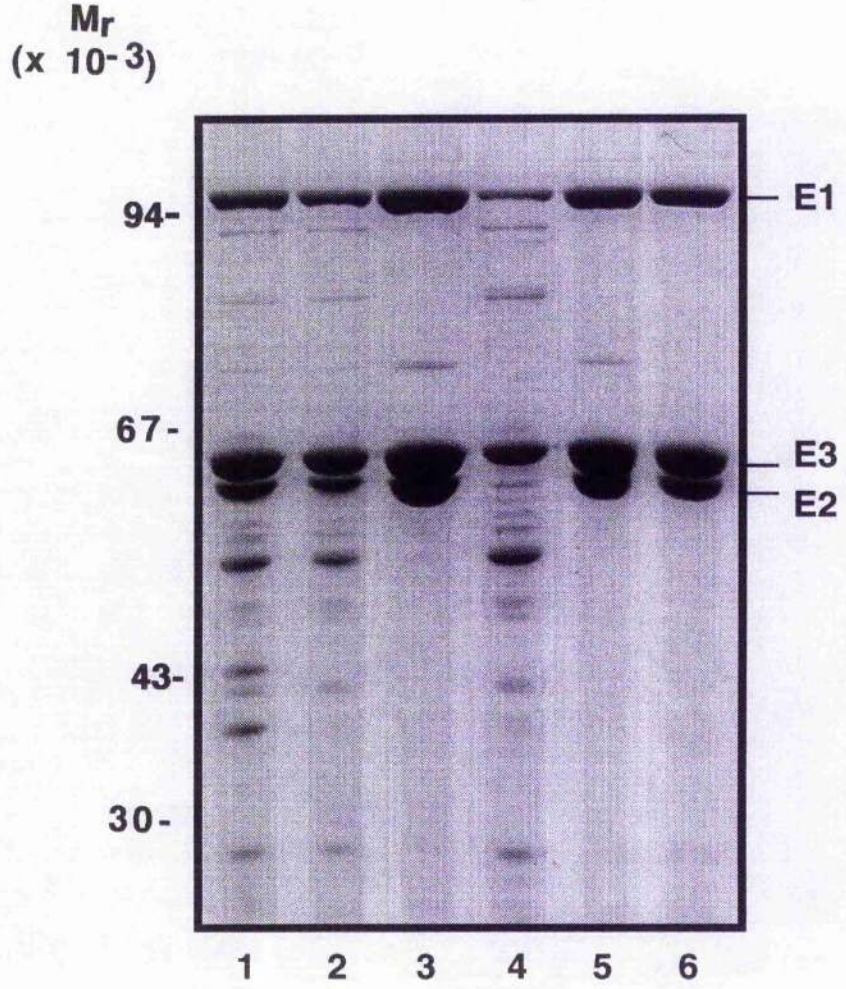
	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	% Recovery	Purification factor
Homogenate	92	6.0	552	29.0	2668	4.83	100	1.00
After clarification spin	87	5.0	435	29.9	2600	5.98	97.5	1.24
Resuspended pellet after 4 hr. spin	11	13.0	143	178	1958	13.7	73.4	2.84
After filtering	8.2	12.0	98.4	180	1476	15.0	55.3	3.11
Resource Q column pooled peaks	32.2	1.6	51.5	25.0	805	15.6	30.2	3.23

Table 3.2 Purification table for bacterial PDC

Samples from each step of the purification were analysed using Bradford and activity assays. The table shows the average data from three separate preparations. A Unit is defined as the amount of enzyme which converts 1 μmol of substrate to product in 1 min.

Figure 3.3 SDS-PAGE analysis of samples taken throughout the *E. coli* PDC purification process

Samples from the various stages of the *E. coli* PDC preparation stated below were diluted to approximately 0.5 mg/ml and 15 μl of each was mixed with an equal volume of Laemmli sample buffer and resolved on a 10% (w/v) SDS-polyacrylamide gel. Lane 1-homogenate, lane 2- after clarification spin, lane 3- resuspended pellet after 4 h ultracentrifuge step, lane 4- supernatant after 4 h ultracentrifuge step, lane 5- resuspended pellet after filtering and lane 6- after Resource Q column step.


Mr
(x 10-3)
94-
67-
43-
30-
E1
E3
E2
1
2
3
4
5
6

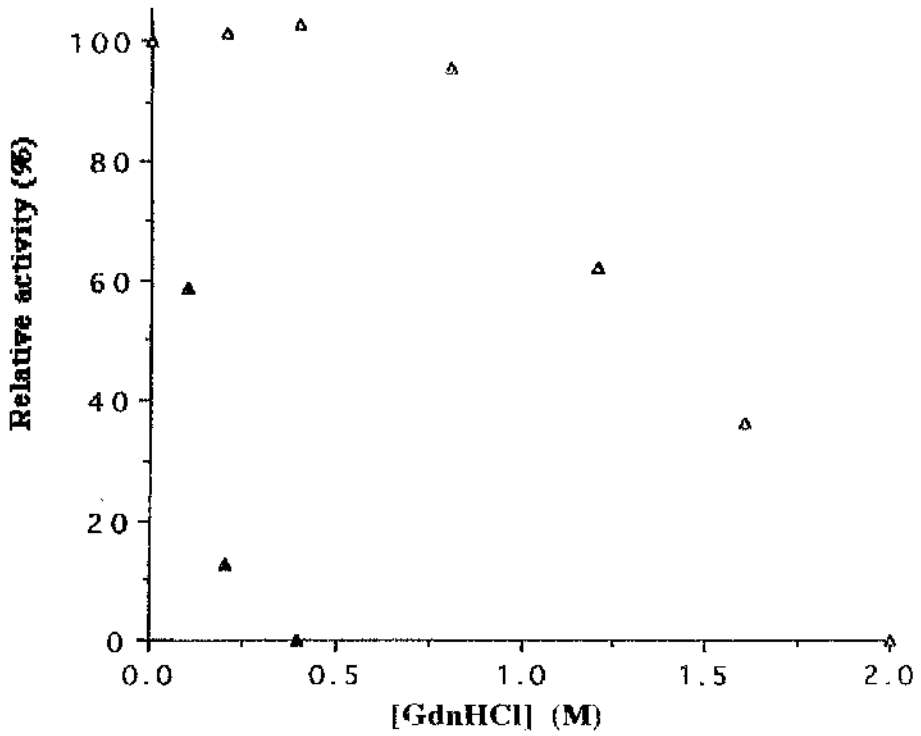


Figure 3.4 Inactivation and reactivation of bacterial PDC

PDC was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (▲, with assay mixture containing the same GdnHCl concentration) or reactivation (Δ, GdnHCl concentration reduced to below 0.03M by dilution) was assayed. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations.

GdnHCl is lost as the concentration of denaturant is increased above 0.7M and this ability is completely lost when incubated at GdnHCl concentrations above 1.5M. The *E. coli* PDC loss of re-activation range is from 0.9-2.0M, with 38% re-activation occurring after incubation in 1.6M GdnHCl, conditions from which the bovine complexes show no significant recovery of activity. Thus, it can be seen then that the bacterial complex is the most resilient of those studied to incubation in GdnHCl .

3.7 Unfolding of bovine heart OGDC and PDC and *E. coli* PDC as monitored by changes in CD properties

Figure 3.5 (**A, B** and **C**) shows the far-uv CD spectra of beef heart OGDC (**A**) and PDC (**B**) and *E. coli* PDC (**C**). The results are shown in Figure 3.6 in terms of the changes of ellipticity at 225nm relative to the total changes occurring between 0 and 6M GdnHCl. Figure 3.6 presents the ellipticity data for the three complexes together and from this it can be seen that the OGDC and PDC from the same source behave in a very similar fashion when incubated in GdnHCl with the majority of secondary structure being lost at GdnHCl concentrations above 4M. PDC from *E. coli* can be seen to be more resistant to secondary structural changes when incubated in low levels of GdnHCl (0-1.5 M) than the bovine complexes. However, at incubations above 2.0 M GdnHCl the three complexes studied show little difference in the pattern of loss of secondary structure.

Figure 3.5A Changes in the far-UV CD spectra on denaturation of bovine heart OGDC with GdnHCl

Spectra were obtained in 50mM potassium phosphate buffer, pH 7.6 at 20°C. Unfolding of OGDC (90μg/ml) was carried out in the presence of GdnHCl for 15 min at 4°C. Spectra were recorded by Sharon Kelly in the laboratory of Prof. Nicholas Price at Stirling University.

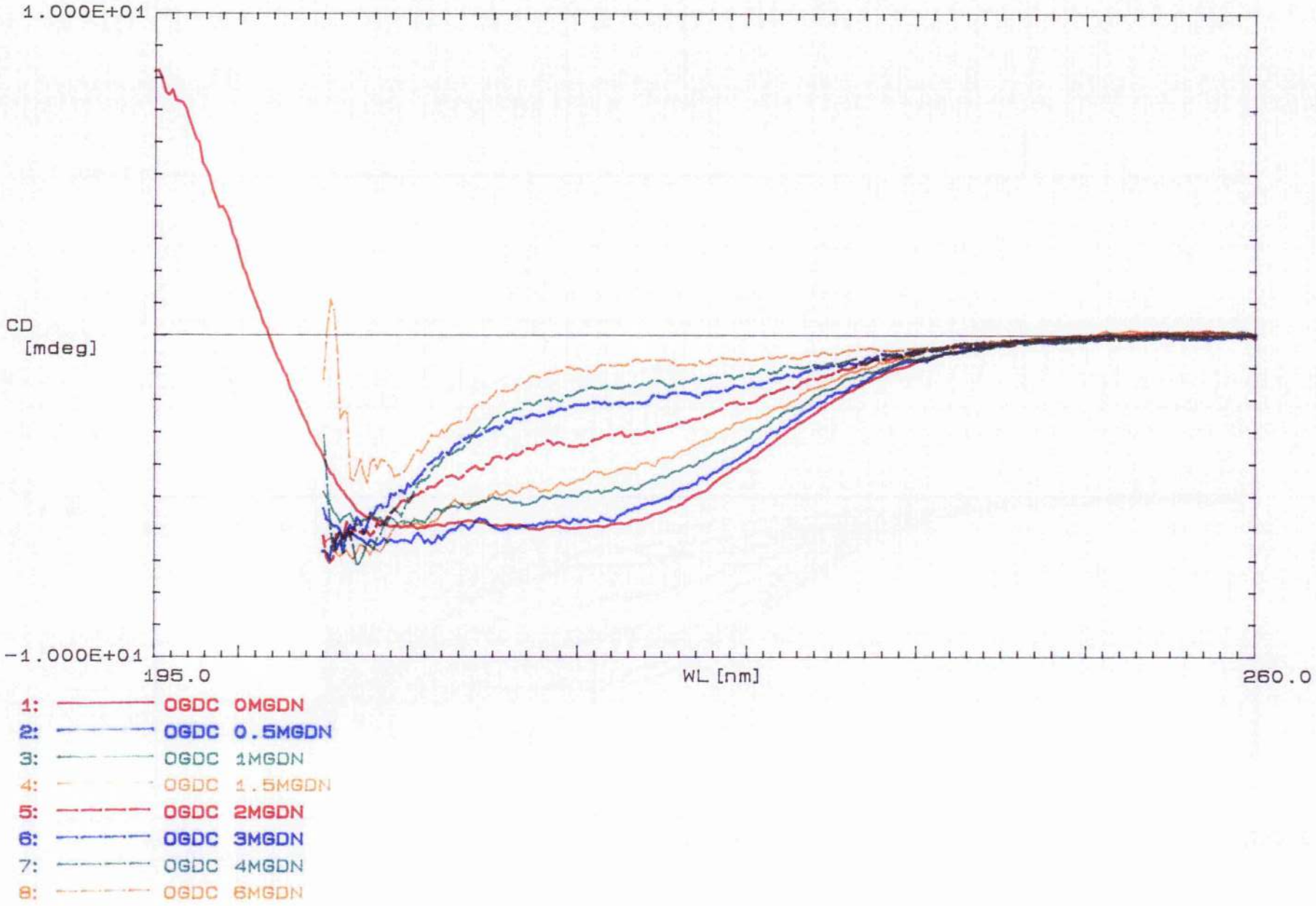

1.000E+01
CD
[mdeg]
-1.000E+01
195.0
WL [nm]
260.0
1: OGDC 0MGDN
2: OGDC 0.5MGDN
3: OGDC 1MGDN
4: OGDC 1.5MGDN
5: OGDC 2MGDN
6: OGDC 3MGDN
7: OGDC 4MGDN
8: OGDC 6MGDN

Figure 3.5B Changes in the far-UV CD spectra on denaturation of bovine heart PDC with GdnHCl

Spectra were obtained in 50 mM potassium phosphate buffer, pH 7.6, at 20°C. Unfolding of PDC (80μg/ml) was carried out in the presence of GdnHCl for 15 min at 4°C. Spectra were recorded by Sharon Kelly in the laboratory of Prof. Nicholas Price at Stirling University.

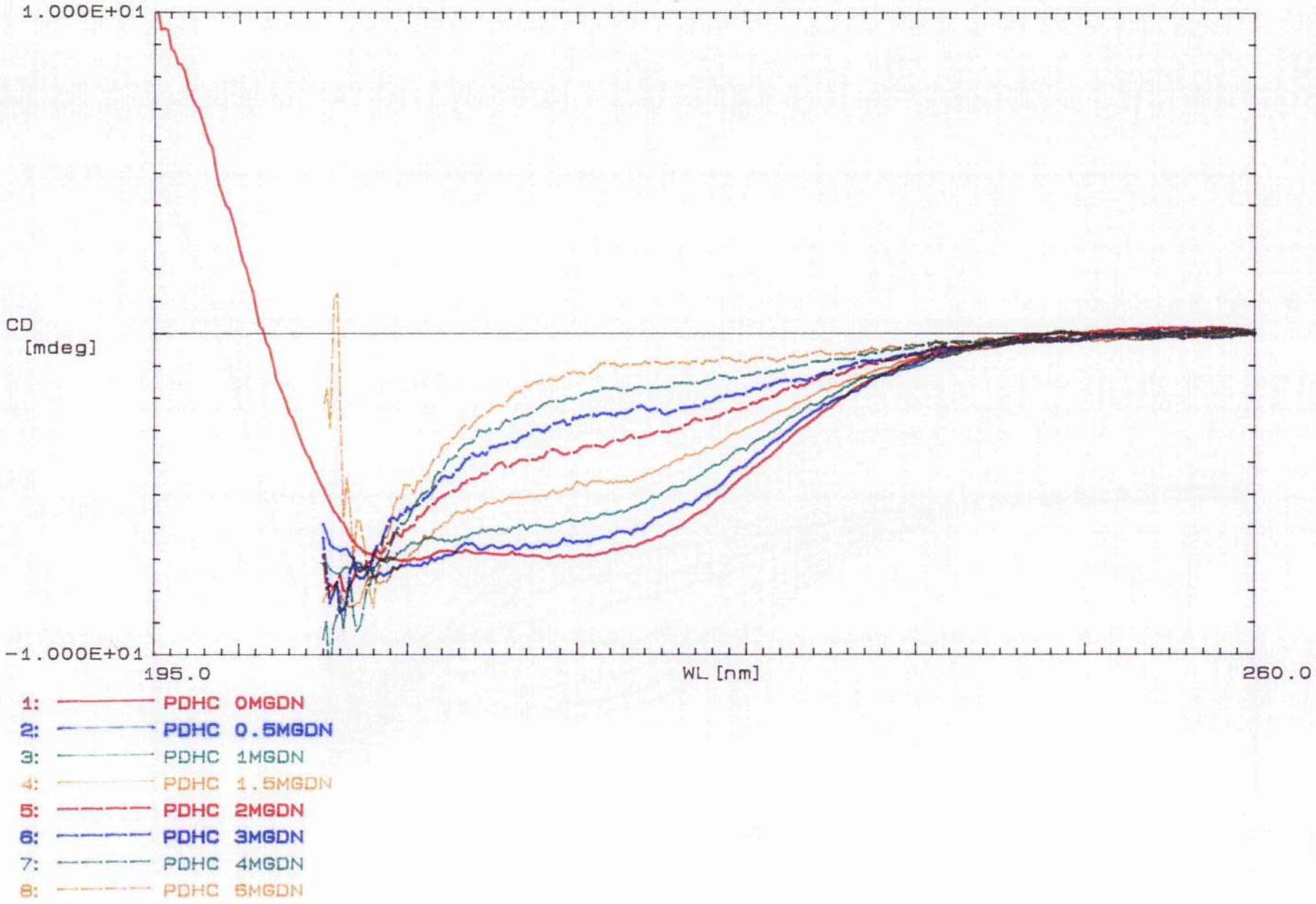
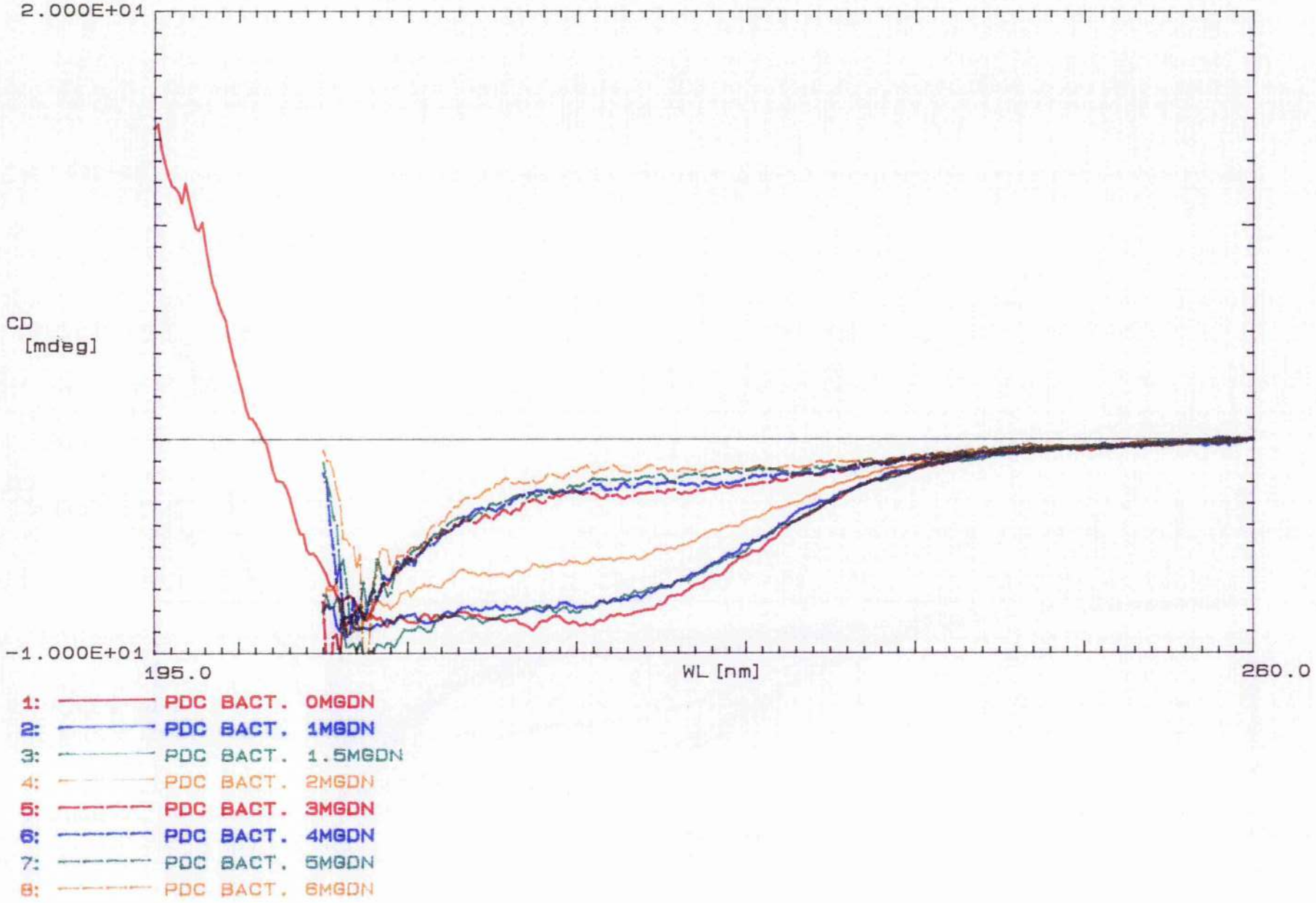

1.000E+01
CD
[mdeg]
-1.000E+01
195.0
WL [nm]
260.0
1: PDHC 0MGDN
2: PDHC 0.5MGDN
3: PDHC 1MGDN
4: PDHC 1.5MGDN
5: PDHC 2MGDN
6: PDHC 3MGDN
7: PDHC 4MGDN
8: PDHC 5MGDN

Figure 3.5C Changes in the far-UV CD spectra on denaturation of *E. coli* PDC with GdnHCl

Spectra were obtained in 50 mM potassium phosphate buffer, pH 7.6, at 20°C. Unfolding of PDC (80μg/ml) was carried out in the presence of GdnHCl for 15 min at 4°C. Spectra were recorded by Sharon Kelly in the laboratory of Prof. Nicholas Price at Stirling University.


2.000E+01
CD
[mdeg]
-1.000E+01
195.0
WL [nm]
260.0
1: PDC BACT. 0MGDN
2: PDC BACT. 1MGDN
3: PDC BACT. 1.5MGDN
4: PDC BACT. 2MGDN
5: PDC BACT. 3MGDN
6: PDC BACT. 4MGDN
7: PDC BACT. 5MGDN
8: PDC BACT. 6MGDN

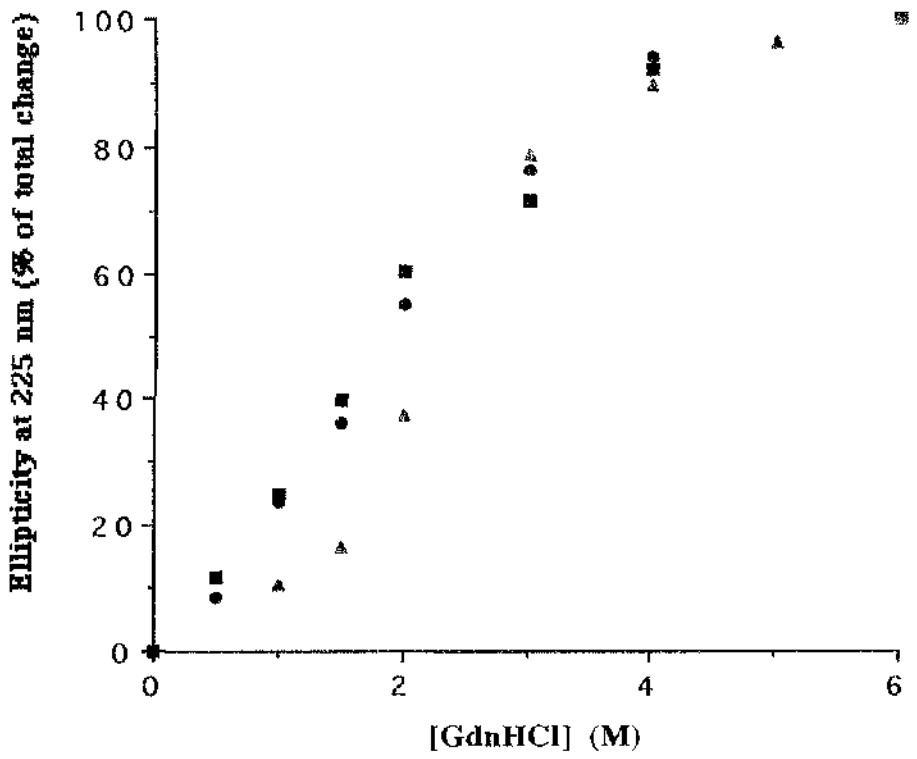


Figure 3.6 Comparing the GdnHCl induced unfolding of *E. coli* PDC, bovine heart PDC and OGDC using circular dichroism

The data shown in Figures 3.5A and 3.5B have been used to calculated the percentage of the total change occurring between 0 and 6M GdnHCl incubations. Ellipticity of *E. coli* PDC (▲), bovine heart PDC (■) and OGDC (●) were measured at 225nm.

3.8 Discussion

This chapter describes the purification and basic characterisation of *E. coli* PDC and bovine heart OGDC and PDC complexes. The *E. coli* PDC purification (detailed in Table 3.2 and Figure 3.3) provided a reproducible method for PDC overproduction with around 30% recovery. It has previously been shown that PDC purified using this method has an excess of E3 subunits and a reduced amount of E1 subunits and it is thought that hyper production of E3 may limit the extent of E1 binding (Russell *et al.*, 1992). As can be seen from Figure 3.3, PDC forms a major part of the original homogenate. Two of the contaminating polypeptides whose M_r are between those of the E1 and E3 subunits can be seen in Figure 3.3 in lanes 3 and 4. The upper band (lane 4) has been shown to be lysine decarboxylase. The lower band (lane 3) was first thought to be a proteolytic fragment of E1 because a tryptic fragment of this subunit migrates with approximately the same mobility (Russell and Guest, 1990). However, sequencing has shown that this lower band is identical to the chaperonin, GroEL, over 30 residues (Russell *et al.*, 1992). The presence of GroEL is interesting as it has been implicated in the folding of many proteins and is investigated further in Chapter 5. However, it is unclear whether its appearance here is due to the increased number of PDC molecules requiring folding assistance or stress that may be caused by the addition of IPTG.

The *E. coli* PDC unfolding and renaturation after GdnHCl incubation is shown in Figure 3.4. From this it can be seen that the complex activity is perturbed by relatively low levels of GdnHCl (in the range 0-0.3M). The ability to regain activity on dilution from GdnHCl is lost gradually after incubation in GdnHCl solutions between 0.9-2.0M. This shows that while PDC activity can be recovered after its total loss during GdnHCl incubation, refolding from a totally unfolded state is not possible under these conditions. Bovine OGDC and PDC both behave similarly to the *E. coli* PDC on incubation in GdnHCl, with activity being lost in the range 0-0.3M, again relatively low levels (Figure

3.2A). For bovine PDC it has been demonstrated that this loss of activity is due to the dissociation of the E1 and E3 components from the E2/X core (West *et al.*, 1995). As this dissociation occurs in full at 0.1M GdnHCl, it is clear that on dilution spontaneous reassociation of the subunits occurs. Both bovine complexes appear to be less resilient to dilution from GdnHCl incubation (Figure 3.2B). It is clear from Figure 3.6 that only a small percentage of the total secondary structural change has taken place after incubation of bovine OGDC and PDC in 1.0M GdnHCl. It appears that after around 15% of total secondary structure has been lost during GdnHCl incubation, activity can not be recovered on dilution. This loss of complex activity may be due to the misfolding of one of the subunits causing its lack of activity and thus inactivity of the whole complex. The unfolding and reactivation of the individual subunits of each of the complexes is investigated in detail in Chapter 4.

CHAPTER 4

INVESTIGATION OF THE COMPONENT ENZYMES

4.1 Introduction

Following the unfolding and reactivation of OGDC and PDC by monitoring the effects of GdnHCl on overall complex activity and CD and fluorescence properties gives an overview of the effects on the individual components. Since the individual folded chains might be expected to expose more hydrophobic side-chains prior to association, it seems reasonable to assume that a multi-subunit assembly is likely to lead to stabilisation of each subunit. Other features such as loops which might offer a suitable target for proteolysis *in vivo* may also be concealed during the association process adding to the increased stability. Although in some cases dissociation precedes unfolding, quantitative data on the increased stability afforded by association are in other cases difficult to obtain because conditions promoting dissociation can lead to partial unfolding of the subunits themselves, particularly where tightly associated subunits, such as the bovine OGDC E1 and E2 components, are concerned. Work by Chan and Mosbach (1976) is in general agreement with the assumption made above. This has shown that single matrix-bound subunits are more susceptible to denaturation than the same subunit assayed within the oligomeric complex, although the possibility that the matrix environment is complicating the interpretation of the results cannot be excluded. The 2-oxoacid dehydrogenase multienzyme complexes provide an interesting model to investigate this association stabilisation as the individual components can be assayed in the presence or absence of the rest of the complex.

In this chapter the stability of the individual components of the complexes are studied. The results for the component enzymes are compared between complexes. Isolation of E3 from various sources has been carried out to enable comparison of its deactivation and reactivation as free enzyme or in the intact complex. Results in this

chapter are obtained using assays of the individual components of the complex and by monitoring CD and fluorescence changes after incubation in the presence of denaturant.

4.2 Unfolding and reactivation of the E1 component

A clearer picture of the unfolding events which occur during whole complex inactivation can be obtained by monitoring the loss and regain of activity of the individual components of the complex upon treatment with denaturant and subsequent dilution. Figure 4.1 shows the effects of GdnHCl treatment where the activity of complex-bound E1 from bovine heart OGDC is monitored. It can be seen that 98% of E1 activity is lost after incubation and assaying in 1.0M GdnHCl. The ability to regain activity is progressively lost over the range 0.8-2.2M GdnHCl. The unfolding and reactivation of E1 isolated from bovine heart PDC is shown in Figure 4.2. It has been shown previously that there is no significant difference between unfolding and reactivation experiments performed on isolated and complex intact bovine PDC E1 (S. M. West, unpublished work). From Figure 4.2 it can be seen that all E1 activity is lost after incubation and assaying in 1.0M GdnHCl and that reactivation upon dilution of denaturant decreases progressively between 0.8 and 1.6M. Figure 4.3 shows similar data for PDC E1 from *E. coli*. E1 from this source appears to be marginally more sensitive to GdnHCl inactivation with no activity remaining after incubation and assaying in 0.75M GdnHCl. The ability to regain activity after incubation is progressively lost in this case over the range 0.6-2.1M GdnHCl.

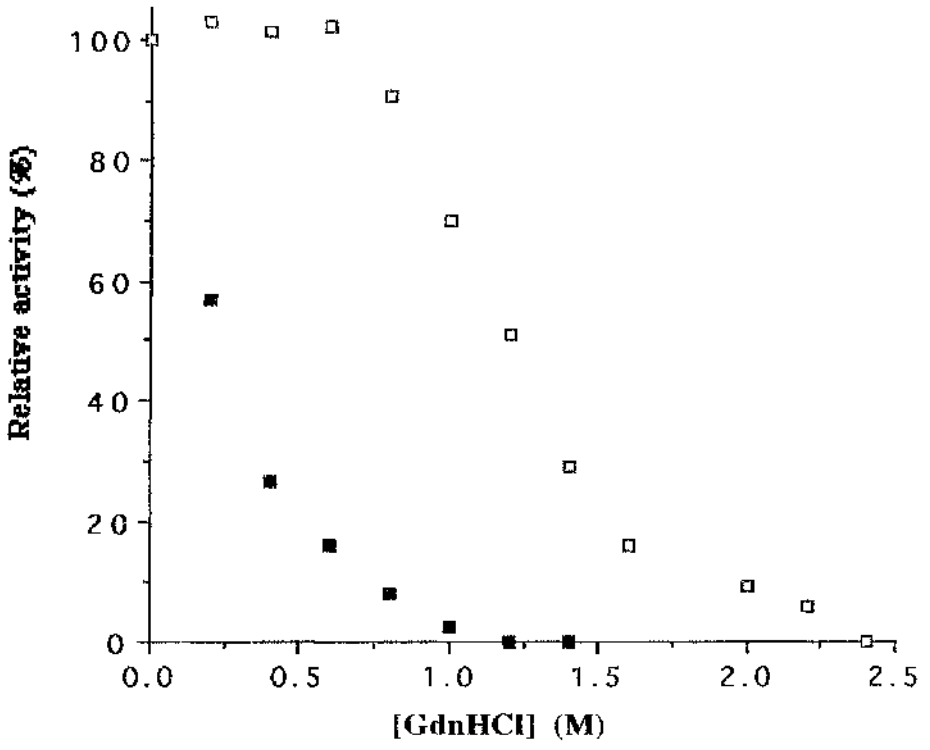


Figure 4.1 Deactivation and reactivation of bovine heart OGDC E1

Bovine heart OGDC (1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration, ■) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution, □) was assayed for E1 activity as outlined in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%.

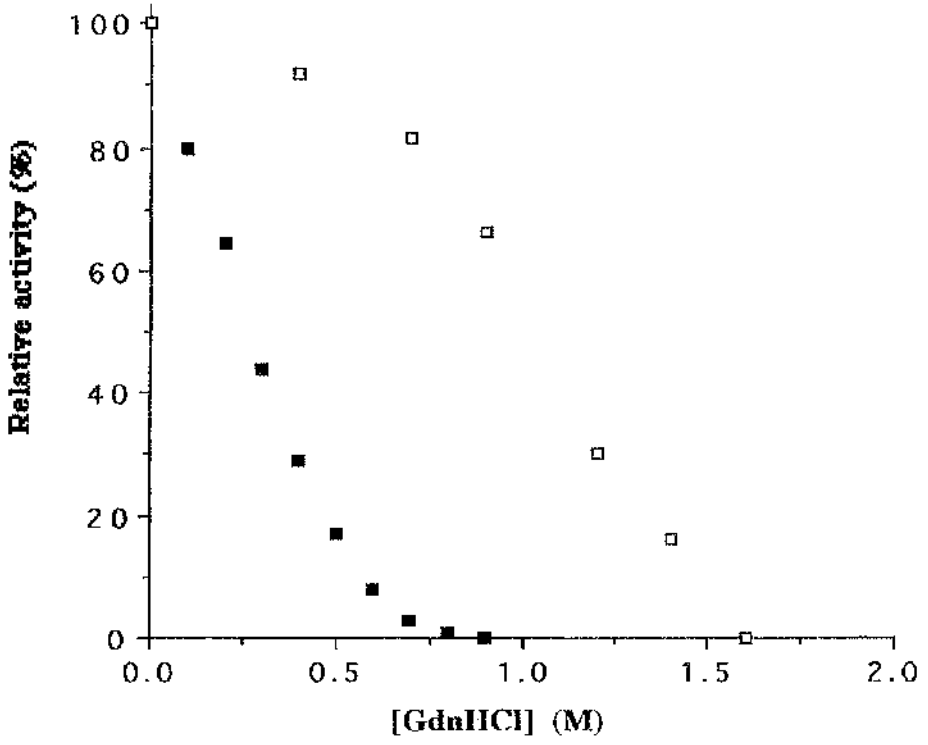


Figure 4.2 Deactivation and reactivation of bovine heart PDC E1

E1 was isolated from bovine heart PDC and was incubated at 1 mg/ml at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration, ■) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution, □) was assayed for E1 activity as outlined in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%. These data are in agreement with those of West *et al.* (1995).

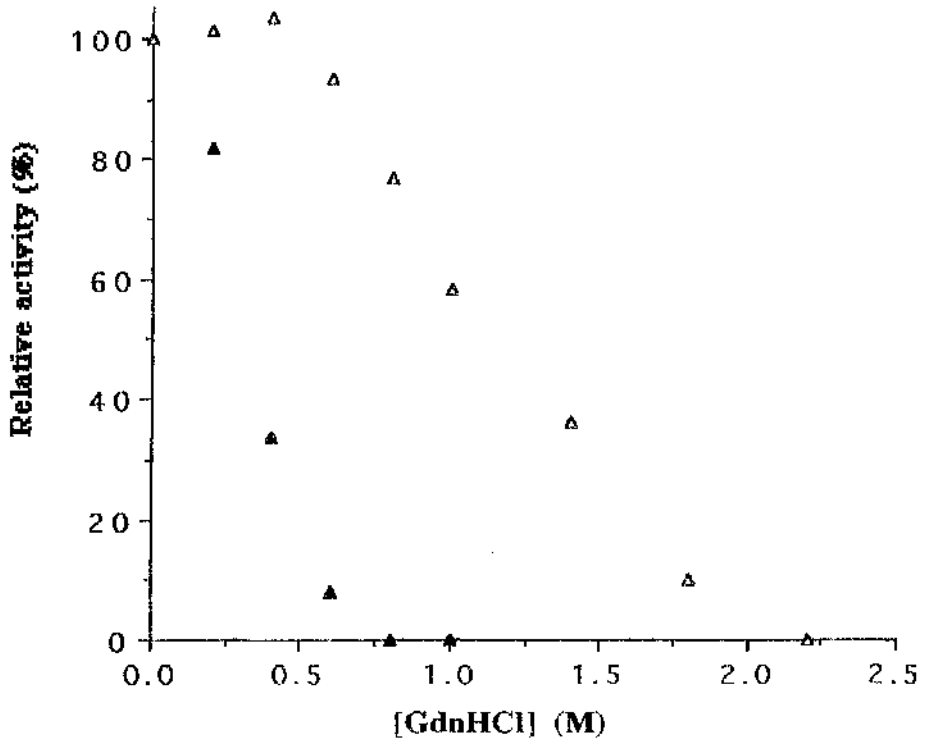


Figure 4.3 Deactivation and reactivation of *E. coli* PDC E1

E. coli PDC (1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration, ▲) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution, Δ) was assayed for E1 activity as outlined in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%.

4.3 Tryptic digestion of bovine OGDC

To investigate whether the other complex components are contributing to the stability of the bovine OGDC E1 component it was necessary to dissociate E1 from the E2 core and conduct similar unfolding and reactivation experiments to those shown in Figure 4.1. As the E1 and E2 components of OGDC are tightly associated and there is no published procedure to date to separate these components without denaturation, a proteolytic method was employed. Low levels of trypsin have been shown to promote a rapid, highly-selective cleavage of E1 as can be seen in Figure 4.4. This cleavage produces a large stable, 100 000 M_r fragment, designated E1', and a small peptide with an estimated M_r value of 10 000 which is not detected in the gel system used in Figure 4.4. Previous work has shown that the cleaved N-terminal region of bovine OGDC E1 exhibits significant sequence similarity with corresponding sequences from the lipoyl domains of the E2 and protein X components of eukaryotic PDCs, (Rice *et al.*, 1992). This region of bovine OGDC E1 has been found to be highly immunogenic as it induces the majority of the antibody response to intact E1. Rice *et al.* (1992) have also observed that tryptic digestion of bovine OGDC causes loss of overall OGDC activity due to the dissociation of the E1' and E3 components, although the E1' fragment retains full catalytic activity.

4.4 Unfolding and reactivation of E1'

Figure 4.5 compares the unfolding and reactivation of E1' to E1 assayed in the intact complex. From these data it can be seen that the tryptic digestion of E1 does not affect the loss or regain of E1 activity. Both the undigested and digested E1 show a complete loss of activity after incubation and assaying in 1.2M GdnHCl at which concentration 50% of activity can be recovered on dilution of the denaturant. As in the

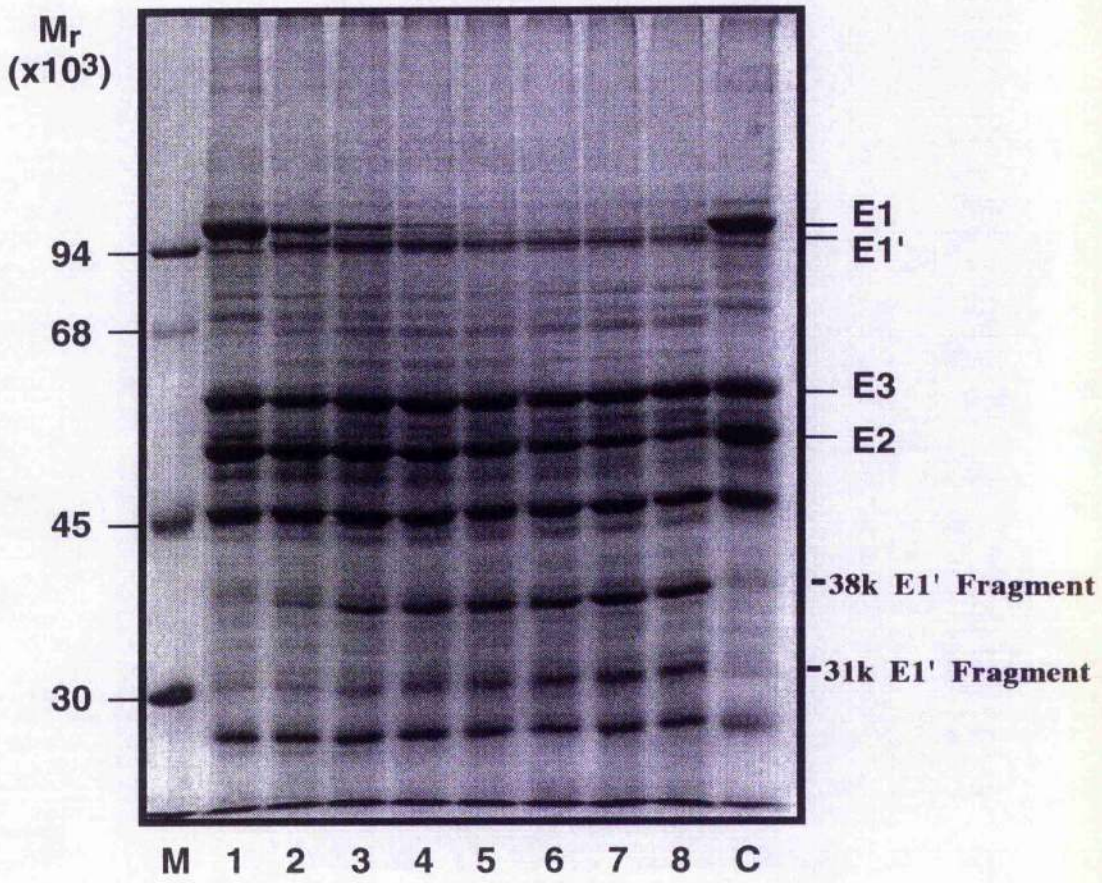
intact E1, ability to recover activity reduces progressively over the range 0.8-2.2M GdnHCl.

4.5 Investigating the effect of calcium on E1 and E1' of bovine heart OGDC

To investigate whether the tryptic digestion of E1 to E1' affects the calcium requirements of the component, calcium was removed from the complex by dialysis against 3 x 100 volumes of 20 mM TrisHCl, pH 7.0 containing the calcium chelator EGTA at 0.1 mM. This procedure removed the calcium from the solution and no changes were observed when dialysis was carried out with 6 x 100 volumes of buffer over 6 days, although it is possible that tightly associated calcium ions remained with the complex. The dialysed OGDC was then selectively digested with trypsin as described in section 4.3. The digestion was found to proceed more slowly than that carried out with calcium present and trypsin levels were increased to 0.15% (w/w) to avoid incubating the complex at 30°C for longer than was necessary to minimise possible structural damage to other constituent enzymes. The results displayed in Figure 4.6 were carried out on intact or digested OGDC at a concentration of 1 mg/ml. In the digested complex the E1' and E3 components have been shown previously to dissociate from the complex while the smaller 10 000 M_r N-terminal fragment yielded by the tryptic digestion remains tightly bound to the core (Rice *et al*, 1992). From Figure 4.6 it can be seen that there is a distinct difference in K_m from 4.4×10^{-5} to 1×10^{-3} M when E1 activity is assayed in the presence and absence of calcium respectively. For E1' the change in K_m is similarly distinct, from 8×10^{-5} to 1×10^{-3} M when assayed in the presence and absence of calcium respectively.

Figure 4.4 Selective digestion of OGDC E1 with trypsin

Bovine OGDC was diluted to a final concentration of 1 mg/ml with 50mM potassium phosphate buffer, pH 7.6 containing 0.2 mM ThDP, 3 mM NAD^+ and 1 mM $MgCl_2$. Digestion of OGDC was initiated at 30°C by adding 0.1% (w/w) trypsin. Aliquots (20 μg)were withdrawn at the times indicated below, mixed with an equal volume of Laemmli sample buffer and resolved on a 10% (w/v) SDS-polyacrylamide gel. Lane M, low M_r standards (10 μg), lanes 1-8, OGDC digested with trypsin for 0, 1, 3, 5, 7, 10, 20 and 40 min, and lane C control OGDC incubated for 40 min in the absence of trypsin.


Mr
(x10³)
94
68
45
30
E1
E1'
E3
E2
38k E1' Fragment
31k E1' Fragment
M 1 2 3 4 5 6 7 8 C

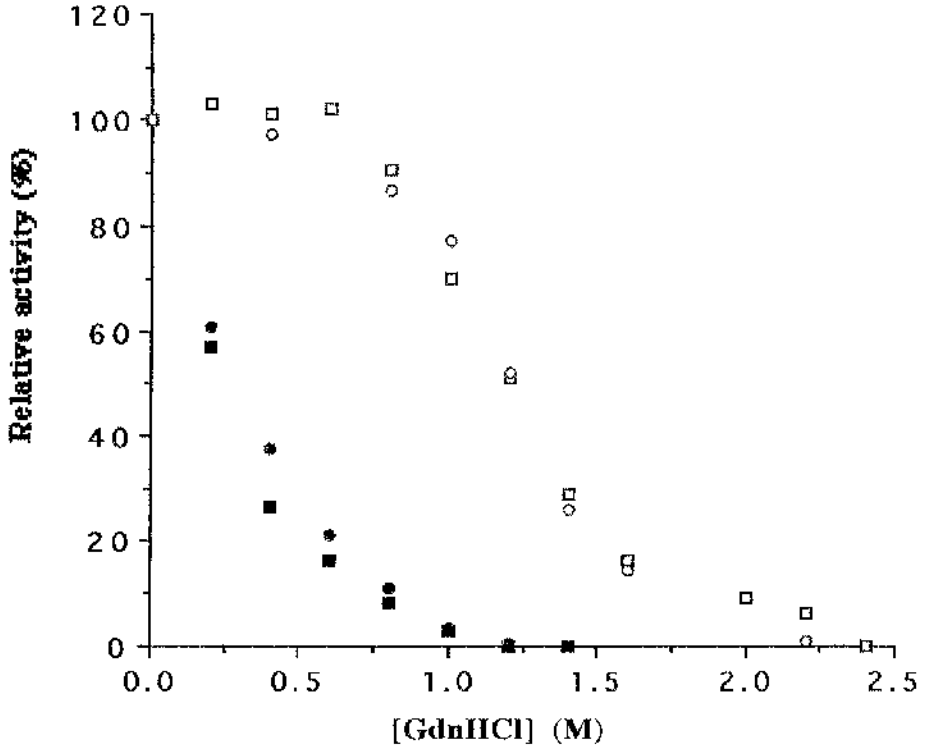


Figure 4.5 Comparing the deactivation and reactivation of bovine heart OGDC E1 and E1'

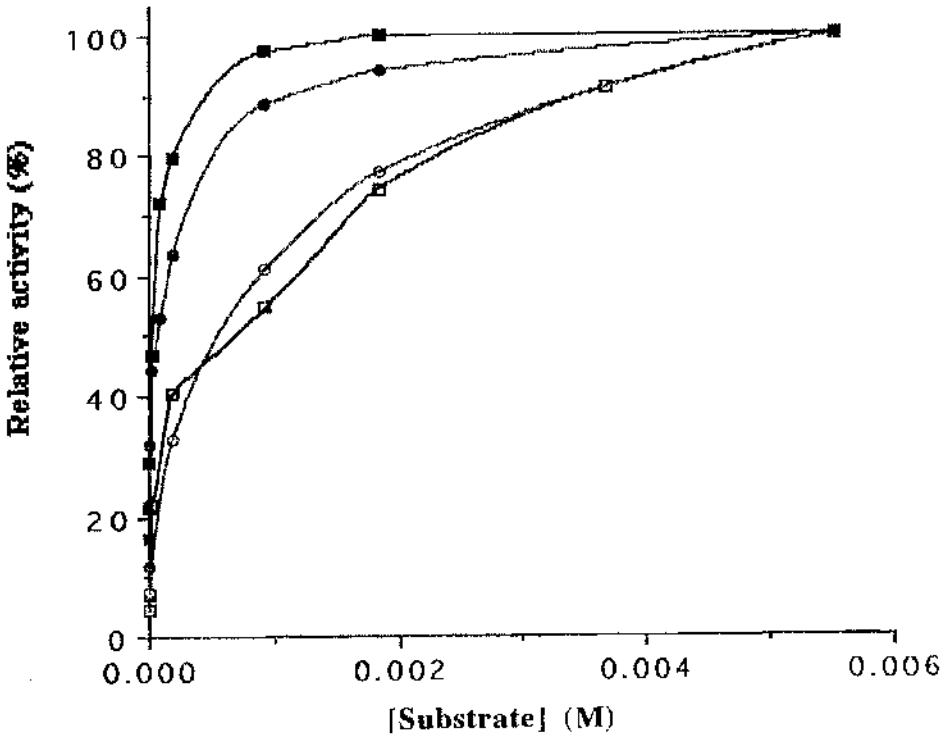
Intact or digested OGDC (1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution) was assayed for E1 activity as outlined in Materials and Methods. Intact E1 is represented by the symbols ■ and □ for inhibition and reactivation respectively. E1' is represented by the symbols ● and ○ for inhibition and reactivation respectively. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%.

4.6 Comparing unfolding and reactivation of E2 from bovine heart and *E. coli*

Figure 4.7 compares the unfolding and reactivation of E2 from bovine heart PDC and *E. coli* PDC after incubation for 15 min with increasing concentrations of GdnHCl. The activity of the *E. coli* E2 was measured as described in Materials and Methods. The bovine heart PDC E2 data are taken from West *et al.* (1995) and activity was found to be the same when measured by two independent methods. The first is a. spectrophotometrical determination comprising a coupled assay employing phosphotransacetylase to generate acetyl-CoA from acetyl phosphate and CoA as a substrate for the E2-driven acetylation of free dihydrolipoamide to the S-acetyl derivative. This was detected in a colorimetric assay as the ferric acetohydroxamate complex. The second is the isotopic conversion of [1-^{14}C]acetyl-CoA to the radio labelled S-acetyl dihydrolipoamide intermediate which was then extracted into toluene as in the method used to measure E2 activity in *E. coli* PDC. While the data shown in Figure 4.7 compare isolated bovine PDC E2 to complex intact *E coli* PDC E2, it has been shown previously that in the bovine heart PDC the E2 has an identical response to GdnHCl-induced unfolding and reactivation whether it is within the complex or isolated (West, unpublished work). From Figure 4.7 it can be seen that E2 from these two sources has a similar response to GdnHCl incubation. In both cases inactivation of E2 is complete after incubation and assaying in 1.0M GdnHCl. Reactivation of E2 is 100% after incubation in 1.0M GdnHCl but gradually decreases as the concentration of GdnHCl increases with 50% recovery occurring after 3.0M GdnHCl incubation in both cases. From the data it would appear that the bovine heart PDC E2 is marginally more sensitive to incubation with 3.0-5.0M GdnHCl.

Figure 4.6A Comparing the effect of calcium ions on the activity of intact E1 and E1'

Calcium was first removed from the complex by dialysing against 3 x 100 volumes of 20 mM TrisHCl, pH 7.0 containing 0.1 mM EGTA. Digestion was then carried out as described in Figure 4.4 while monitoring both whole complex and E1 activity as outlined in Materials and Methods. Whole or digested OGDC (both 1 mg/ml) were then assayed for E1 activity without calcium present in solution A containing 1 mM EGTA and with calcium present by adding a 5-fold molar excess of $CaCl_2$ to the solution A. The concentration of 2-oxoglutarate was varied as shown in the figure. E1 results are represented by ■ (with calcium)and □ (without calcium). E1' results are represented by ● (with calcium) and ○ (without calcium). The results shown are averages taken from 2 different preparations differing by no more than plus or minus 7%.


Relative activity (%)
100
80
60
40
20
0
0.000
0.002
0.004
0.006
[Substrate] (M)

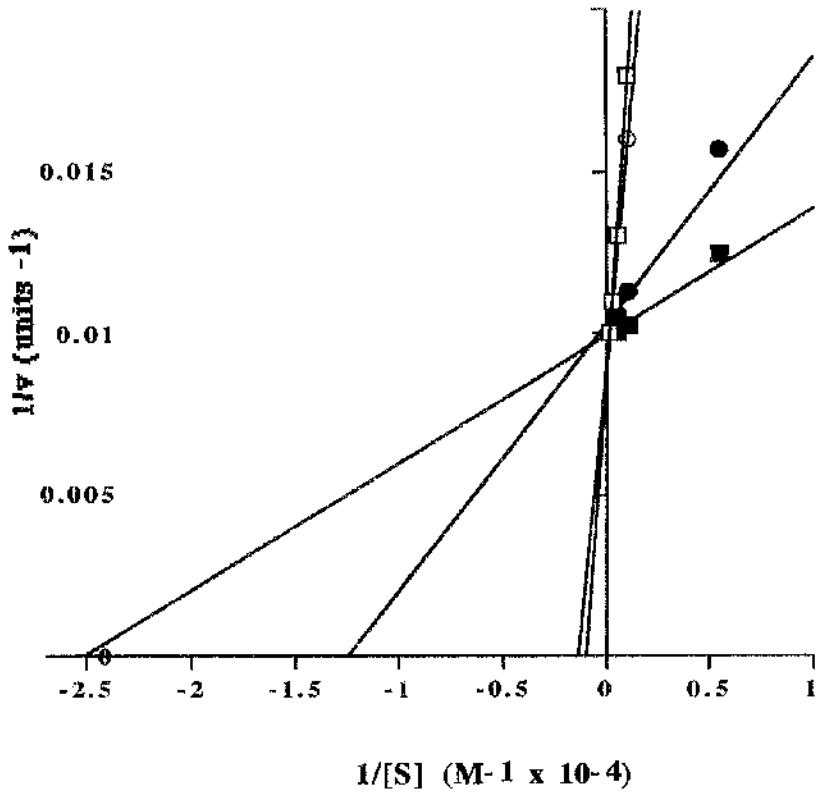


Figure 4.6B Lineweaver-Burk plot of E1 and E1' assayed in the presence and absence of calcium

These data are those represented in Figure 4.6A and the previous legend reports the procedures employed to obtain them. E1 results are represented by ■ (with calcium) and □ (without calcium). E1' results are represented by ● (with calcium) and ○ (without calcium).

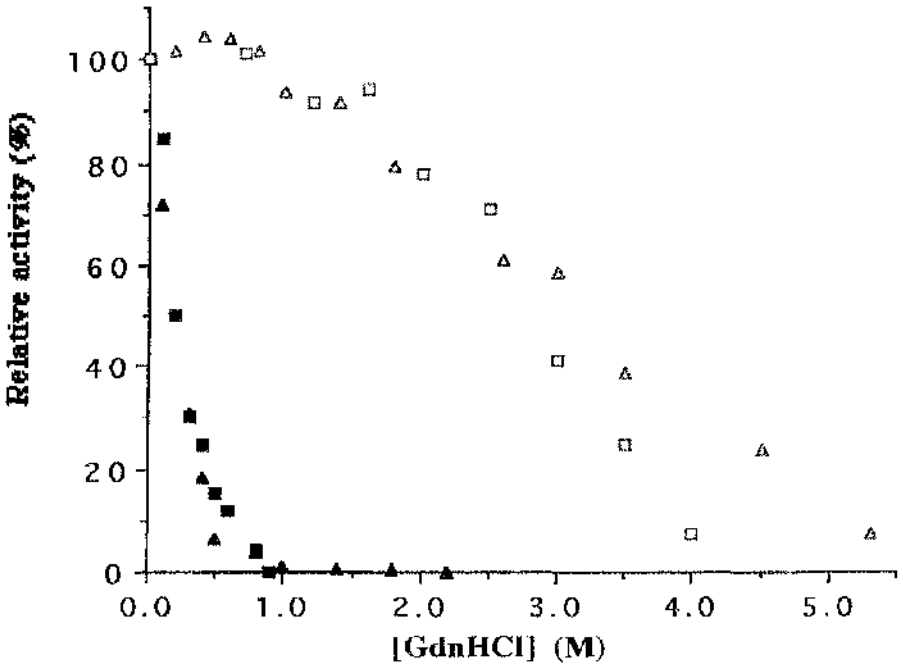


Figure 4.7 Comparing the deactivation and reactivation of bovine heart and E. *coli* PDC E2

E. coli PDC (1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration, ▲) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution, Δ) of E2 was assayed isotopically via the conversion of [1-C^{14}] acetyl-CoA to the radiolabelled S-acetyl dihydrolipoamide intermediate as outlined fully in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 2 readings taken from different preparations. The extent of inhibition (■) and reactivation (□) of isolated bovine heart PDC E2 results are taken from West *et al.* (1995).

4.7 Deactivation and reactivation of the E3 component

Dihydrolipoamide dehydrogenase (E3) is the common component of the three 2-oxoacid dehydrogenase complexes oxidising pyruvate, 2-oxoglutarate and the branched-chain 2-oxoacids. Investigating the E3 component is particularly interesting because of the high degree of sequence homology observed between species for this component. Figure 4.8 shows the unfolding and reactivation of bovine heart and *E. coli* PDC E3. The assays have been performed in the presence of the whole complex. From this figure, it can be seen that both E3s behave similarly when incubated and assayed in GdnHCl. Inactivation proceeds gradually over the range of 0-1.2M GdnHCl with 97% of activity lost after incubation and assaying in 1.0M GdnHCl. Figure 4.8 also shows, however, that there is a striking difference in the recovery of activity after GdnHCl incubation of the E3 from these two sources. For the bovine heart E3, recovery of activity decreases progressively when the complex is incubated in 1.4-2.8M GdnHCl, with 50% of activity lost after incubation with 1.8M GdnHCl. The recovery of E3 activity of *E. coli* PDC decreases progressively after incubation in 3.1-4.5M GdnHCl, with 50% of activity lost after incubation with 4.0M GdnHCl.

4.8 Isolation of the E3 component

To investigate the possible stabilising effect of the presence of the rest of the complex and to enable CD and fluorescence data to be collected, E3 was isolated from bovine heart and *E. coli* PDC. For the bovine heart complex, isolation was carried out as described in Materials and Methods. This method of heat treatment was adapted to isolate E3 from *E. coli* PDC. During purification of the complex it was found that a

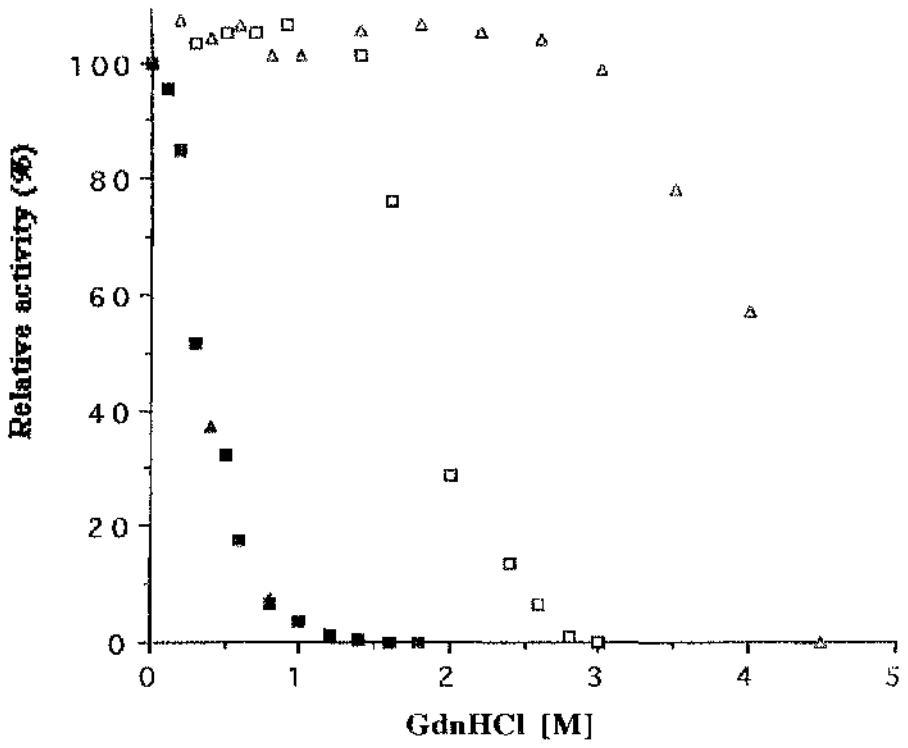


Figure 4.8 Deactivation and reactivation of bovine heart and *E. coli* PDC E3

Bovine heart OGDC or *E. coli* PDC (both 1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution) was assayed for E3 activity as outlined in Materials and Methods. Inhibition results are represented by ■ and ▲ for bovine heart and *E. coli* E3 respectively. Reactivation results are represented by □ and Δ for bovine heart and *E. coli* E3 respectively. In each case, the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%.

considerable amount (ranging from 28-40%) of E3 activity remained in the supernatant of the final ultracentrifugation step. Figure 4.9 shows heat treatments carried out on this supernatant at 65-85°C and from this it can be seen that at 65°C the E3 still retains 100% of its activity after a 30 min incubation. Increasing the temperature above this gradually increased the rate of activity loss of E3. Figure 4.10 shows the SDS-PAGE analysis of samples taken during the 65°C heat treatment. Using both the E3 activity assays and SDS-PAGE analysis, an incubation of 20-30 min at 65°C was chosen to isolate the E3 component from the supernatant although the treatment varied slightly between preparations and was monitored closely each time it was performed.

4.9 Comparing the unfolding and reactivation of E3 within the complex to isolated E3

Figure 4.11 compares the unfolding and reactivation of isolated and complex bound bovine heart E3. In both cases it can be seen that when incubated and assayed with GdnHCl E3 activity is progressively lost between 0 and 1.2M GdnHCl with 97% of activity lost after incubation in 1.0 M GdnHCl. The recovery of activity after GdnHCl incubation is also very similar in both the isolated and complex intact data. Full activity is recovered on dilution after incubation in 1.4M GdnHCl. As the denaturant concentration is increased to 2.8M GdnHCl recovery progressively decreases to 0% in both cases. Figure 4.12 compares the unfolding and reactivation of *E. coli* E3 in the presence of the rest of the PDC complex to isolated E3. From this figure it is clear that there is a striking difference in the recovery of E3 activity on dilution after GdnHCl incubation between the two situations. When E3 recovery is assayed in the presence of the complex, recovery is 100% when incubation is at 3.0M GdnHCl and decreases to 0% after incubation in 4.5M GdnHCl. Isolated *E. coli* PDC E3 loses the ability to recover after GdnHCl incubation in the range 1.4-2.8M, a concentration at which the E3 assayed

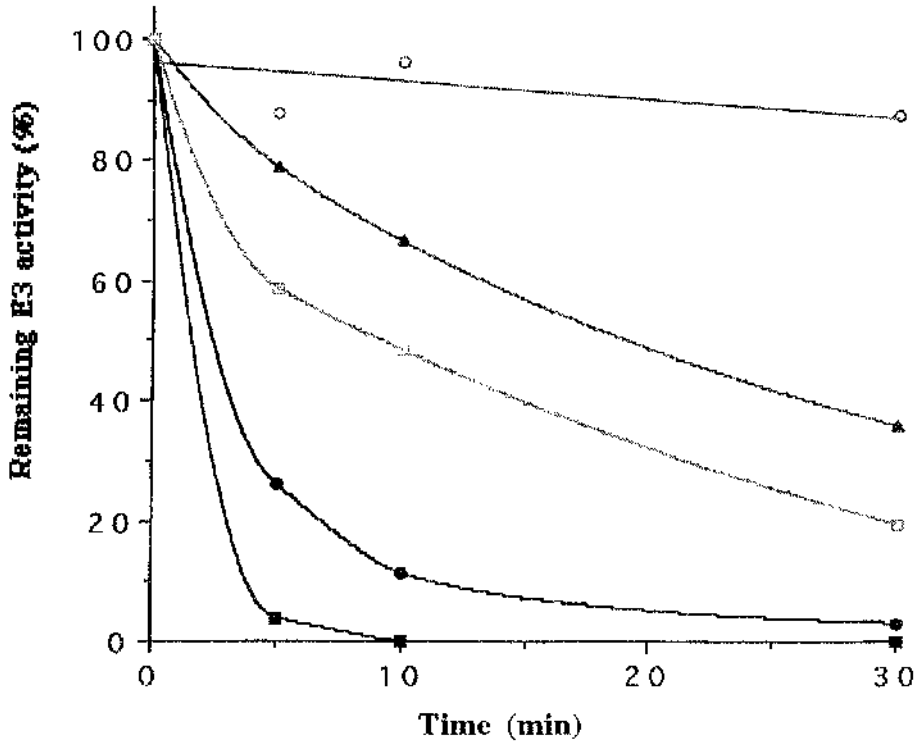
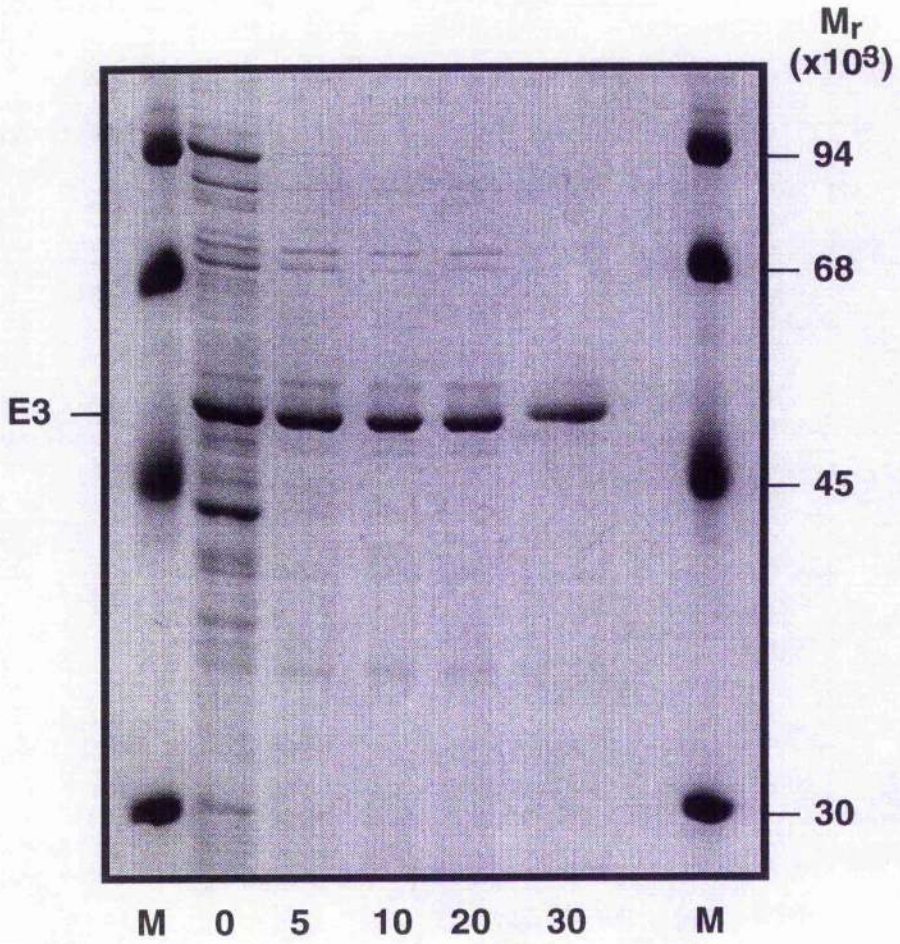


Figure 4.9 Monitoring the stability of the *E. coli* PDC E3 component during heat treatment

Bacterial PDC (2 mg/ml) was pre-incubated in 50mM potassium phosphate buffer, pH 7.6, containing 2.0M NaCl for 15 min on ice before heat treatment began. After the heat treatment at the stated temperature for the stated length of time, the sample was removed from the water bath and spun for 10 min at 15000g. The supernatant was then assayed for E3 activity as described in Materials and Methods. Heat treatments were carried out at 65°C (○), 70°C (▲), 75°C (□), 80°C (●) and 85°C(■).

Figure 4.10 SDS-PAGE analysis of samples taken during the 65°C heat treatment of *E. coli* PDC E3

The supernatant from the final high speed centrifugation step of the *E. coli* PDC preparation was incubated in 50mM potassium phosphate buffer, pH 7.6, containing 2.0M NaCl for 15 min on ice before the 65°C heat treatment began. At the times stated 10μg aliquots were withdrawn and mixed with an equal volume of Laemmli sample buffer and resolved on a 10% (w/v) SDS-polyacrylamide gel. Both lanes labelled M are low M_r standards (10μg). Lanes 1-5 are supernatant containing E3 treated for 0, 5, 10, 20 and 30 min.


Mr
(x10³)
94
68
E3
45
30
M
0
5
10
20
30
M

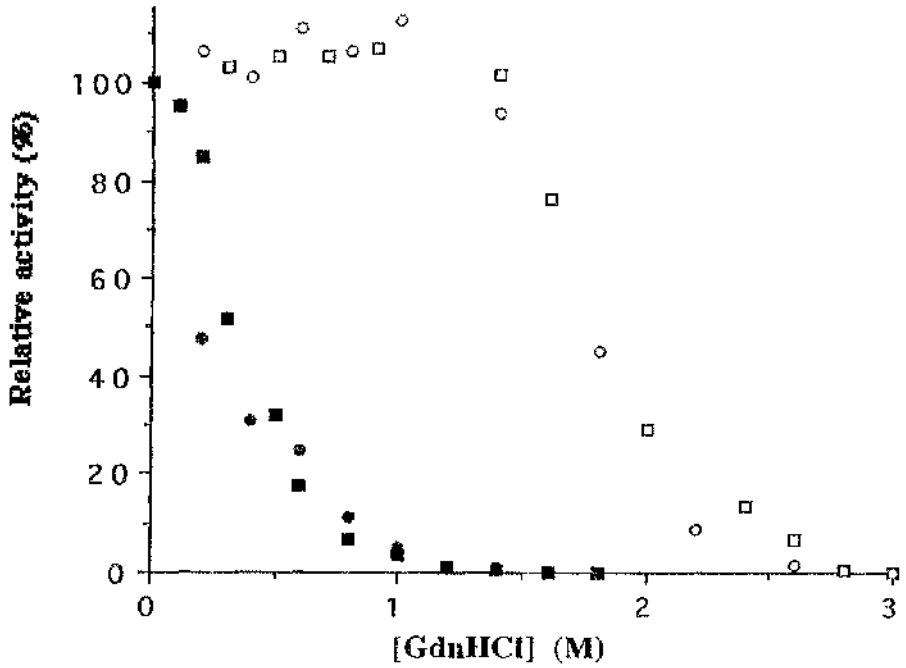


Figure 4.11 Comparing the deactivation and reactivation of isolated and complex bound bovine heart E3.

Bovine PDC (1 mg/ml) or purified bovine E3 (0.1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration, complex and purified E3 are represented by ■ and ● respectively) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution, complex and purified E3 are represented by □ and ○ respectively) was assayed for E3 activity as outlined in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of two readings taken from different preparations differing by no more than plus or minus 5%.

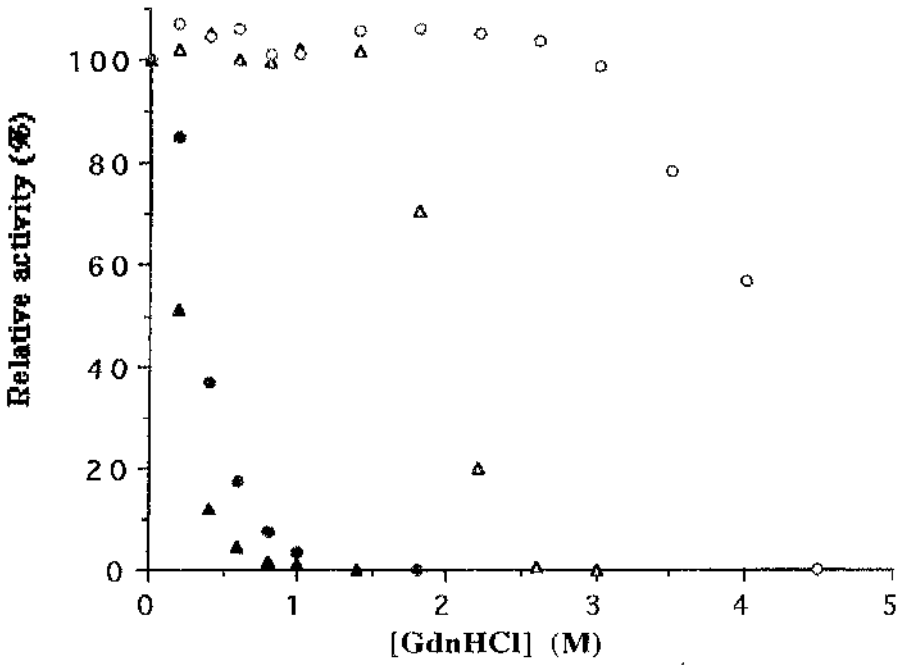


Figure 4.12 Comparing the deactivation and reactivation of isolated and complex bound *E. coli* E3.

E. coli PDC (1 mg/ml) or purified *E. coli* E3 (0.1 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (with assay mixture containing the same GdnHCl concentration) or reactivation (GdnHCl concentration reduced to below 0.03M by dilution) was assayed for E3 activity as outlined in Materials and Methods. Inhibition results are represented by ● and ▲ for complex and purified E3 respectively. Reactivation results are represented by ○ and Δ for complex and purified E3 respectively. In each case the changes are expressed relative to control sample from which GdnHCl was omitted. Results shown are averages of 3 readings taken from different preparations differing by no more than plus or minus 5%.

with the complex still recovers 100% of activity on dilution. The presence of the E2 component has also caused a minor decrease in susceptibility to GdnHCl unfolding of E3. From Figure 4.12, it can be seen that 50% of E3 activity is lost after incubation and assaying in 0.2 and 0.3M GdnHCl for isolated and complex associated E3 respectively.

4.10 Comparing the unfolding and reactivation of E3 isolated from 3 different sources

Figure 4.13 shows the results of GdnHCl induced unfolding and subsequent reactivation of E3 isolated from bovine heart, yeast and *E. coli*. E3 from these 3 sources can be seen to behave in a similar way when incubated and assayed in GdnHCl, (A). Activity is lost over the range 0-1.2M GdnHCl with 50% of activity lost at around 0.2M GdnHCl. The ability to regain activity after dilution from GdnHCl incubation is also lost over the same range for the different E3s, (B), this range being 1.0-2.2M GdnHCl, with 50% of activity regained at 1.8, 1.7 and 1.5M GdnHCl for *E. coli*, bovine heart and yeast E3 respectively.

4.11 Monitoring changes in fluorescence and CD properties on GdnHCl induced unfolding of E3 from 3 different sources

The unfolding of the isolated E3 components by GdnHCl was also monitored by changes in protein fluorescence and far-UV CD. The results are showing Figures 4.14 and 4.15, in terms of the changes in these parameters relative to the total changes occurring between 0 and 6M GdnHCl although in both cases very little change at all was detected between 4 and 6M GdnHCl. In each case the fluorescence emission maximum and the size of the ellipticity at 225nm indicated that E3 was completely unfolded in 6M GdnHCl. For the 3 E3s the changes in CD (reflecting the loss of secondary structure)

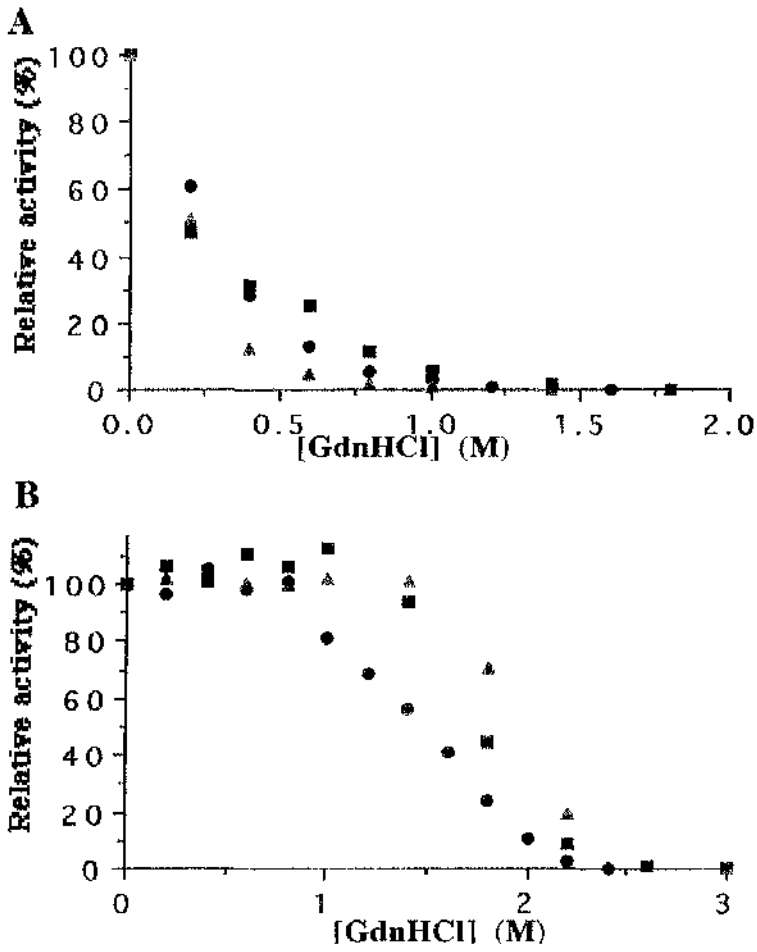


Figure 4.13 Deactivation (A) and reactivation (B) of purified E3 from yeast, *E. coli* and bovine sources.

E. coli E3, ▲, (0.1 mg/ml), bovine E3, ■, (0.1 mg/ml) or yeast E3, ●, (0.2 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min before the extent of inhibition (shown in **A** where assay mixture contained the same GdnHCl concentration as incubation) or reactivation (shown in **B** where GdnHCl concentration has been reduced to below 0.03M by dilution) was assayed for E3 activity as outlined in Materials and Methods. In each case the changes are expressed relative to control sample from which GdnHCl was omitted.

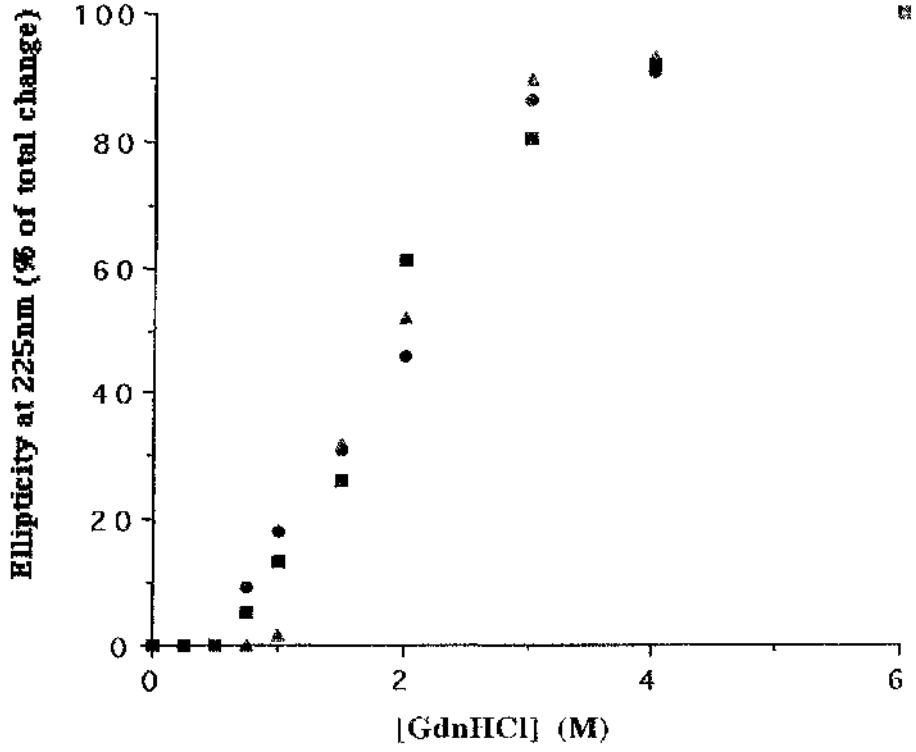


Figure 4.14 Unfolding E3 from bovine heart, *E. coli* and yeast PDC as monitored by changes in circular dichroism

E. coli E3, ▲, (0.1 mg/ml), bovine E3, ■, (0.1 mg/ml) or yeast E3, ●, (0.17 mg/ml) was incubated at 4°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min prior to the changes in ellipticity at 225nm being recorded as outlined in the Materials and Methods section.

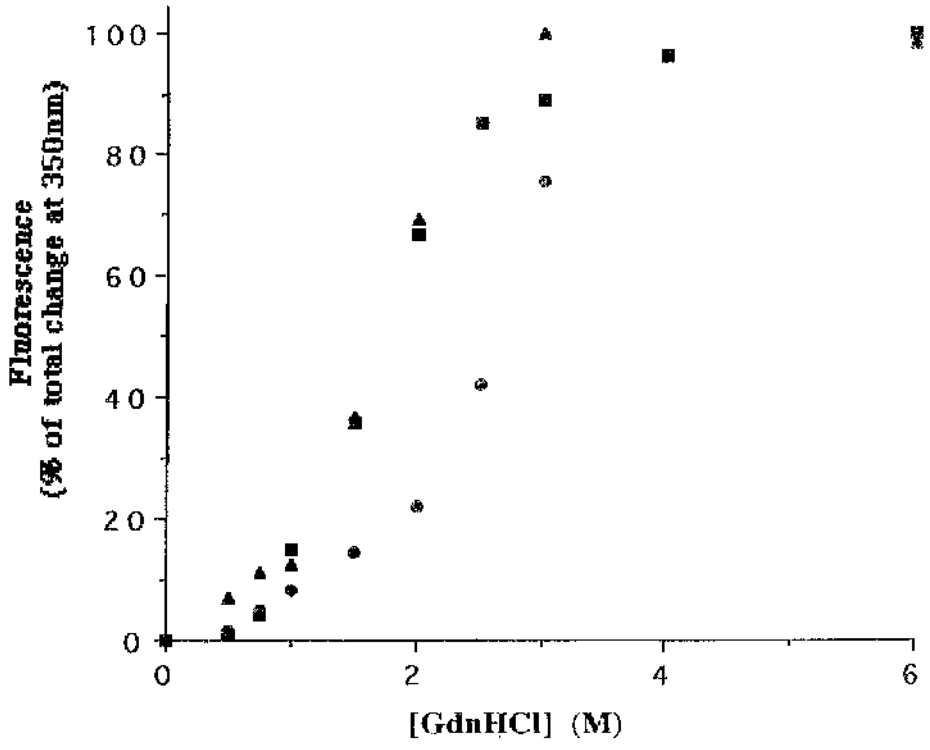


Figure 4.15 Unfolding E3 from bovine heart, *E. coli* and yeast PDC as monitored by fluorescence properties

E3 was incubated at 20°C in 50mM potassium phosphate, pH 7.6 containing GdnHCl for 15 min prior to measurement. In each case the changes are expressed relative to the total change observed between 0 and 6M GdnHCl. The excitation wavelength in each case was 290 nm and the emission wavelength was 350 nm. The concentrations of bovine heart (■), *E. coli* (▲), and yeast (●) E3 were each 80μg/ml.

and fluorescence (reflecting the loss of tertiary structure as reported by the exposure of internally located tryptophan residues to the external solvent) occur over comparable ranges of GdnHCl incubation concentration (1-3 M). From Figure 4.15 it can be seen that the yeast E3 has increased stability to tertiary structural changes measured in this way with 50% of the total change occurring at 2.6M compared to 1.8M for both *E. coli* and bovine heart E3.

4.12 Discussion

The results described in this chapter shed light on the properties of the component enzymes of PDC and OGDC from bovine heart, *E. coli* and *S. cerevisiae*. Comparing the data for individual component assays to those for the whole complex shown in Chapter 3 (Figures 3.2 and 3.4), it is clear that loss of complex activity on GdnHCl incubation occurs at lower GdnHCl concentrations than the loss of activity of the most sensitive component enzyme. The E1 component catalyses the only irreversible reaction in the multistep activity of the 2-oxoacid dehydrogenases, the initial decarboxylation of the 2-oxoacid, which is the rate limiting step of the overall reaction (Walsh *et al.*, 1976). While a common ThDP binding motif has been identified on all known E1 components (Hawkins *et al.*, 1989), sequences available for E1 of PDC and OGDC show little overall homology even when extracted from the same source (Darlison *et al.*, 1984). The quaternary structure of the E1 component is another difference between sources. The E1 components of bacterial and mammalian OGDC and Gram negative bacterial PDC are homodimers (Koike and Koike, 1976). In contrast, the E1 component from eukaryotic and Gram positive bacterial PDC is composed of two non-identical subunits , α and β, which form $\alpha\ 2\beta 2$ tetramers (Reed *et al.*, 1985). Despite this the 3 E1 components investigated in this chapter show very similar unfolding and reactivation curves shown in Figures 4.1, 4.2 and 4.3. To investigate the

possible contribution of the complex core to the stability of the E1 component, the protease trypsin was used to cleave bovine heart OGDC E1 into 2 stable fragments. The smaller M_r 10 000 fragment has been found to have significant similarities with equivalent lipoyl domain regions of protein X and E2 sequences of bovine heart PDC. The larger M_r 100 000 fragment, E1', is released from the core on cleavage, as is the E3 component (Rice *et al.*, 1992). An observation worthy of further investigation is noted from Figure 4.4. The gradual appearance of two stable bands during the tryptic digestion of approx. 38 000 Da and 31 000 Da occurs concurrently with the disappearance of the E1' fragment. This limited digestion of E1' apparently does not affect the E1 activity as this was routinely between 90 and 95% after a 10 min tryptic digestion, and at this time on Figure 4.4 it can be seen that the intensity of the E1' band has reduced to around half that of the 3 min sample. Figure 4.5 shows that E1' (or the E1' fragments) behaves identically to the undigested E1 component when unfolding and reactivation are assayed after GdnHCl incubation. The smaller product of the trypsin cleavage of bovine heart OGDC has previously been found to be highly immunogenic and as yet the reason for this characteristic is uncertain. Figures 4.6A and 4.6B show data investigating whether this area of the protein may be involved in calcium control of the complex. Figure 4.6B shows that the K_m for E1 rises from 4.4×10^{-5} to 1×10^{-3}M when calcium is removed. For E1' a similar rise of 8×10^{-5} to 1×10^{-3}M is observed. These values indicate that calcium plays an important role in complex regulation as physiological levels of calcium are generally in the range 10-100μM. However, it is unlikely, given these results, that the small fragment cleaved from E1 during trypsin digestion is involved in this regulation.

It has previously been shown that for bovine heart PDC, E2 loses the ability to regain activity at substantially higher initial GdnHCl concentrations (2-4M) than the E1 and E3 components (West *et al.*, 1995). Figure 4.7 compares these data with similar measurements collected for *E. coli* E2 and here it can be seen that the core component is

found to recover activity after GdnHCl incubations over the range 2-5M with 50% of activity being recovered after a 3.0M GdnHCl incubation. As the E2 components form the structural core of the complexes, it is possible that this ability to regain activity from higher GdnHCl concentrations *in vitro*, may reflect a physiological role of the E2 component, that of providing a framework which helps the other components to attain their native structure. To investigate whether there is a difference between reactivation results from isolated and complex associated components, purification of E3 from bovine heart and *E. coli* PDC were carried out. Figure 4.11 confirms that for bovine heart E3 there is no significant difference in reactivation between the isolated and complex associated sates. This is to be expected as it has previously been shown that the E3 component is completely dissociated from the bovine heart PDC complex after incubation in 0.1M GdnHCl (West *et al.*, 1995). The method of purification of the *E. coli* PDC complex, in particular the Resource Q column step, indicates that the E3 component in this complex is more tightly bound to the central core. Figure 4.12 shows the striking difference found between the isolated and complex associated forms of E3 in the *E. coli* PDC complex. Recently it has been shown that the binding to E2 occurs across the E3 dimer interface (Mande *et al.*, 1996b). The structure of E3 from *B. stearothermophilus* present within the complex shows very little deviation from that of uncomplexed E3. Similarly, the E3 binding domain on E2 is almost structurally identical when either free or complexed with E3 (Mande *et al.*, 1996b). As both components appear to undergo little overall conformational change upon complex formation, recognition is thought to occur via a 'lock and key' rather than an 'induced fit' mechanism. From the data presented here it is clear that E3 is stabilised by association with the E2 core. This stabilisation is probably almost entirely due to electrostatic interactions between the binding domain and residues from the interface domain of both E3 monomers.

From Figures 4.14 and 4.15 it can be seen that for E3, enzyme activity is lost at GdnHCl concentrations where no secondary or tertiary structural changes have occurred as monitored by far-UV CD and protein fluorescence respectively. This is consistent with the proposal that the precise architecture of the active site of an enzyme is much more easily perturbed than the overall structure (Tsou, 1986). The ability to regain activity on subsequent dilution of GdnHCl to below 0.03M has previously been shown, for bovine heart PDC, to correlate with the integrity of the secondary structure (West *et al*, 1995). Comparing Figures 4.13 and 4.14 it is evident that this is also the case for the three E3s investigated here. On incubation and assaying E3 in GdnHCl, 95% of activity is lost over the range 0-0.8M. At these low concentrations of denaturant no secondary structural changes are detected using circular dichroism. Recovery of activity on dilution decreases over the range 1-2M GdnHCl and Figure 4.14 shows that 47%, 52% and 60% of the secondary structure has been lost at 2.0M GdnHCl by yeast, *E. coli* and bovine E3 respectively. Dilution after incubation above 2.2M GdnHCl does not result in any regain of activity suggesting that under these conditions E3 cannot refold after loosing around 70% of its native secondary structure.

This correlation between regain of activity and loss of secondary structure has been reported previously for a number of other imported mitochondrial enzymes such as glutamate dehydrogenase (West and Price, 1988), fumarase (Kelly and Price, 1991) and citrate synthase (West et al, 1990). This is in contrast to the situation with some cytoplasmic enzymes, which appear to refold and reassemble with at least moderate efficiency after denaturation (Jaenicke, 1987). Indeed in some cases comparing the mitochondrial and cytosolic isoforms of the same enzyme has revealed clear differences in recovery of activity after GdnHCl incubation. West and Price (1990) have shown that for aspartate aminotransferase while the cytosolic isoform can recover activity on dilution from incubation in 6M GdnHCl, the mitochondrial isoform looses the ability to regain activity on dilution over the incubation range 1.0-2.0M GdnHCl. Examples such

as these led to the suggestion that the reason why many mitochondrial enzymes are significantly less stable than comparable cytosolic enzymes was associated with the unfolded state which is required for translocation of protein across the mitochondrial and other membranes. The requirement of chaperone proteins in the folding and assembly process of these imported mitochondrial proteins has also been suggested to help to explain their inability to refold from a completely unfolded state under the conditions used *in vitro*. More recently though, a number of mitochondrial proteins have been shown to reassemble after denaturation, including bovine heart PDC E2 (De Marcucci *et al.*, 1995), suggesting that the ability to refold proteins *in vitro* may have less to do with the proteins cellular location than was once thought.

CHAPTER 5

INVESTIGATION OF THE E3 REFOLDING ENVIRONMENT

5.1 Introduction

The assembly of complexes as large as the 2-oxoacid dehydrogenases poses interesting questions as to how this process occurs *in vivo*. As all the integral polypeptides of the mammalian complexes are nuclear-encoded and are synthesised as larger-M_r precursors on cytoplasmic ribosomes (Hunter and Lindsay 1986; De Marcucci *et al.*, 1988; Jackman *et al.*, 1990; Clarkson and Lindsay 1991), it is clear that import across both mitochondrial membranes and proteolytic cleavage of the extended presequences must occur before assembly into mature complexes. The precursor form of E2 from OGDC has been found not to be recognised by antibodies against native E2, suggesting that the precursor is present in the cytoplasm in an unfolded state with no conformational similarity to the mature E2 (Hunter and Lindsay, 1986). It has become clear that several additional factors are involved in maintaining this unfolded state in the cytoplasm, presentation and translocation of the mitochondrial membranes and attaining functional maturity. These factors include mitochondrial receptors, proteases and chaperone proteins. In the cytoplasm, chaperone proteins are responsible for newly synthesised subunits remaining in a translocation-competent state for delivery to the mitochondrial surface. The mitochondrial chaperone protein hsp70 appears to have a dual role in facilitating the movement of the translocation intermediate through mitochondrial contact sites into the matrix and then presenting this intermediate to the hsp60 system which is implicated in the functional maturation of the intact complex (Gething and Sambrook, 1992). Stuart *et al* . (1994) have recently shown that in *Saccharomyces cerevisae* folding of the mitochondrial precursors *in vivo* is mediated by sequential interactions with a series of chaperones which are homologues of the *E. coli* dnaK, dnaJ, grpE, groEL and groES proteins. Although chaperone proteins are believed to be involved in assisting the folding of the individual components of the 2-oxoacid dehydrogenase complexes, the association of the folded subunits is thought to be an

entirely spontaneous process, as is demonstrated by the reversibility of the loss of complex activity after dilution from low concentration denaturant incubations (Figures 3.2 and 3.4).

To investigate the potential interaction between chaperone proteins and the 2-oxoacid dehydrogenase complexes, this chapter is involved in examining the effect of groEL and groES on the refolding of the purified E3 component from 3 different sources. Also in this chapter, the requirement of the presence of non-covalently bound E3 cofactor, FAD, for optimal renaturation is investigated. The effect of its concentration and time of addition to the refolding protein are reported. In addition, the effects on the refolding yield of protein concentration and the inclusion of a reducing agent in the refolding buffer have been studied.

5.2 Investigating the effect of the FAD cofactor on the *E. coli* E3 refolding yield

Pilot studies revealed at an early stage that no significant recovery of bacterial E3 activity was obtained without the addition of exogenous FAD. Figures 5.1 and 5.2 illustrate the effects of FAD at various concentrations on the *E. coli* E3 refolding process. Figure 5.1 shows that there is an increase in the regain of activity on inclusion of a twofold molar excess of FAD to the refolding buffer, in addition to the endogenous FAD present. This regain appears to be maximal at a 5 molar excess of FAD as increasing the concentration further did not produce a significantly higher yield of E3 activity. Figure 5.2 shows a time course of *E. coli* E3 recovery after GdnHCl-induced unfolding. It can be seen that the activity recovers steadily between 0 and 120 min and a maximum of 24% of activity is recovered compared to a non-denatured control incubated at the same temperature. No recovery of activity was detected with endogenous FAD alone under these conditions.

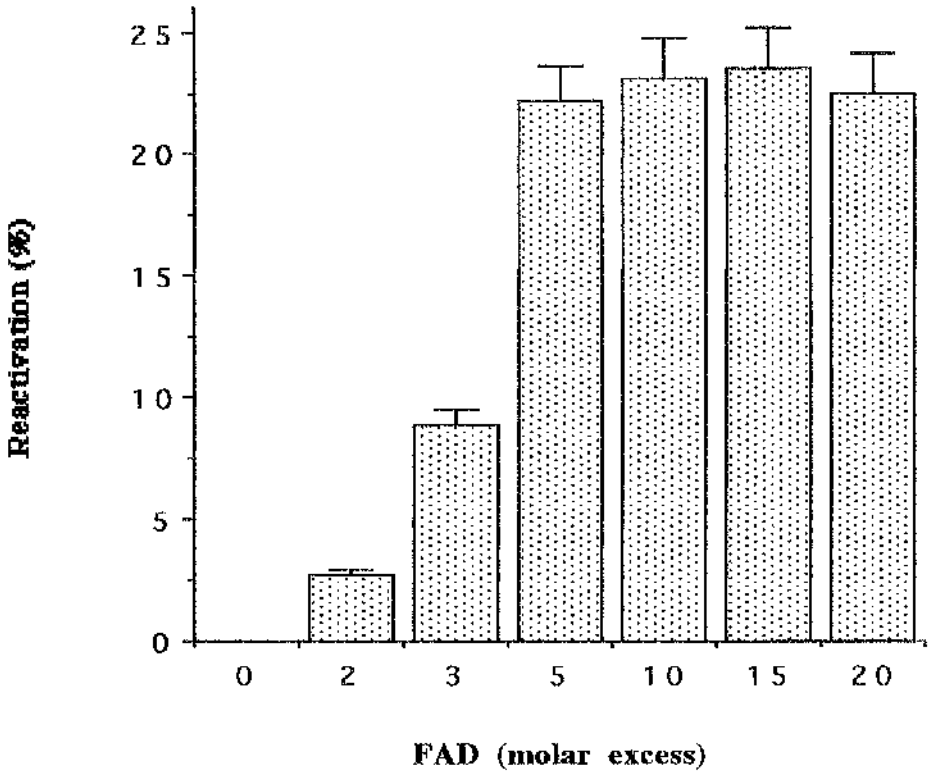


Figure 5.1 Investigating the effect of FAD concentration on the refolding of *E. coli* E3

E. coli E3 was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Refolding was initiated by 60-fold dilution into 50mM potassium phosphate pH 7.6, containing the stated added molar excess of FAD. E3, final concentration after dilution 10μg/ml, was assayed as described in Materials and Methods after 180 min of incubation at 25°C after dilution.

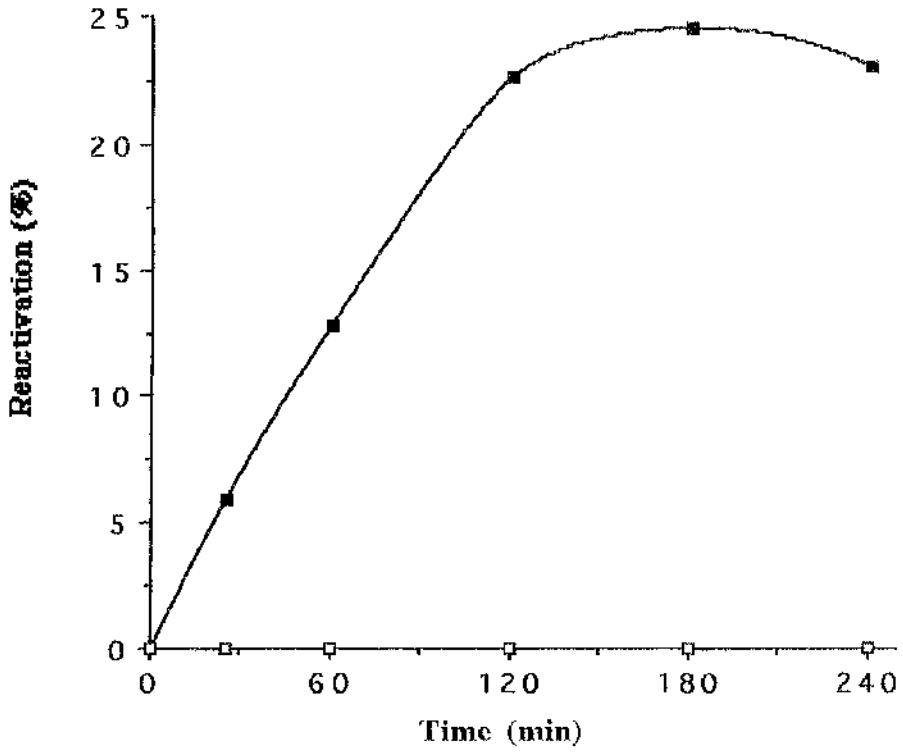


Figure 5.2 Comparing reactivation of *E. coli* E3 in the presence and absence of FAD

E. coli E3 was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Refolding was initiated by 60-fold dilution into 50mM potassium phosphate pH 7.6 with (■), and without (□), a 5 x molar excess of FAD. E3, final concentration after dilution 10μg/ml, was assayed as described in Materials and Methods.

5.3 Investigating the effect of GdnHCl concentration on the ATPase activity of groEL

Before investigating the effect of GroEL on the refolding of E3, Figure 5.3 shows the results of an experiment to determine what effect if any the residual GdnHCl concentration, after dilution, would have on the ATPase activity of GroEL. ATPase activity readings were taken for GroEL incubated in a range of GdnHCl concentrations between 0-0.7M. From Figure 5.3 it can be seen that there is a steady decrease in the ATPase activity of GroEL as the concentration of GdnHCl incubation increases in this range with no remaining activity detectable at 0.7M. The final concentration of GdnHCl after dilution in the E3 refolding experiments is 0.07M at which, it can be seen from Figure 5.3, 94% of GroEL ATPase activity remains. From this determination, it was concluded that the residual GdnHCl concentration would not significantly interfere with potential E3 refolding by the chaperone protein.

5.4 Chaperone involvement in the E3 refolding process

Table 5.1 displays the results of various refolding experiments investigating the effect of a number of agents on the yield of E3 activity after GdnHCl-induced unfolding. Attempts to refold yeast and bovine E3 under this selection of conditions have proved to be unsuccessful. *E. coli* E3 refolding, however, can be achieved routinely to varying degrees from a completely unfolded state. Surprisingly, BSA was able to promote similar limited E3 refolding at the same protein concentration as GroEL, GroES and ATP. This effect is probably due to the external hydrophobic surface of BSA interacting with individual E3 molecules, effectively reducing the chances of aggregation occurring between folding E3 monomers. FAD, in conjunction with various combinations of the other agents, can be seen to be an important factor in E3 refolding, increasing the yield

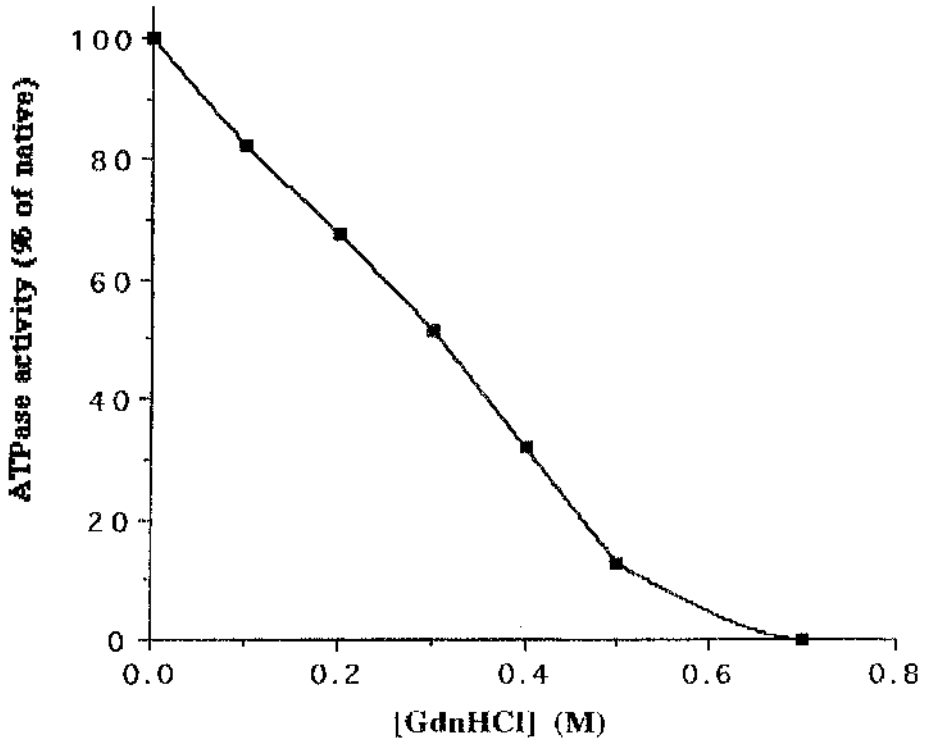


Figure 5.3 Changes in the ATPase activity of groEL in the presence of GdnHCl

GroEL (0.7 mg/ml) was incubated in 50 mM triethanolamine containing 10 mM magnesium acetate and the corresponding GdnHCl concentration (pH 8.0) for 15 min at 20°C before the ATPase assays were carried out as described in Materials and Methods. The k_{cat} values calculated are expressed here as a percentage of the value for groEL incubated under the same conditions with no GdnHCl. Results shown are the averages of two sets of data taken from different groEL preparations.

Table 5.1 Investigation of the refolding of *E. coli*, yeast and bovine E3

E3 was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Refolding was initiated by 60-fold dilution into 50mM potassium phosphate pH 7.6 containing the stated additions. The final concentration of E3 after dilution was 15μg/ml in each case. A 4-fold molar excess of GroEL was present where stated so that four 14-mer GroEL molecules were present for each refolding E3 subunit. Similarly a 4-fold molar excess of GroES 7-mers were present where stated. FAD and FMN were present at 5-fold molar excess compared to E3 monomers. ATP was present at 3μM, a 5 fold molar excess compared to GroEL 14-mers. BSA was present at 1.0 mg/ml, a concentration identical to that of the GroEL. DTT was present at 1 mM. After 180 min refolding at room temperature E3 was assayed as described in Materials and Methods and the results displayed here are relative to a non-denatured control sample incubated in the same way. Figures shown are averages of 3 runs for each set of refolding conditions for *E. coli* using E3 from different preparations and results were all within +/- 10% of the stated values.

Additions to refolding buffer	Reactivation at 180 min (%)		
	E. coli	Yeast	Bovine
None	0.0	0.0	0.0
GroEL, GroES, ATP	15.2	0.0	0.0
BSA	14.1	0.0	0.0
FAD, GroEL, GroES, ATP	61.0	0.0	0.0
FAD, BSA	41.5	0.0	0.0
FAD, GroEL	44.2	-	-
FAD, GroEL, ATP	51.2	0.0	0.0
DTT	15.5	-	-
BSA, FAD, DTT	62.0	-	-
FMN	0.0	0.0	0.0

from 15.2 to 61% when included in the GroEL, GroES and ATP experiment. These results support the idea of FAD creating a folding nucleation site at the N-terminal FAD binding site on E3. FMN, however, was unable to replace FAD in promoting the E3 refolding process.

5.5 Effect of time of FAD addition to the refolding E3

As inclusion of FAD in the refolding buffer has been shown to have a major influence on the refolding yield of *E. coli* E3, the time of its addition after dilution has been investigated. Figure 5.4 shows the results of this experiment where FAD (5-fold molar excess) has been added at between 0 and 75 min after dilution from 4M GdnHCl. From this figure, it is apparent that the recovery at 180 min, when FAD is added at 10 or 20 min is at a similar level to the 0 min value, 24%. By 30 and 40 min the recovery has fallen to 16 and 7% respectively at 180 min. No recovery is detected when the FAD is added to the refolded mixture at 75 min and the activity monitored for 240 min.

5.6 Effect of varying the concentration of E3 on the refolding yield

Figure 5.5 shows the results of E3 refolding experiments where the concentration of E3 has been varied between 0.5 and 30 μg/ml. In each case FAD was included in the refolding buffer in a 5-fold molar excess with respect to E3 monomers. Figure 5.5 shows clearly that for E3 the concentration of the refolding protein influences the yield of activity regain after 180 min. At the E3 concentrations investigated the regain of activity varied between 24 and 36% of a non-denatured sample incubated in otherwise the same conditions. The optimum concentration from these data, with 36% reactivation, occurred with an E3 concentration of 20μg/ml.

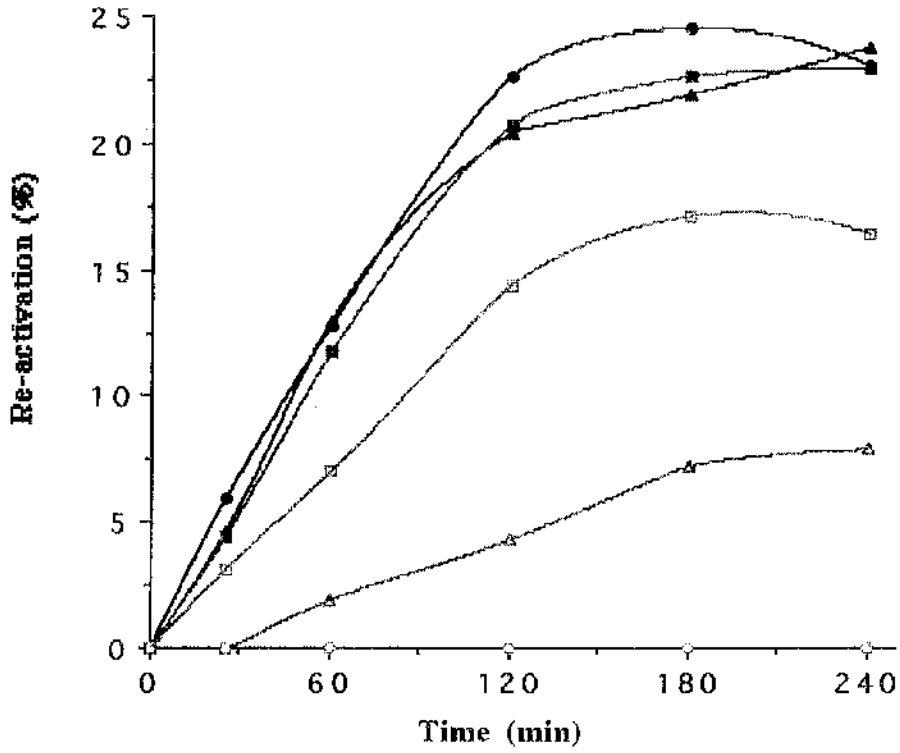


Figure 5.4 Investigating the effect of the time of FAD addition on the refolding of *E. coli* E3

E. coli E3 was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Refolding was initiated by 60-fold dilution into 50mM potassium phosphate pH 7.6 and E3 assayed as described in Materials and Methods at the times shown above after the addition of 5 times molar excess of FAD in each case. The final concentration of E3 after dilution was 5μg/ml. FAD was added 0 (●), 10 (■), 20 (▲), 30 (□), 40 (Δ) and 75 (○) min after dilution out of GdnHCl.

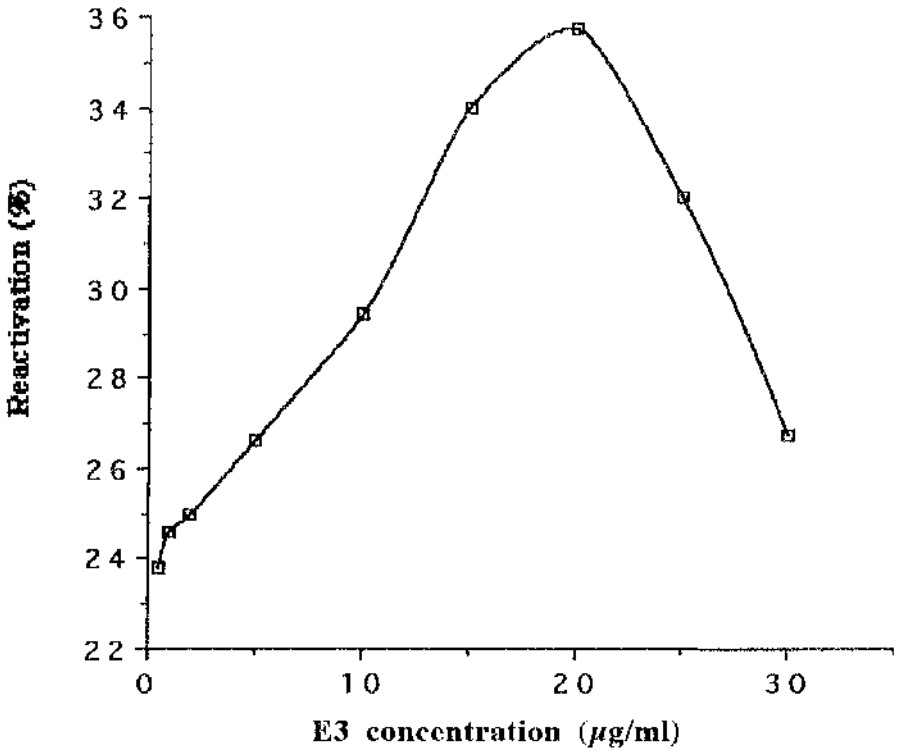


Figure 5.5 Investigating the effect of E3 concentration on reactivation yield

E. coli E3 (2 mg/ml) was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Refolding was initiated by the appropriate dilution (minimum 60-fold) to give the stated E3 concentrations in buffer containing a 5 fold molar excess of FAD in each case. E3 activity was assayed at 180 min as described in Materials and Methods. The results shown are an average of 2 sets of data taken from different E3 preparations.

5.7 Investigating FAD release during *E. coli* PDC E3 unfolding

To investigate at what point during GdnHCl-induced unfolding FAD is released from E3, incubation was first carried out in a range of GdnHCl concentrations for 15 min at 4°C. Centrifugation using Centricon tubes with a 10 000 M_r cut-off enabled the released FAD in each of these incubations to be separated from that still associated with E3. Fluorescence readings taken on the run through from the Centricon spins are shown in Figure 5.6. From the trace it can be seen that there is a rapid increase in fluorescence in the samples obtained from the 1.5-2.4M GdnHCl incubations, indicating that it is in this range in which FAD is released from E3 unfolded under these conditions. Figure 5.7 shows the results of far-uv CD scans on E3 incubated with 0-3M GdnHCl to investigate FAD release in this way. From these results it is evident that there is little change between the 0M and 1.0M GdnHCl scans, indicating that no FAD has been released after incubation in 1.0M GdnHCl. Between 1.0M and 3.0M GdnHCl both the amplitude and the wavelength of the peak are observed to change with the largest change in amplitude occurring between 2.5M and 2.6M GdnHCl incubations. The peak wavelength shifts from 370 nm to 380 nm for 0M and 3.0M GdnHCl incubations, respectively.

5.8 Structural changes on dilution from 4M GdnHCl incubation

Figure 5.8 shows the fluorescence scans of *E. coli* E3 after three different treatments. Trace A shows the native protein, trace B the denatured protein in 6.0 M GdnHCl and trace C 10 min after dilution out of 6.0 M GdnHCl. From this figure it is clear that the fluorescence scans of denatured and native E3 are distinct. The denatured

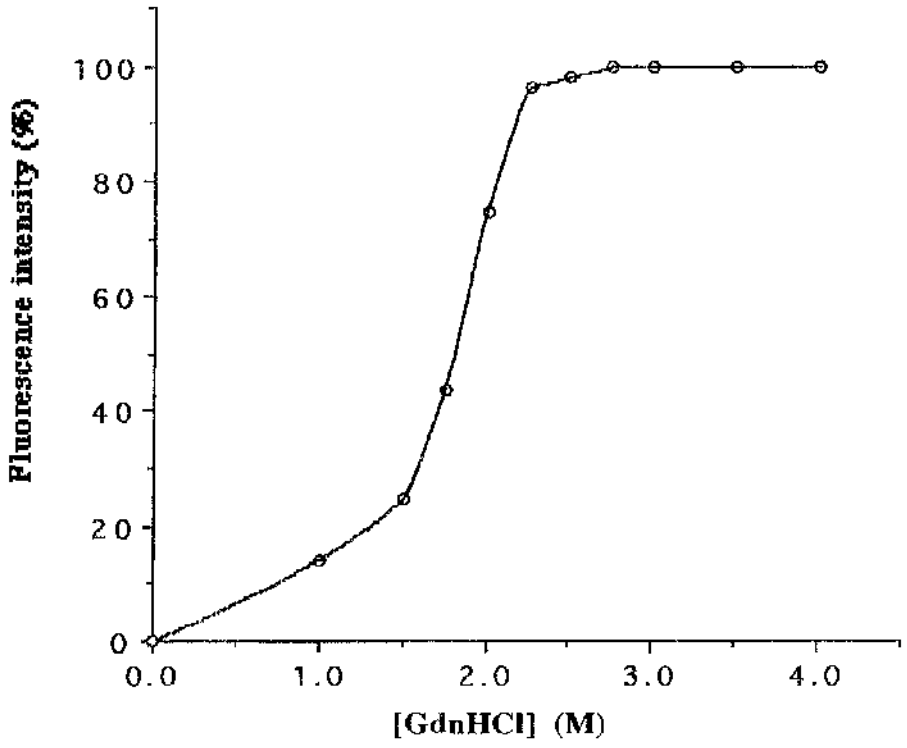
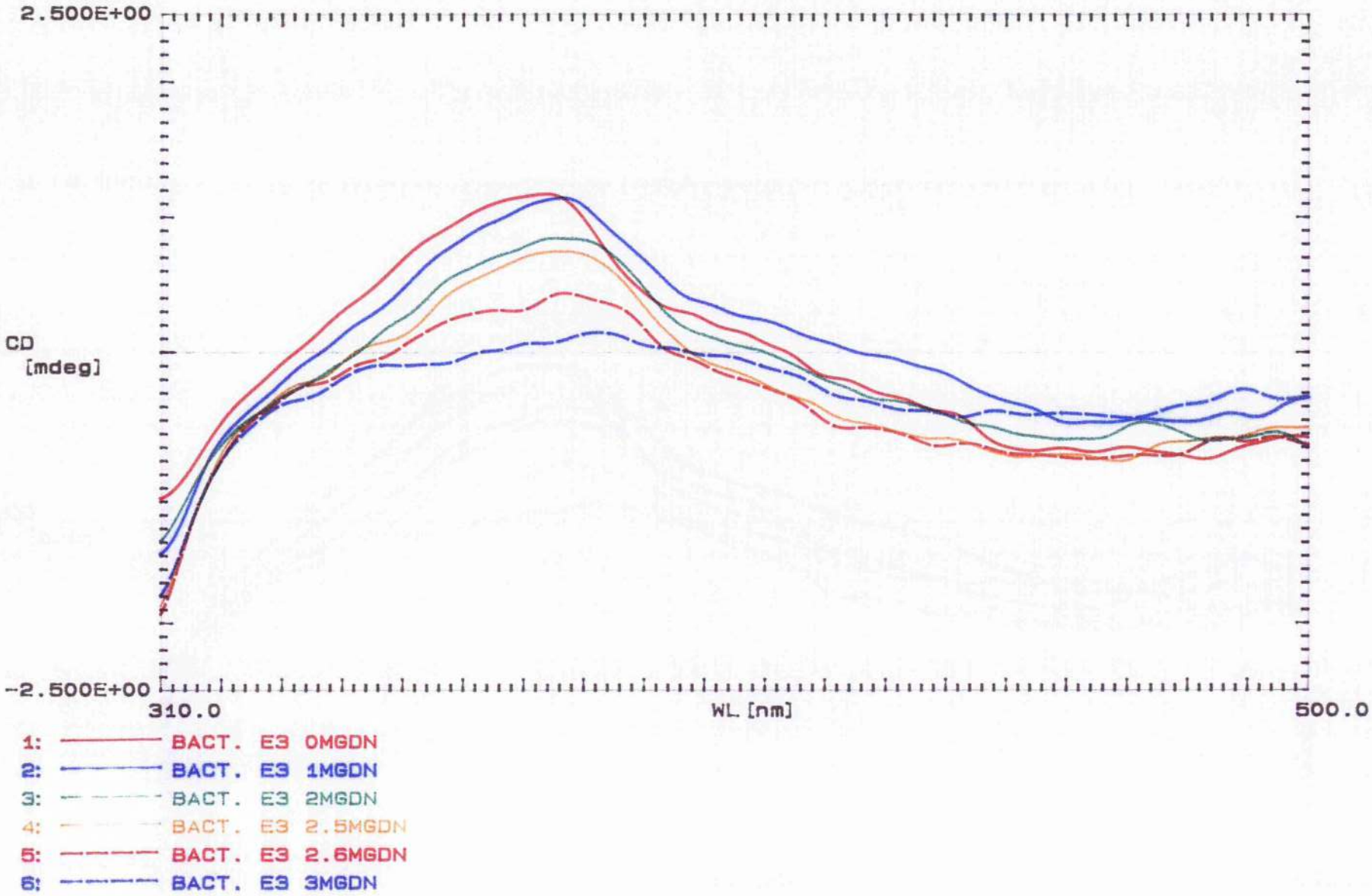


Figure 5.6 Fluorescence studies to investigate the GdnHCl concentration at which FAD is released from E3

E3 (1 mg/ml) purified from *E. coli* was incubated for 15 min at 4°C at the appropriate GdnHCl concentration. These solutions were then spun for 30 min at 10K using Centricon tubes with a 10 000 Mr cut-off, allowing free FAD to be separated from FAD remaining bound to E3. Fluorescence readings were then taken of this run-through, exciting at 450 nm and measuring emission at 535 nm, with the excitation time being limited to 1 sec to reduce the occurrence of photobleaching. Results are expressed as a percentage of the changes that occurred between 0 and 4M GdnHCl.

Figure 5.7 CD scans investigating the dissociation of the flavin group from *E. coli* E3

E3 (0.75 mg/ml) purified from *E. coli* PDC was incubated for 15 min at 4°C at the appropriate GdnHCl concentration. Scans were then taken between 310 nm and 500 nm with a 0.5 cm pathlength.


2.500E+00
CD
[mdeg]
-2.500E+00
310.0
WL [nm]
500.0
1: BACT. E3 0MGDN
2: BACT. E3 1MGDN
3: BACT. E3 2MGDN
4: BACT. E3 2.5MGDN
5: BACT. E3 2.6MGDN
6: BACT. E3 3MGDN

protein shows a peak at 352 nm of 74 arbitrary units, while the native protein shows a peak at 337 nm of 87 arbitrary units. The denatured sample diluted out of GdnHCl shows an intermediate peak at 341 nm with an amplitude of 78 arbitrary units, suggesting that while the protein has not reached its native state, some folding, or possibly aggregation, has occurred. To follow the rate of this structural change, Figure 5.9 shows a time-drive experiment where the intensity of fluorescence at 337 nm is monitored in three E3 samples. Trace A shows native E3, trace B denatured E3 in 6.0 M GdnHCl and trace C denatured E3 diluted out of GdnHCl at time -5 sec. The figure shows that the majority of the change occurs while the dilution and mixing of the sample is taking place (between -5 and 0 sec), although during the first 60 sec a continued increase in fluorescence intensity occurs. After this time no further change is detected up to 90 min. Figure 5.9 illustrates the rapidity of the folding events which are occurring upon dilution of the denatured E3.

Light scattering is a useful tool in detecting changes in particle size. Oligomerisation or aggregation can be followed by monitoring increases in the light scattered by a given sample over time. Emission readings are detected at the same wavelength, here 350 nm, but perpendicular to the excitation beam. Table 5.2 shows light scattering results for *E. coli* E3 denatured at 4°C for 15 min in 4M GdnHCl. From these data, it can be seen that there is a distinct difference between the native and denatured readings. Over the 2 h of monitoring the denatured E3, no significant light scattering change was detected indicating that aggregation did not occur.

Figure 5.8 Fluorescence scans of *E. coli* E3

Trace A shows native *E. coli* E3. Trace B shows denatured *E. coli* E3, unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 6M GdnHCl. Trace C shows *E. coli* E3, unfolded in GdnHCl in the same way as trace B, then refolding was initiated by a 60-fold dilution into the same buffer without GdnHCl. Trace C has been taken 10 min after dilution from GdnHCl. The final concentration of E3 in each case was 15 μg/ml. The excitation wavelength was 290 nm and the samples were scanned between 310 and 400 nm. The slit widths during this experiment were set at 10 nm.

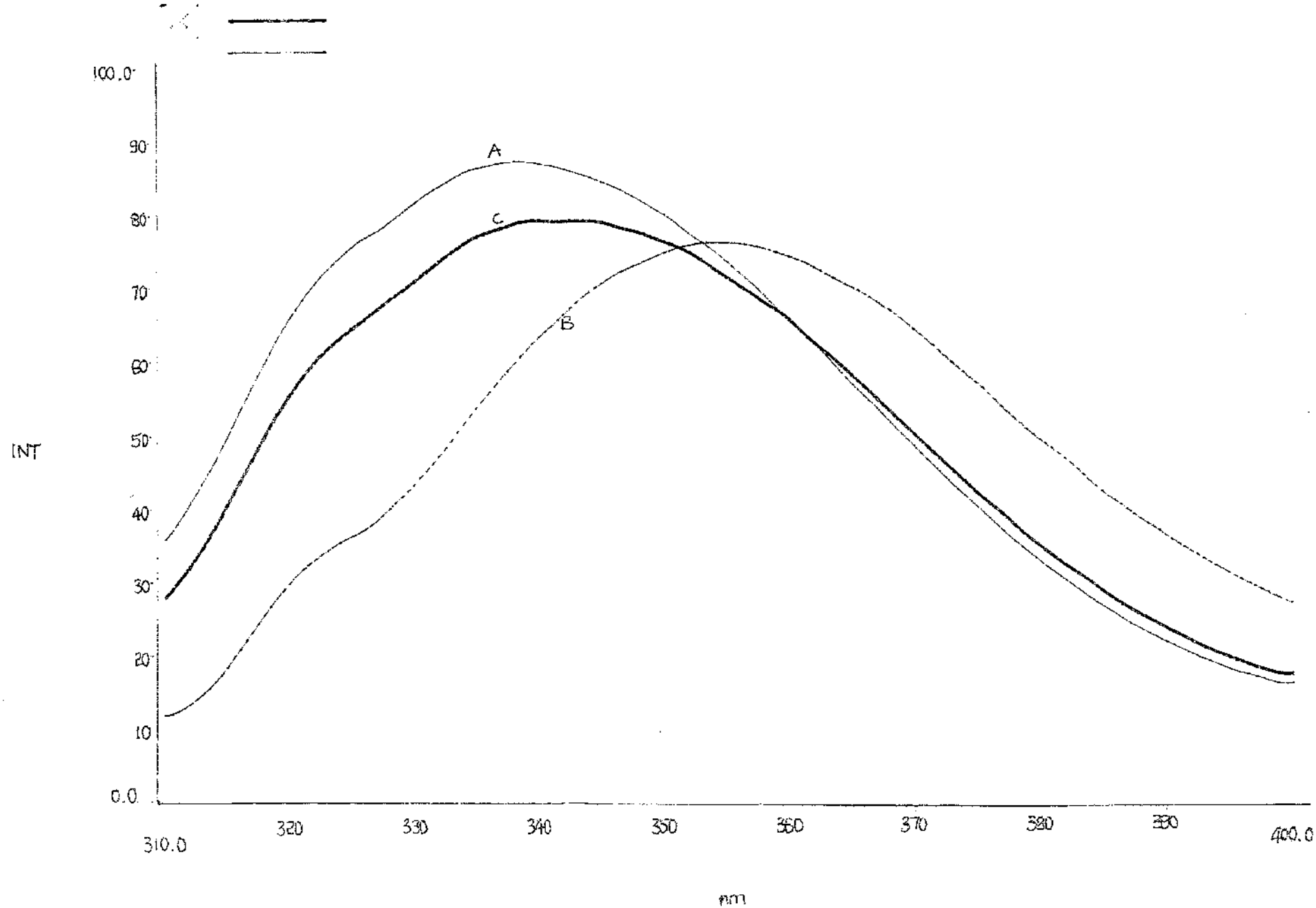
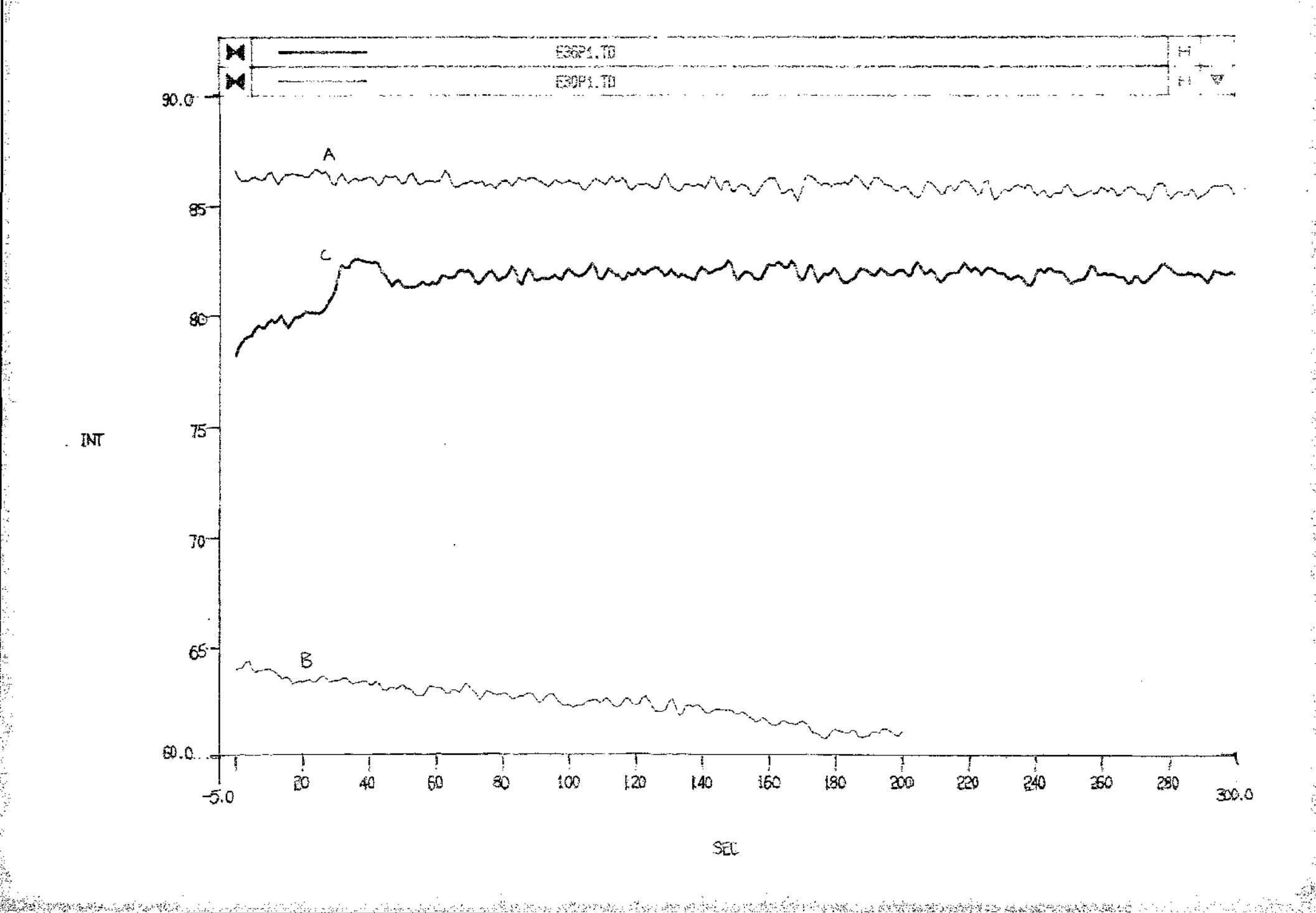

100.0
90
80
70
60
50
40
30
20
10
0.0
INT
A
B
C
310.0
320
330
340
350
360
370
380
390
400.0
nm

Figure 5.9 Fluorescence time-drive following the structural changes on dilution of *E. coli* E3 from 6 M GdnHCl

Trace A shows native *E. coli* E3. Trace B shows denatured *E. coli* E3, unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6, containing 6M GdnHCl. Trace C shows E3 denatured in the same way, then diluted into the same buffer without GdnHCl at time -5 sec. The final concentration for each trace was 15 μg/ml. The excitation wavelength was 290 nm and readings were taken of each sample at 337 nm at 1 sec intervals for 5 min. The slit widths during this experiment were set at 10 nm.


E36P1.TD
E30P1.TD
90.0
85
80
75
70
65
60.0
INT
A
B
C
-5.0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300.0
SEC

Time (min)	350 nm emission (arbitrary units)
0	202
1	195
2	193
5	191
10	184
45	186
90	191
105	190
120	190

Table 5.2 Light scattering data from *E. coli* E3 after GdnHCl incubation

E. coli E3 was unfolded by incubating for 15 min at 4°C in 50mM potassium phosphate, pH 7.6 containing 4M GdnHCl. Dilution by 60-fold was carried out at 0 min to give 80 μg/ml E3. Light scattering readings were taken at the times stated with the excitation and emission wavelengths at 350 nm and both slit widths at 2.5 nm. Native E3 under the same conditions gave a reading of 284 units.

5.9 Discussion

The E3 component is a member of the pyridine nucleotide disulphide oxidoreductase family, a group of homodimeric FAD-dependent enzymes with considerable versatility. Crystal structures determined for E3 from *Azotobacter vinelandii* (Schierbeek *et al.*, 1989; Mattevi *et al.*, 1991), *Pseudomonas putida* and *Pseudomonas fluorescens* (Mattevi *et al.*, 1992) reveal the peculiar arrangement of the E3 dimer, which has a characteristic butterfly-like shape. The structure of E3 comprises four distinct domains: an FAD binding domain, an NAD binding domain, a central domain and an interface domain. The FAD and NAD binding domains have similar topologies, suggesting that they may have evolved from the duplication of a common ancestor. The catalytic centre has been found to be located at the interface between the two chains, with one molecule of FAD bound to each subunit in an extended conformation. Figures 5.1 and 5.2 show the effect of additional FAD in the refolding buffer on the regain of E3 activity.

The requirement of additional FAD for any detectable regain of activity for *E. coli* PDC E3 refolding from a completely unfolded state under these conditions is very interesting. One explanation of this finding is that the binding of FAD is stabilising segments of secondary and tertiary structure around the FAD binding site, thus enabling other areas away from this site to proceed along the folding pathway. The fluorescence scans shown in Figure 5.8, however, show that some folding is taking place in the absence of additional FAD. The light scattering data in Table 5.2 suggest that this fluorescence quenching is not caused by E3 aggregation although even after 90 min the native state has not been reached and from Figure 4.12 it can be seen that the ability to regain activity is lost after incubation and dilution from much lower GdnHCl concentrations. The effect of additional FAD on the refolding of yeast and bovine E3 has also been investigated. In Table 5.2, it is reported that both with and without additional

FAD no activity is recovered after 180 min with either bovine or yeast E3. Figures 5.6 and 5.7 show experiments designed to determine at what GdnHCl concentration the FAD is released from E3 using visible and near-uv adsorption spectra and fluorescence data. CD spectra of flavoproteins in the visible and near ultraviolet flavin light-absorbing region are very characteristic and have previously shown a good correlation between the type of CD spectrum observed and the particular function of that flavoprotein (Edmondson and Tollin, 1971; D'Anna and Tollin, 1972; Van Berkel and Müller, 1989). From Figure 5.7, it is observed that under the conditions used the FAD appears to be released when incubated in 2.0-2.6M GdnHCl. This correlates well with the steepest change in the fluorescence intensity measurements shown in Figure 5.6 which occurs over the range 1.5-2.5M GdnHCl.

The *E. coli* chaperone protein GroEL and its mitochondrial and plastid homologues have been implicated in playing central roles in the folding and/or assembly of a variety of different polypeptides (Martin *et al.*, 1991; Fisher, 1992; Zheng *et al.*, 1993; Wynn *et al.*, 1994). The exact mechanism of chaperonin mediated protein folding still remains to be fully elucidated. GroEL, however, possesses a weak ATPase activity ($k_{cat} \approx 0.1$ sec^{-1} per GroEL subunit) and it has been demonstrated that ATP hydrolysis is involved in the folding and/or release of polypeptide chains from the GroEL protein, usually in conjunction with its co-chaperonin GroES. Figure 5.3 shows that the low level of GdnHCl which is present after dilution out of the 4.0M incubation (maximum 0.07M) has only a very minor effect on ATPase activity reducing the value to 94% of the native 100% value. This effect has not been taken into consideration when calculating the chaperone-assisted refolding yields shown in Table 5.1. From Table 5.1 it is observed that the addition of groEL, groES and ATP to the refolding buffer gives a refolding yield of 15.2% for the *E. coli* E3. To investigate if this effect was specifically mediated by the added chaperone proteins, or due to the increased protein concentration in the refolding buffer, regain of activity was also measured with BSA in the refolding buffer to give the

same protein concentration without the potential chaperone action. The inclusion of BSA gave a 14.1% regain of activity, suggesting that the increased protein concentration, rather than the chaperone activity of groEL and groES, was responsible for the activity regained under these refolding conditions. However, a difference is observed when comparing the chaperone and BSA refolding buffers when FAD is included. Table 5.1 shows that when FAD is included the refolding yields are 61.0% and 41.5% for the chaperone and BSA refolding buffers respectively. It appears that under these refolding conditions GroEL and GroES are causing the regain of more activity than can be explained by the effect of increased protein concentration in the refolding buffer, suggesting chaperoning was taking place under these conditions. From these data the significance of groES in the E3 refolding process is difficult to identify. Table 5.1 also shows that FMN does not induce refolding unlike FAD. This implies that the entire FAD cofactor, not only the isoalloxazine and ribitol portions, have a role to play in stabilising structural elements of the *E. coli* E3, presumably around the FAD binding site on each monomer.

All attempts to refold the yeast and bovine E3 after GdnHCl-induced unfolding have been unsuccessful as shown in Table 5.2. It is not clear why this is so, although the refolding of yeast and bovine E3 may well be possible *in vitro* under different conditions. The inclusion of 1 mM DTT with FAD in the refolding buffer gave a 62% recovery of activity after 180 min as shown in Table 5.1. This suggests that the oxidation state of the refolding buffer is another important factor affecting the yield of regained activity and that the chaperone experiments described here may not have been carried out under ideal conditions.

CHAPTER 6

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