STUDIES ON THE UNFOLDING AND REFOLDING OF MULTI-SUBUNIT PROTEINS

by

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CONTENTS

CHAPTER ONE: INTRODUCTION

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: UNFOLDING AND REFOLDING OF GLUTAMATE DEHYDROGENASES

CHAPTER FOUR: UNFOLDING AND REFOLDING OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

CHAPTER FIVE: UNFOLDING AND REFOLDING OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
AAT	aspartate aminotransferase
CM	carboxymethyl
c.d.	circular dichroism
CS	citrate synthase
DEAE	diethylaminoethyl
DTT	dithiothreitol
GDH	glutamate dehydrogenase
GdnHCl	guanidinium chloride
n.m.r.	nuclear magnetic resonance
Nbs_2	5,5'-dithiobis-(2-nitrobenzoic acid)
PAGE	polyacrylamide gel electrophoresis
PLP	pyridoxal 5'-phosphate
QAE	diethyl(2-hydroxypropyl)aminoethyl
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine

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ABSTRACT

Studies on the unfolding and refolding of multi-subunit proteins have been carried out and the results obtained for translocated proteins have been compared where possible with those obtained for non-translocated proteins. This comparison should indicate the way in which translocation affects the folding and assembly of such proteins. The enzymes studied were the glutamate dehydrogenases from bovine liver, baker's yeast and *Clostridium symbiosum*; the cytoplasmic and mitochondrial aspartate aminotransferase isoenzymes from pig heart and citrate synthase from pig heart.

The unfolding of the enzymes by guanidinium chloride (GdnHCl) was studied by monitoring loss of catalytic activity and changes in structure by fluorescence, circular dichroism and exposure of reactive thiol groups. It was found that loss of enzyme activity occurs at lower concentrations of GdnHCl than any major changes in the structure. The refolding of the enzymes was studied by measuring the regain of catalytic activity on dilution of the GdnHCl. It was found that only the cytoplasmic aspartate aminotransferase enzyme was able to regain activity from the unfolded state. In comparison, the translocated proteins studied; bovine liver glutamate dehydrogenase, mitochondrial aspartate aminotransferase and citrate synthase could not be refolded after denaturation. This inability of translocated proteins to refold from their isolated, unfolded subunits implies that other factors are involved in the folding and assembly of translocated proteins *in vivo*. It is possible that chaperone proteins may be involved in this process.

CHAPTER ONE

INTRODUCTION

1.1. Protein Folding

The term protein folding includes all the events occurring from translation to the formation of a functional three-dimensional protein structure. The mechanism by which a polypeptide chain folds to reach its native, functional structure remains an intriguing problem. The first significant works relating to the protein folding problem showed the correlation between biological activity of a protein and the so called 'native structure' (Northrop, 1932; Anson and Mirsky, 1934a, 1934b). The reversibility of the denaturation of proteins was demonstrated at this time, but only for a few proteins. Subsequent work by Anfinsen (1966) on the reduction and reoxidation of ribonuclease showed that the amino acid sequence contains the necessary information for regaining the native three-dimensional structure. This work initiated the use of protein refolding as a model for *in vivo* protein folding. Refolding refers to the regain of the native structure of a protein after denaturation. The *in vitro* reconstitution may differ from the *in vivo* folding process, as refolding starts from the complete denatured polypeptide chain, while folding of the nascent protein might be a step-wise process accompanying the growth of the polypeptide chain.

Studies on protein refolding aim to determine the pathway of folding which should reveal the mechanism of folding and help in elucidating the 'code' by which the amino acid sequence of a protein specifies its three-dimensional structure. This folding code will aid in engineering changes in protein structure using recombinant DNA techniques and provide a tool to specify hypothetical functions for given amino acid sequences deduced from DNA sequence analysis

(Jaenicke and Rudolph, 1986). At the present time there is a large amount of protein sequence data which cannot be followed up by the elucidation of the three-dimensional structure of the corresponding proteins by X-ray diffraction. Since the biological function of proteins is totally determined by the spatial arrangement of the polypeptide chain, it would be advantageous to derive the folded structure from the amino acid sequence. It has been observed that amino acid substitutions, which relate to changes in the code, are allowed at almost all residue positions in a protein without drastic changes in the folding pattern. Another observation was that the folding of some related proteins is similar, though the amino acid sequences differ significantly. Thus, the code for folding is not a simple code like the mRNA triplet code (Kim and Baldwin, 1982).

1.2 Structure prediction methods

A variety of methods has been used to predict the conformation of a protein based solely on its amino acid sequence. The difficulties associated with structure prediction methods arise from the large size and complexity of protein molecules, as well as from uncertainties about the nature and magnitude of the forces acting between the atoms especially in the presence of solvents (Jaenicke, 1987). One approach used for structure prediction was ab initio calculations of the most stable structure (i.e. of lowest free energy) but this has only been of use for short oligopeptides. For most proteins of biological interest 'semi-empirical' methods have been developed in which proteins of known three-dimensional structure are examined in order to categorise amino acids or short sequences of amino acids into the

types of secondary structure in which they are most likely to occur. Predictive algorithms can then be deduced and applied to predict secondary structure elements in other proteins.

Three of the most widely used algorithms for prediction of secondary structure from the amino acid sequence are those by Lim (1974) based on the physicochemical characteristics of amino acids and Chou and Fasman (1978) and Garnier et al. (1978) which are based on statistical analysis of proteins of known structure. An assessment of these three prediction methods on 62 proteins of known structure found that no method predicts better than 56% of the residues correctly, for three states: helix, sheet and loop (Kabsch and Sander, 1983).

More recently a method has been described for protein secondary structure prediction based on a 'neural network' (Holley and Karplus, 1989). This involved a training phase which was used to teach the network to recognise the relation between secondary structure and amino acid sequences on a sample set of 48 proteins of known structure. For a test set of 14 proteins of known structure, the method achieved a maximum overall predictive accuracy of 63% for three states: helix, sheet and coil. To allow comparison with other prediction methods, the 62 proteins used were those used previously by Kabsch and Sander (1983). This 'neural network' method was found to be generally more accurate than previous methods when compared on identical proteins. The limited accuracy of the prediction methods is believed to be due to the small size of the data base and/or the fact that the secondary structure is determined by tertiary interactions not included in the local sequence (Holley and Karplus, 1989).

1.3 Hierarchy of protein structure

In looking at the problem of protein folding a brief description of the levels of protein structure is required. The pathway of protein folding in which the polypeptide chain acquires its three-dimensional structure reflects the hierarchy of protein structure. This hierarchy of levels of protein structure was first defined by Linderstrøm-Lang (1952) and added to by Bernal (1958).

The primary structure refers to the specific amino acid sequence along the covalent polypeptide chain. The secondary structure refers to the local spatial arrangement of the polypeptide backbone which forms regular structures such as αhelix and βsheet. An extension of this level is supersecondary structure which refers to clusters of helices and β-structures. The tertiary structure refers to the three-dimensional chain fold, with structural or functional 'domains' as spatially separate entities. Proteins which contain more than one polypeptide chain have another level of protein structure, quaternary structure. Each polypeptide chain is a subunit and the quaternary structure describes the stoichiometry and geometry of subunit assembly.

In the formation of oligomeric and multimeric proteins, subunit association has to be considered in addition to folding. Both folding and association must be properly co-ordinated as subunit assembly requires the surfaces of the monomers to be preformed in the correct way in order that specific recognition can occur (Jaenicke and Rudolph, 1986).

1.4 Studies of protein folding

Studies of protein folding are mainly carried out *in vitro* rather than *in vivo*, as many of the physical methods for studying the conformation of proteins cannot be applied to protein synthesising systems and require relatively large quantities of well- characterised material. Some studies have been carried out *in vivo* and these indicated that folding can occur during biosynthesis, but there is little indication of the extent or rate of folding. *In vitro* studies can examine the folding process in more detail by using physical techniques which can monitor protein conformation. Other techniques which can be used to look at protein folding *in vitro* include the biological activity of proteins and immunological techniques.

In the *in vitro* method, denaturing conditions are used to cause unfolding and dissociation of a protein, the subsequent removal of the denaturing conditions allows the refolding and reassociation of a protein to occur. The unfolding of proteins can be achieved by a number of treatments including extremes of pH, increase of temperature, high pressure, organic solvents, detergents and organic solutes, such as guanidinium chloride (GdnHCl) or urea. The extent of unfolding can vary for different denaturing conditions (Ghélis and Yon, 1982; Jaenicke, 1987) from local distortions of the polypeptide backbone to a complete, randomly coiled polypeptide chain.

The denaturing agents guanidinium chloride (GdnHCl) and urea are most commonly used, as they are able to disrupt the folded protein structure to a great extent. Concentrations of 6 M GdnHCl

generally lead to complete unfolding of globular proteins. The mechanism by which GdnHCl denatures proteins is not known but it appears that the denaturing power of GdnHCl is due to its ability to make water a better solvent for non-polar side chains (Hibbard and Tulinsky, 1978). At relatively low ($\sim$1 M) concentrations of GdnHCl limited unfolding of oligomeric proteins can occur. This results in dissociation of the protein but not the total loss of secondary and tertiary structure. This allows reassociation of the protein to be studied separately from refolding. Urea has the general disadvantage compared with GdnHCl that isocyanate which is in equilibrium with aqueous urea, can cause covalent modifications of amino or thiol groups (Stark et al., 1960).

The refolding of proteins is initiated by removal of the structure-perturbing agent, by dilution or dialysis in the case of GdnHCl and urea, by a pH jump or by a change in pressure. The refolding of a protein can be studied by a number of techniques as shown in Table 1.1. The references mentioned, relevant to each technique, have been used to follow protein folding. The techniques shown each give information about particular aspects of the folding process and the results taken together build up a model for refolding. For proteins whose native structures have been determined by X-ray crystallography the information on the refolding pathway can be interpreted to a much greater extent (Price, 1990).

1.5 Comparisons of protein folding in vivo and in vitro

In vitro studies of protein folding differ from those *in vivo* as refolding of a protein starts from a denatured but complete polypeptide chain, whereas *in vivo* folding of the nascent chain may

Table 1.1 Techniques which can be used to study protein refolding

Property studied	Technique	Reference
Biological activity	Kinetics of reactivation	Chan et al. (1973)
Protein conformation	Fluorescence	Teipel and Koshland (1971)
	Circular dichroism	Labhardt (1986)
	Chemical modification of amino acid side chains.	Ghélis (1980)
	Hydrogen-tritium exchange	Kim (1986)
	Hydrogen-deuterium exchange	Roder et al. (1986)
		Udgaonkar and Baldwin (1986)
	Susceptibility to proteolysis	Girg et al. (1981)
	Trapping of intermediates in disulphide-bonded proteins	Creighton (1978)
State of association	Chemical cross-linking	Hermann et al. (1981)
	Hybridisation	Bothwell and Schachman (1980a,b)

occur as a co-translational event. *In vitro* studies of protein folding have provided a lot of information, but to what extent can the results be used to explain folding *in vivo*? Two criteria which have been used in comparing folding *in vitro* to folding *in vivo* are the rate of folding and the final product.

The rate of folding *in vitro* for many proteins is fast (within seconds or minutes) which is compatible with the rate of folding of nascent polypeptide chains *in vivo*. The monomeric fructose 1,6-bisphosphate aldolase from *Staphylococcus aureus* has been shown to regain activity after denaturation in guanidinium chloride within 10 seconds (Rudolph *et al.*, 1983). This can be compared with estimates of 30 seconds or less for the folding of proteins *in vivo* (Tsou, 1988). In some cases, the refolding of proteins *in vitro* is much slower, taking several minutes or hours. It was found the recovery of about 85% of creatine kinase activity after dilution of the enzyme takes around 30 minutes and recovery of full activity takes many hours (Zhou and Tsou, 1986). This would appear to be incompatible with the rate of folding *in vivo*. However, this slow rate is still faster than the time it would take for a protein to obtain its most stable conformation by a random search pathway (Karplus and Weaver, 1976). The final product of refolding has generally been shown by a variety of physicochemical and enzymological methods to have structural and functional characteristics which are similar, if not identical, to the native protein (Jaenicke and Rudolph, 1986).

1.5.1 Co-translational folding

Some approaches used to study the folding of polypeptide chains presume that folding is entirely post-translational, but the folding of a completed sequence would be different from that of a growing polypeptide chain (Bergman and Kuehl, 1979). It has been found that isolated N-terminal fragments of some proteins have significant amounts of secondary structure. The conformation of these peptides resemble the corresponding peptide segment within the native protein (Wetlaufer, 1981). Other experimental evidence has found that some of the disulphide bonds of certain proteins are formed co-translationally (Bergman and Kuehl, 1979; Peters and Davidson, 1982). From these results it appears that the nascent peptide chain folds while it is being synthesised. This could explain why the rate of protein folding *in vivo* is faster than has been found for protein refolding from the complete polypeptide chain. As the peptide chain folds, the folding of the segment already synthesised should affect the subsequent folding of the rest of the molecule. Also, the conformation of that part of the molecule already synthesised should be continuously affected by the newly synthesised segments and undergo constant adjustments. But folding is not entirely co-translational as folding of the peptide chain can continue after the completion of synthesis. For some proteins post-translational covalent modifications are required. (Tsou, 1988).

1.5.2 Factors involved in directing protein folding

Two proteins which are involved in protein folding *in vivo* are protein disulphide isomerase (PDI) and peptidyl-prolyl *cis-trans*

isomerase (PPI). Both of these proteins increase the rate of folding *in vivo* for certain proteins compared to the rate of folding found *in vitro*. The catalytic activities of these two enzymes are different and act independently of one another as was shown by Lang and Schmid (1988). PDI catalyses thiol: disulphide interchange reactions for a wide range of protein substrates whereas PPI catalyses the *cis-trans* isomerisation of proline peptide (X-Pro) bonds.

The proteins which contain disulphide bonds are generally secreted or are cell surface proteins. The formation of disulphide bonds has been shown to be an early step in the post-translational modification of secreted proteins which occurs in the endoplasmic reticulum. PDI is located in the endoplasmic reticulum of secretory tissues such as the liver and pancreas. It is a very abundant protein and amounts to about 2% of the entire protein content in rat liver microsomal fractions. Depending on the nature of the protein substrate and the overall thiol: disulphide redox potential, PDI can catalyse net formation, net isomerisation and net reduction of protein disulphides (Freedman, 1984). Thus, the function of PDI was thought to be as a catalyst for correct formation of disulphide bonds in protein biosynthesis.

Evidence to show that PDI was capable of catalysing correct disulphide bond formation in a protein and that it could accelerate *in vivo* protein folding was provided by Koivu and Myllylä (1987). They studied the process of disulphide formation in the assembly of type I and type II procollagen, a protein comprising of three polypeptide chains linked by disulphide bonds at the carboxy termini. The results indicated that PDI is able to accelerate the

formation of native interchain disulphide bonds in these procollagens. For type I procollagen, the optimal half-time for chemically catalysed trimer formation was found to be 11 minutes whereas in the presence of PDI it is as short as 4 minutes. The rate of trimer formation in the presence of PDI corresponds to the *in vivo* rate. The assembly of type II procollagen is very slow in the absence of PDI *in vitro*, but in the presence of PDI it assembles with a half-time of 12 minutes. The assembly and folding of type II procollagen is slower than that of type I procollagen which agrees with the situation observed *in vivo*.

The next step in establishing the role of PDI was to show that PDI was present in a cell that actively synthesises and secretes a single major disulphide protein. This was approached by Roth and Pierce (1987) who studied proteins which were directly associated with PDI in intact lymphocytes to see if PDI had a role in immunologlobulin assembly. They found that newly synthesised immunoglobulin heavy and light chains can both be cross-linked to PDI in intact hybridoma cells which are actively synthesising and secreting immunoglobulins. These results supported the hypothesis that PDI functions in the *in vivo* synthesis of immunoglobulins.

Evidence that PDI is involved in co-translational formation of disulphide bonds was provided by Bulleid and Freedman (1988). They depleted dog pancreas microsomes of soluble proteins, including PDI. These microsomal preprations were still able to translocate and process proteins synthesised *in vitro*. The formation of intramolecular disulphide bonds during the *in vitro* synthesis of γ- gliadin, a wheat storage protein, was studied and it was found that these microsomes were defective in the co-translational

formation of disulphide bonds. Addition of purified PDI to these microsomes restored the capacity to generate disulphide bonds. These depletion and reconstitution data show that PDI can catalyse disulphide bond formation, not only when acting on reduced and denatured mature proteins, but also on the immediate products of translation. PDI is required for efficient disulphide bond formation in such translation products. From the available evidence it can be concluded that PDI acts within the cell as a catalyst of native disulphide bond formation associated with initial protein folding in the lumen of the endoplasmic reticulum (Freedman *et al.*, 1989).

The slow, rate-determining step in the *in vitro* refolding of some proteins is due to the slow rate of isomerisation of incorrect proline peptide bonds (Lang *et al.*, 1987). The enzyme peptidyl-prolyl *cis-trans* isomerase (PPI) was found by Fischer and coworkers (see Fischer and Bang, 1985), and it was shown to accelerate efficiently the *cis-trans* isomerisation of prolyl peptide bonds in short oligopeptides. The catalysed reaction is a 180° rotation about the C-N linkage of the peptide bond preceding proline. PPI has a widespread distribution and is found in virtually all tissues and organisms.

The effect of PPI on the refolding of denatured ribonuclease A was studied by Fischer and Bang (1985) and they found an enhanced rate of refolding when PPI was present. In another study which looked at the effect of PPI on the refolding of immunoglobulin light chain from mouse, it was found that the slow folding reaction was catalysed by PPI (Lang *et al.*, 1987). The catlytic effect of PPI on the slow folding reactions of several different proteins has also been studied by Lin *et al.* (1988).

They found that significant catalysis was only observed in proteins which are rate-limited by proline isomerisation. These results show that PPI catalyses slow steps in the refolding of several small proteins for which proline isomerisation was assumed to be the rate-limiting step. The efficiency of catalysis during refolding depends on the accessibility of the particular proline peptide bond for the isomerase. Structure formation in proline containing chain segments may render them inaccessible for PPI (Lang et al., 1987).

In attempts to further characterise PPI it was found that PPI from porcine kidney is identical to bovine cyclophilin, a protein that binds the immunosuppressant cyclosporin A with high affinity. Fischer et al. (1989) found that the first 38 amino acid residues of porcine PPI and bovine cyclophilin were identical and that both proteins have a relative molecular mass of 17 000. It was also found that the catalysis of prolyl isomerisation in oligopeptides and of protein folding by PPI were strongly inhibited the presence of low levels of cyclosporin A. These results were confirmed by Takahashi et al. (1989) who showed that the amino acid sequence of PPI from pig kidney is identical to that reported for bovine cyclophilin. They also found that cyclosporin A has an inhibitory effect on PPI activity. The role of PPI for in vivo protein folding or other transconformational reactions in the cell is not yet known and the effect of the immunosuppressant cyclosporin A is not yet understood (Fischer and Schmid, 1990).

1.5.3 Efficiency of folding

The in vitro folding of proteins, in most cases is less than 100% efficient. This indicates that side reactions, mainly

the formation of aggregates, can occur. The aggregation of proteins is due to the poor solubility of unfolded or partially folded chains in aqueous solvents. Aggregates are formed by intermolecular interactions and are found more significantly in proteins of larger subunit mass, due to the greater number of folded domains present. The refolding of multi-subunit proteins can result in folded or partially folded monomers associating to give 'wrong' aggregates. During the refolding process there is competition between the formation of the native structure and the irreversible formation of aggregates (Zettlmeissel et al., 1979). The extent of aggregation can be reduced by lowering the protein concentration during refolding. Protein folding in vivo is a more efficient process and the problem of aggregation which occurs during in vitro folding is avoided by the occurrence of co-translational folding. This could allow domains of a polypeptide chain to form in a specific order and thus prevent interactions occurring between groups of amino acid residues which occur in different domains.

It has been proposed that the folding of some proteins in vivo is affected by the rates at which regions of their polypeptide chains are translated. The gene sequences of these protein may have evolved to control the rate of translation so that synthesis of defined portions of their polypeptide chains is separated temporally. Thus, the inability of some proteins to refold in vitro might indicate that the temporal separation of folding events during synthesis in vivo is important in helping some of these proteins to achieve their native conformation (Purvis et al., 1987). It was proposed that yeast pyruvate kinase, which contained a consecutive sequence of five non-preferred codons, could have a

translational pause in this region to allow correct folding of the multi-domain structure. More recently, it was shown that the gene sequence does not contain a string of non-preferred codons. Thus pyruvate kinase is not an example of the class of genes which possibly exhibit translational control (McNally et al., 1989), although there may be evidence for translational control in other yeast genes such as ARO1 (which codes for the Arom protein) and FAS1 (which codes for four different enzymes).

1.6 The folding of monomeric proteins

The folding of monomeric (single chain) proteins into native three dimensional structures can be divided into two groups, single domain and multi-domain proteins. These groups can be sub-divided into proteins with or without disulphide bonds.

1.6.1 Single domain proteins

The folding of a globular protein involves a complex series of rearrangements beginning with random coil states and ending with the three dimensional native structure. Two single domain proteins which contain disulphide bonds are bovine pancreatic trypsin inhibitor and ribonuclease A.

The refolding of bovine pancreatic trypsin inhibitor (BPTI) has been extensively studied by Creighton (1978). This small protein consists of 58 amino acids and contains three disulphide bonds between cysteine residues 5-55, 14-38 and 30-51. The thiol-disulphide interchange reaction by which protein disulphide bonds are formed, broken or interchanged during protein folding or unfolding may be quenched by blocking all thiol groups of the solution. This allows intermediate states of a protein containing

two or more disulphide bonds in the folded state to be trapped in a stable form with respect to their disulphide bonds. Reaction of thiol groups with iodoacetate is completely irreversible and was therefore used by Creighton to trap intermediates of the BPTI folding pathway. The trapped intermediates were separated and the cysteine residues involved in disulphide bonds were determined for each of the trapped intermediates.

Creighton (1978) proposed a folding pathway based on the kinetics of formation of the disulphide intermediates, as shown in Fig.1.1. The pathway shows the order of disulphide bond formation. First, is the formation of the one-disulphide intermediates with species (30-51) comprising 60-70% of the one-disulphide intermediates and (5-30) about 20-30%, with the remaining 10% consisting of minor species such as (5-51) and (30-55). These observations suggested that the one-disulphide intermediates were rapidly interconverted by intramolecular thiol-disulphide exchange. All further species in the folding pathway contain the 30-51 disulphide bond and it appears only this predominant one-disulphide intermediate forms a second disulphide. The three two-disulphide intermediates formed are (30-51, 5-14), (30-51, 5-38) and (30-51, 14-38). The (30-51, 14-38) intermediate is favoured over the other two intermediates but it is then converted to one of the other two intermediates. The three two-disulphide intermediates formed cannot readily form a third disulphide bond. The intermediates (30-51, 5-14) and (30-51, 5-38) rearrange to form (30-51, 5-55). This fourth two-disulphide intermediate is the most stable two-disulphide species, to which all

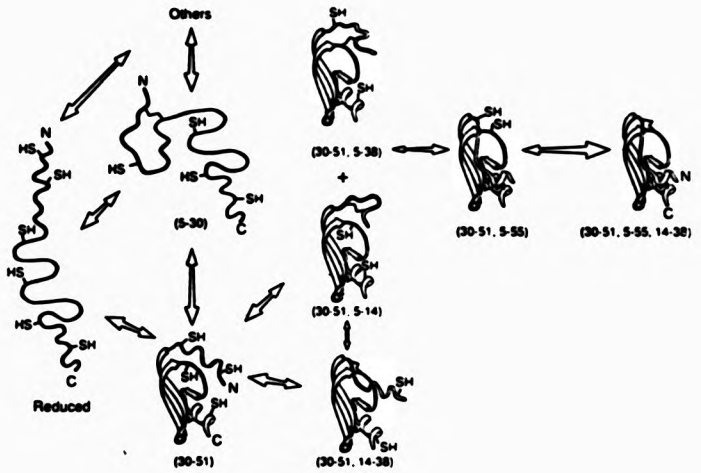


Fig. 1.1 Refolding pathway of bovine pancreatic trypsin inhibitor (from Creighton, 1990)

The polypeptide backbone of the protein is depicted by a smooth open line when its conformation is not regular or well-defined, by arrows for β-strand conformation, and by a coil for α-helix. The positions of the six cysteine residues are indicated.

others are converted by intramolecular rearrangements. This (30-51, 5-55) intermediate readily forms the third disulphide between Cys 14 and Cys 38, which are in close proximity, consistent with (30-51, 5-55) having native-like conformation.

The conformations of the trapped intermediates of BPTI have been studed by n.m.r (States, 1987). The structures proposed for the different intermediates have been used to provide a description of the folding of BPTI in terms of specific conformational transitions. Intermediates trapped during reoxidation and consequent refolding of reduced BPTI by alkylating free thiols were studied by n.m.r. It was found that conformational features of the native protein was present in the intermediate containing just one of the three normal disulphide bonds (30-51). In this intermediate the central β-sheet structure exists in a similar conformation to that of the native BPTI but there are differences in details of the conformation, resulting in a less tightly folded structure. As the intermediate (30-51) possesses native-like structure, this accounts for its importance in folding such that its predominance in the folding process can be explained by energetically favourable conformational features which direct the protein towards the correctly folded state. No additional native-like structure is introduced with formation of either 5-14 or 5-38 disulphide bonds, neither of which are found in the native structure.

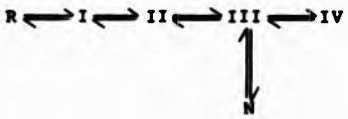
The intermediate (30-51, 14-38) which contains two correct disulphide bonds is distinctly more similar to native BPTI than (30-51), (30-51, 5-14) or (30-51, 5-38). In this intermediate the N-terminal portion and some of the helical regions of structure are formed but the N-terminal is not as stable as in native BPTI due to

the 5-55 disulphide bond not being present. The intermediate (30-51, 5-55) which is the most stable two-disulphide species is very similar to the native protein which suggests that all regions of structure must be present.

The folding pathway of BPTI has also been studied using a mutant form of BPTI in which Cys-14 and Cys-38 have been mutated to Ser (Goldenberg, 1988). The altered cysteines are normally paired in a disulphide bond in the native protein but also form disulphide bonds with Cys-5 in two disulphide intermediates during folding. The mutant protein was shown to fold efficiently, though the kinetics of folding and unfolding were altered. The observed rate of unfolding of the mutant was lower than the wild type, demonstrating that the altered cysteines are involved in the intramolecular rearrangements that are the rate-determining steps in the unfolding of the wild type protein.

Bovine pancreatic ribonuclease A is probably the protein most extensively studied with regard to protein folding. It consists of 124 amino acids and contains four disulphide bonds which stabilise the native structure. The refolding of reduced (S-S bonds broken), denatured ribonuclease A has been studied by Creighton (1978) using the quenching technique described for BPTI. The folding pathway involved the separation of the native conformation with four disulphide bonds from all other species by a high energy barrier, slowing down formation of the final two disulphide bonds after formation of the first and second one.

Thus, the disulphide bonds formed initially in reduced ribonuclease do not favour further disulphide bond formation, but rather hinder it (Creighton, 1978). The overall mechanism may be summarised as follows:-



where R and N are fully reduced and native protein respectively, and I, II, III and IV represent all the intermediates with the respective numbers of intramolecular disulphide bonds. These intermediates appear to have little non-random conformation (Jaenicke, 1987).

Ribonuclease A in its non-reduced (S-S bonds intact), denatured form has also been used to study the role of proline isomerisation in protein folding. The existence of fast- and slow-folding species, U_F and U_S, was first detected for ribonuclease A by Garel and Baldwin (1973). A model was proposed by Brandts et al. (1975) based on the biphasic kinetics observed during denaturation which suggested that the fast phase represented unfolding, whereas the slow phase represented cis-trans isomerisation of proline residues in the denatured protein. Ribonuclease A contains four proline residues, two of which (Pro-93 and Pro-114) are cis in the native state. At equilibrium ribonuclease A in its unfolded state consists of a mixture of $\sim$ 20% of a fast-folding species, U_F and $\sim$ 80% of a slow-folding species, U_S (Garel and Baldwin, 1973).

There are two main slow folding species of ribonuclease A, a major one, U_S^n (65–70% of all unfolded molecules) and a minor one, U_S' (10–15%). The U_S^n molecules have an incorrect proline isomer only at Pro-93 whereas U_S' molecules have additional incorrect proline residues. Thus most, if not all, slow-folding molecules contain an incorrect trans Pro-93 (Schmid et al., 1986). The refolding of U_S^n involves structural intermediates I_I and I_N. I_I is an open, hydrogen bonded intermediate which is formed rapidly from U_S^n and I_N is a native-like, enzymatically active species which is separated from the fully native state by a slow isomerisation process. The U_S^n molecules have a wrong proline isomer only at Pro-93 and under favourable conditions they can refold with a trans Pro-93 to the native-like I_N state. The final slow $I_N \longrightarrow N$ step consists of a trans $\longrightarrow$ cis isomerisation of Pro-93 which is accelerated by prior structure formation (Schmid et al., 1986). The refolding pathway for U_S^n can be written as

$$U_S^n \xrightarrow[\text{folding}]{\text{rapid}} I_I \xrightarrow{\text{folding}} I_N \xrightarrow[\text{Pro 93}]{\text{trans} \rightarrow \text{cis}} N$$

The isomerisation of Pro-93 on the minor $U_S' \longrightarrow N$ pathway is significantly slower. The presence of additional incorrect isomers could impair the formation of I_N-like structure on this pathway and thereby decrease the rate of refolding and trans $\longrightarrow$ cis isomerisation at Pro-93.

The presence of two kinetic intermediates on the folding pathway for ribonu ease A has been shown, but little was known of the structure and stability of these intermediates. The early hydrogen-bonded intermediate was stud d by using a two-dimensional n.m.r. technique to monitor the exchange of protons between the peptide chain amide groups and solvent (Udgaonkar and Baldwin, 1988). A stable secondary structural intermediate was found to be formed rapidly, providing the framework necessary for the subsequent formation of the complete tertiary structure.

1.6.2 Multi-domain proteins

Many larger monomeric proteins consist of domains which act as independent folding units. The independence refers to *in vitro* folding, as well as *in vivo* translation experiments where nascent multi-domain proteins have been shown to 'fold by parts' in a co-translational fashion. This enables large polypeptide chains to speed up folding and at the same time minimize wrong intramolecular long-range interactions (Jaenicke, 1987). Several multi-domain proteins have been studied, these include α-lactalbumin, phosphoglycerate kinase, thermolysin and penicillinase which is discussed below.

Penicillinase (M_r 29 000) consists of three domains which can be prepared by limited proteolysis. Two intermediates have been observed during unfolding as in the following scheme.

$$N \rightleftharpoons I \rightleftharpoons H \rightleftharpoons U$$

H is a substantially ordered state and I is a 'collapsed'

native-like state, which is in slow equilibrium with N and fast equilibrium with H (Hitchinson and Pain, 1985). During refolding after denaturation the native secondary structure is formed fast whereas recovery of the native tertiary structure is slow. It has also been shown by fragmentation and subsequent reconstitution of penicillinase that domain pairing participates in the overall folding mechanism. Cleavage by cyanogen bromide produces three inactive fragments of similar size which can associate with each other. This complex is enzymatically inactive but it contains native secondary structure and antigenic specificity. The complex also undergoes reversible unfolding in guanidinium chloride, showing the importance of domain pairing in the overall folding mechanism.

Folding units that form subdomains or domains may be considered obligatory kinetic intermediates on the folding path, even if their individual structure formation cannot be neatly separated along the time scale (Jaenicke, 1987). Apart from eliminating 'wrong aggregation' they enhance the folding rate of large protein molecules, thus avoiding proteolytic degradation of the nascent polypeptide chain.

1.6.3 A general model for the folding of monomeric proteins

Protein folding does not occur by a random search pathway as the time scale of protein refolding is too rapid. Thus, protein folding must be directed kinetically such that a limited number of pathways are accessible to the unfolded (nascent) polypeptide chain. A number of refolding pathway models have been proposed in order to limit the number of possible conformations into which a protein may fold. In the sequential folding model, folding occurs

in a unique and definite sequence of steps with populated intermediates. To demonstrate sequential folding, it is necessary to show that there are specific, well populated intermediates. These have been found in the major folding reaction of Ribonuclease A ($U^{n}_{S} \longrightarrow N$) and in the refolding/reoxidation of reduced BPTI. In the presence of guanidinium chloride some proteins (i.e. α-lactalbumins) were shown to have two conformational transitions, suggesting the existence of intermediate forms of proteins with a secondary structure but without the specific tertiary structure. The sequential folding model may be considered a general folding mechanism for small proteins or individual compact domains of more complex structures (Kim and Baldwin, 1982; Goldberg, 1985).

Two working models are currently popular, the framework model, in which protein folding begins with the formation of secondary structure in an unfolded chain and the modular assembly model, in which protein folding begins with the independent folding of separate parts of the protein molecule. These two models may be combined. Formation of secondary structure may precede tertiary interactions, as in the framework model, while separate subdomains (each capable of forming its own structure) may fold at different times as in the modular assembly model (Kim and Baldwin, 1982; Ptitsyn, 1987).

The majority of studies on the refolding of proteins support a framework model of folding in which the secondary structure is formed at an early stage. However, the secondary structure even of a small protein may be formed in distinct stages. The framework model was first suggested by the finding that the secondary structures of some small proteins are more

resistant to unfolding by denaturants than their tertiary structures. According to this model, protein folding consists of at least three stages (as shown in Fig. 1.2): (1) the formation of native secondary structure in an unfolded chain, (2) the merging of pre-existing blocks with secondary structure to an intermediate globular structure, and (3) local adjustment of this intermediate structure to the tightly packed native tertiary structure. The essential feature of this model is that different states are stabilised by different interactions: (1) secondary structure, by peptide hydrogen bonds; (2) intermediate globular structure, by hydrophobic interactions; and (3) specific tertiary structure, by van der Waals interactions.

Initially there is a collapse from the denatured random coil conformation to a secondary structure state. This is followed by further collapse to a compact intermediate state. This intermediate has a globular conformation with a dense interior and a high secondary structure content, which is qualitatively similar to that of the native state. However, the amino acid side chains are less tightly packed and are more mobile than in the native state. The mobility of the side chains occurs as most of the van der Waals intramolecular interactions present in the native state are not yet formed. This intermediate state has been termed a 'molten globule' (Dolgikh *et al.*, 1981; Ohgushi and Wada, 1983) or 'compact intermediate' (Creighton, 1990). The characteristics of this state have been studied in conditions in which it is stable, at acid pH, high temperatures and intermediate concentrations of strong denaturants. Studies of several proteins have shown that the secondary structure and compactness of proteins are restored when

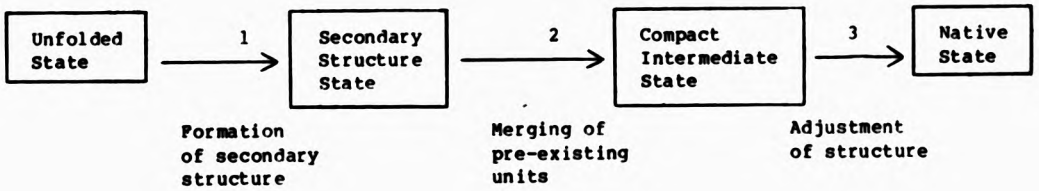


Fig. 1.2 A general model for the folding of monomeric proteins. Stages 1, 2 and 3 involve predominantly hydrogen bonds, hydrophobic interactions and van der Waals interactions respectively.

they undergo folding much more rapidly than their specific tertiary structure and their activity. It has also been shown that amide hydrogen bonds corresponding to native secondary structures in ribonuclease A and cytochrome c are formed early in folding, thus directly confirming the framework model (Udgaonkar and Baldwin, 1988; Roder et al., 1988). Recently, it has been found that the molten globule state is formed within 0.1 - 0.2 seconds (Ptitsyn et al., 1990). As the molten globule has been found in proteins belonging to different structural types, with and without disulphide bridges, and includes proteins with different times of complete folding, it has been proposed that the formation of the transient molten globule state occurs early on the pathway of refolding of all globular proteins (Ptitsyn et al., 1990).

The formation of this 'compact intermediate' or molten globule state limits the number of folding reactions accessible to a protein and thereby 'channels' and accelerates the protein folding towards the native state. The subsequent adjustments of structure generally form the rate-limiting steps in folding and involve further folding processes, the making and breaking of disulphide bonds and non-covalent interactions.

1.7 The folding and assembly of multi-subunit proteins

The folding of multi-subunit (oligomeric) proteins is more complex than the folding of monomeric proteins as it involves both folding and association reactions. The early stages during the refolding of oligomeric proteins are presumably similar to those involved in the refolding of monomeric (single chain) proteins.

Thus, a subunit polypeptide chain will first fold into subdomains or domains. These will then merge to form the 'structural monomer' with native-like tertiary structure. The final step is association and further folding to yield the native state. It is possible to study the association steps of a protein by using denaturing conditions which cause dissociation of the protein subunits but not loss of native secondary and tertiary structure. The formation of structural intermediates can be achieved by exposure of the native protein to low temperature, high pressure, low ($\sim$ 1 M) guanidinium chloride concentrations, that are close to the oligomer-monomer transition, or addition of stabilizing ions. The presence of 1 M Na_2SO_4 at low pH stabilizes the 'structural monomer' and prevents complete unfolding (Jaenicke, 1987). The most versatile method of investigating structured intermediates on the pathway of folding and association is the kinetic approach using reassociation instead of dissociation.

The refolding of monomeric proteins is determined by sequential or parallel first-order folding reactions as rate-limiting steps. In the refolding of oligomeric proteins second-order association steps may be rate-determining. Folding steps must precede association in order to provide the surface areas for correct subunit recognition but they must also succeed association as a consequence of the exclusion of water from the subunit interfaces that serve to stabilize the native quaternary structure (Jaenicke and Rudolph, 1986).

The kinetics of reactivation of a protein can be studied by using the concentration dependence of the rate of regain of activity to indicate whether or not association reactions are involved. For

a dimeric protein reactivation follows second-order kinetics when folded monomers are inactive and the rate-limiting step is association to produce an active dimer. If the folded monomers were found to possess activity then reactivation would follow first-order kinetics. The kinetics of reactivation of a dimeric protein only involve one association step but with more complex multi-subunit proteins such as tetramers and hexamers more than one association reaction is involved. For a protein that has more than one association step it can be difficult to distinguish which association step is involved in the regain of full activity. In order to analyse the kinetics of association in more detail, isolation and characterisation of the intermediates involved in association is necessary.

The technique of chemical cross-linking using glutaraldehyde as a bifunctional reagent followed by SDS-polyacrylamide gel electrophoresis allows the detection and quantitative evaluation of intermediates of association (Hermann et al., 1981; Jaenicke and Rudolph, 1986). To provide an accurate analysis the following requirements have to be fulfilled: (1) intramolecular cross-linking of associated particles must be quantitative; (2) the cross-linking reaction must be fast compared to the assembly process under consideration; (3) the cross-linking conditions and the reaction itself must not interfere with the assembly and (4) intermolecular cross-linking of particles must be negligible. The conditions to fulfil these requirements have to be optimized for each oligomeric protein (Jaenicke, 1987). This method cannot be applied to all oligomeric proteins as for some proteins only partial cross-linking occurs and for some proteins no cross-linking occurs. In general incomplete cross-linking may be

explained by unfavourable distribution of reactive lysine side chains (Jaenicke and Rudolph, 1986).

Another method which can be used to determine the intermediates of association is hybridisation. This method involves stopping reassociation of a certain oligomer by a chase with an excess of electrophoretically different subunits. These may be chemically modified subunits or isoenzyme subunits of the same oligomer. At various reassociation times these different subunits are added in excess and form hybrids with 'incomplete' intermediates present in the refolding mixture. The various species formed can be separated and quantified by gel electrophoresis. The results are correct only if the modified subunits do not affect the assembly mechanism e.g. by subunit exchange (Jaenicke and Rudolph, 1986).

Many studies have been carried out on the refolding of multi-subunit proteins and lactate dehydrogenase is one example which is discussed below. The refolding of the tetrameric lactate dehydrogenase from pig heart and skeletal muscle has been extensively studied by Jaenicke and co-workers. To analyse the assembly mechanisms, cross-linking experiments were carried out. The results showed dimer formation predominates in the early phase of reconstitution and the corresponding decrease in monomer concentration obeys first order kinetics. The monomer to dimer equilibrium reaction is rapid ($K=3x10^{8}\ M^{-1}$), with a rate close to that of a diffusion controlled reaction. The scheme for the pathway of refolding may be written

$$4\,M' \xrightarrow{k_1} 4\,M \underset{\text{fast}}{\rightleftharpoons} 2D \xrightarrow{k_2} T$$

where M' and M represent different conformational states of the monomer, D the dimer and T the tetramer. The values of k_1 and k_2 are 0.8×10^{-3} sec and 3×10^{4} M^{-1} sec^{-1} respectively for the skeletal muscle enzyme. The slow M'$\longrightarrow$M transition is preceded by fast folding steps restoring $\geq$ 75% of the native secondary structure, as measured by circular dichroism, within 10 seconds. The above scheme shows that there is one slow-folding step at the monomer level which is rate-limiting for the formation of the dimeric intermediate. The association of the 'structured' monomer to the dimer is fast. Tetramerisation parallels reactivation thus T is only active species. At sufficiently low enzyme concentrations, the association of dimers to tetramer becomes very slow, effectively reaching completion only after 24 hours.

The dimeric intermediate can be trapped in a stable configuration by the addition of thermolysin during refolding (Girg et al., 1981). End group analysis of these dimers showed that the N-terminal acetylation present in the native enzyme was missing. This implied that a number of amino acid residues had been cleaved from the N-terminal region and that these residues were required for stabilisation of the native tetrameric configuration. It has been shown by X-ray crystallography that the dimer of dimers is held together by an N-terminal arm of approximately 20 amino acids (Holbrook et al., 1975). The sites of cleavage in this arm are only accessible during refolding and not in the native enzyme. Further studies on the isolated dimeric intermediate (Girg et al., 1983) showed that it had spectroscopic properties similar to the native enzyme. The dimeric intermediate is inactive in the

standard enzyme assay but in the presence of 'structure-making' ions such as $(NH_4)_2SO_4$ the dimers exhibit approximately 40-50% of the native catalytic activity.

More recently, the proteolytic dimers have been shown to be heterogenous and are comprised of species with cleavage sites additional to the N-terminal arm (Opitz et al., 1987). These internally 'nicked' dimers are able to reassociate following denaturation in guanidinium chloride, indicating that separate domains or fragments of a protein may be able to fold and associate correctly even without interconnecting stretches of polypeptide. This supports the possibility that large proteins may fold by parts with the association of domains as a consecutive process.

1.7.1 General mechanism for the assembly of multi-subunit proteins

The assembly of multi-subunit proteins involves one or more first-order folding steps to generate monomeric intermediates, these can then associate to form dimers and larger assemblies. It is possible that intermediates in the assembly pathway undergo kinetically significant folding steps in order to develop the correct interface for further association. A general mechanism would involve a sequence of folding reactions and association steps as follows:-

$$nM' \longrightarrow nM \longrightarrow \frac{nD'}{2} \longrightarrow \frac{nD}{2} \longrightarrow \frac{nT'}{4} \longrightarrow \frac{nT}{4} \longrightarrow - - -$$

where n = number of subunits and M', D', T' represent different conformations of the monomer (M), dimer (D) and tetramer (T) respectively (Jaenicke, 1987).

The rate constants for the various steps (and hence the nature of the rate-determining step) will vary between different proteins but in all cases there is an initial folding step in which a large proportion of the native secondary structure of the protein is generated. This folding step occurs quickly, within a few seconds, and in some cases the folded monomer may possess biological activity. In a number of cases the rapidly-formed folded monomer must undergo further slow rearrangement before association can occur (Price, 1990).

1.7.2 Specificity of association

The specificity of subunit recognition has been examined as the assembly of multi-subunit proteins *in vivo* occurs in the presence of high concentrations of subunits of other proteins. Early evidence for the specificity of association was obtained by Cook and Koshland (1969) who studied the unfolding and refolding of mixtures of multi-subunit proteins. No cross hybrids between subunits of different enzymes were found which suggested intersubunit binding sites are highly specific. More recently, Gerl *et al.*, 1985), studied the joint 'synchronous' reconstitution of two closely related dimeric, NAD-dependent dehydrogenases, pig mitochondrial malate dehydrogenase and D-lactate dehydrogenase from *Limulus polyphemus*. There was no evidence of hybrid proteins which confirms the conclusion that subunit recognition is highly specific. Thus, even in the heterogeneous system of the cytoplasm it is likely that only the correct quaternary structure of a protein is formed.

1.7.3 Side reactions

The structural and functional properties of an oligomeric protein are hardly ever fully restored after denaturation and subsequent renaturation. The major side reaction in the refolding and reassociation of oligomeric proteins *in vitro* is the formation of high molecular weight aggregates. Circular dichroism studies in the far-u.v. suggested that these aggregates are composed of individual monomeric chains with partially restored secondary structure (Zettlmeissl *et al.*, 1979). The formation of aggregates is favoured at high concentrations as the association reactions leading to aggregate formation are generally of a higher kinetic order than those leading to the formation of the native protein. Thus, renaturation and aggregation compete with each other and their relative proportions depend on both the denaturation and renaturation conditions (Teipel and Koshland, 1971; Jaenicke and Rudolph, 1986).

1.8 Translocated proteins

Many proteins which are synthesised in the cytoplasm are located in intracellular organelles or are secreted from cells. In both cases this involves translocation of proteins across biological membranes. The majority of these proteins are synthesised as precursors with cleavable amino-terminal signal sequences. The signal sequence of a protein directs the nascent polypeptide chain across the appropriate membrane.

Studies of secretory protein translocation across the endoplasmic reticulum membrane seemed to show that proteins were translocated only while they were still being synthesised

(co-translational translocation). The signal sequence of a secreted protein is translated first and this binds to the signal recognition particle which then binds to a receptor in the endoplasmic reticulum membrane. The growing polypeptide chain is then translocated directly across the membrane. Soon after the amino-terminal signal sequence has transversed the membrane, it is removed by a signal peptidase located in the lumen of the endoplasmic reticulum. Further studies showed that co-translational translocation is not obligatory for movement of proteins across the endoplasmic reticulum membrane and post-translational translocation of completed polypeptide chains can occur. Post-translational translocation of proteins was shown to occur across other membranes such as bacterial, mitochondrial and chloroplast. It has been proposed that the mechanism of translocation may be fundamentally similar for all membrane systems (Schatz, 1986).

Mitochondrial DNA codes for less than 10% of the mitochondrial proteins, therefore the majority of mitochondrial proteins are imported. Most mitochondrial proteins are synthesised as precursor proteins on cytosolic polysomes and are released as completed polypeptide chains into the cytosol. Nearly all of these precursors contain amino-terminal extensions which target them to the mitochondria. In some cases targeting information is also contained in non-amino-terminal portions of the precursor protein. The precursors bind to specific receptor proteins on the mitochondrial surface and are then translocated across the mitochondrial membranes. The amino-terminal extensions are cleaved off by one or more processing peptidases in the mitochondrial matrix.

The amino-terminal presequences of mitochondrial precursors range from 10-70 amino acid residues in length. The presequences not only direct the precursors to the mitochondria but are also important for intramitochondrial sorting. The presequences direct proteins to one of four possible locations in the mitochondria; matrix, inner membrane, intermembrane space and outer memberane. Sequence analysis of the cleavable presequences of several mitochondrial proteins showed no significant sequence homology but most share similar overall characteristics (Hurt and van Loon, 1986).

The mitochondrial presequences each contain a mitochondrial targeting domain which is rich in positively-charged and hydroxylated amino acids and lacking in negatively-charged amino acids. The positively-charged amino acids (mainly arginine and lysine) are arranged periodically along the presequence. These mitochondrial targeting signals, as shown by gene fusion experiments, are sufficient to direct non-mitochondrial proteins into the mitochondrial matrix. The presequences of proteins exposed to, or present in, the intermembrane space contain a long stretch of uncharged amino acids adjacent to the amino-terminal matrix. These hydrophobic sequences were proposed to prevent transport of the attached proteins across the mitochondrial inner membrane and were termed 'stop-transport' domains (Hurt and van Loon, 1986). More recently a 'conservative sorting' pathway has been proposed for intermembrane space proteins, in which they first enter the matrix and are then re-translocated across the inner membrane (Hartl et al., 1987). The proteins of the outer membrane lack cleavable presequences but the amino-terminal sequence of the mature proteins resembles presequences of intermembrane space

proteins. The matrix targeting domain is followed by a hydrophobic sequence which anchors the protein in the outer membrane (Hurt and van Loon, 1986).

The mitochondrial targeting signals show a tendency to form amphiphilic α helices, with positively-charged and hydrophobic residues exposed to opposite faces (Roise and Schatz, 1988). This amphiphilicity may be important for the initial membrane insertion or the interaction with specific receptor proteins at the mitochondrial surface, or both (Hartl and Neupert, 1990).

The mitochondrial precursors bind to specific receptor proteins on the surface of the mitochondrial outer membrane. The receptor proteins are distributed over the surface of the outer membrane, but are enriched at contact sites between the inner and outer membranes where translocation occurs. The receptor-bound precursors are then thought to interact with a general insertion protein (GIP) present in the outer membrane (Pfaller et al., 1989). After interaction with GIP the import pathways of the precursors diverge to direct proteins to the outer membrane or to contact sites and thus to other mitochondrial compartments (Hartl and Neupert, 1990).

Precursors of outer membrane proteins are inserted into the outer membrane after binding to the specific receptors and are then assembled to their functional forms. Translocation of precursors into the inner membrane or matrix occurs at translocation contact sites, where the outer and inner membranes come so close together that they can be spanned by the precursor polypeptide chains (Schleyer and Neupert, 1985). Entry into mitochondria via translocation contact sites is probably used by most proteins of the

intermembrane space, although their sorting and assembly pathways are more complicated. Generally, intermembrane space proteins seem to be completely translocated across both mitochondrial membranes. They are then redirected from the matrix back across the inner membrane to the intermembrane space (conservative sorting). In some cases the precursors contain a bipartite presequence in which the matrix targeting signal and the sorting signal for retranslocation of the intermediate are linearly arranged and act sequentially (Hartl et al., 1989). One exception to this sorting pathway for intermembrane space proteins is cytochrome c, which has no cleavable presequence and its import is independent of a membrane potential across the inner membrane. A number of inner membrane proteins seem to be sorted via the matrix compartment. It cannot be excluded, however, whether some integral membrane proteins diffuse laterally from translocation contact sites into the inner membrane (Hartl et al., 1989).

The amino-terminal presequences of mitochondrial proteins are proteolytically removed by one or more matrix located processing peptidases during or after translocation across the mitochondrial membranes. Two structurally related components cooperate in the proteolytic processing, the mitochondrial processing peptidase (MPP) and the processing enhancing protein (PEP) (Hawlitschek et al., 1988). PEP stimulates the catalytic activity of MPP. It is not known how MPP and PEP interact with their substrate and with each other. PEP may bind to the presequences of incoming proteins, thus exposing the cleavage site toward MPP (Hartl and Neupert, 1990).

Most of the intermembrane space proteins and inner membrane proteins which protrude into the intermembrane space are

proteolytically processed in two steps. In the first step the processing peptidase in the matrix cleaves the precursors to an intermediate form. The second processing step is carried out by an additional protease located on the intermembrane space surface of the inner membrane.

The initial insertion into, and translocation of, the presequence across the inner membrane requires a membrane potential. The electrical potential ($\Delta\psi$) across the mitochondrial inner membrane is negative inside and it is assumed that the positively-charged presequence may cross the inner membrane electrophoretically. Once the amino-terminal presequence is translocated into or across the inner membrane the remaining part of the precursor protein is translocated independently of the membrane potential (Schleyer and Neupert, 1985). Precursors of the outer membrane proteins do not require a membrane potential for import.

Import of proteins into mitochondria also requires nucleoside triphosphates (NTPs) either ATP or GTP in addition to the membrane potential (Pfanner and Neupert, 1986). This NTP dependence is not unique to the mitochondrial system as protein translocation across bacterial, chloroplast and endoplasmic reticulum membranes have also been found to require ATP (Zimmermann and Meyer, 1986). NTPs are required for both the initial interaction of precursor proteins with the mitochondrial surface and for the completion of translocation into the mitochondria (Chen and Douglas, 1987a). For completion of translocation higher levels of NTPs are necessary than for the initial binding interaction. Import of precursors having identical presequences, but different mature protein parts, have been found to require different levels of NTPs.

Non-hydrolysable ATP analogues are not able to fulfil the NTP requirement indicating that cleavage of a phosphodiester bond is necessary. It was demonstrated that the sensitivity of precursor proteins in reticulocyte lysate to proteases is greater in the presence of NTPs (Pfanner et al., 1987). Thus, it was proposed that the hydrolysis of NTPs is involved in modulating the folding of precursors in the cytosol, thereby conferring an import-competent, loosely folded, conformation.

Post-translational import of precursor proteins into mitochondria involves at least partial protein unfolding to allow transport across the mitochondrial membranes. This was shown by Eilers and Schatz (1986) who studied a fusion protein in which the first 22 residues of the presequence of yeast cytochrome oxidase subunit IV (an imported protein) was fused to the N-terminus of mouse dihydrofolate reductase, DHFR, (a cytosolic protein). The resulting fusion protein is imported and proteolytically processed by isolated yeast mitochondria or by mitochondria in living yeast cells. Addition of methotrexate, a folate antagonist, stabilises the DHFR moiety and prevents unfolding of the protein, thus blocking import of the fusion protein.

Another study which showed that unfolding of mitochondrial precursors is required for membrane translocation was carried out by Chen and Douglas (1987b). Addition of copper blocked import of the F_1 β-subunit precursor when its carboxyl-terminal 129 residues were replaced with 61 residues of yeast copper metallothionein. Concentrations of the cation which inhibited the βCuMP hybrid had no effect on the post-translocational import of the wild type β-subunit. These data indicate that the binding of copper by

βCuMT renders it refractile for partial unfolding which is required for translocation into the mitochondria.

The role of NTPs in keeping precursor proteins in an import competent conformation is supported by two observations. The first is that the import of *in vitro* synthesised porin needs NTPs whereas the import of water-soluble porin prepared by acid-base treatment of the purified membrane form is independent of NTPs. Other import properties such as binding to a specific receptor protein, two-step insertion into the outer membrane and formation of specific membrane channels are identical for both precursors. Thus, the acid-base treatment, which destabilizes the conformation of proteins, can replace the NTP-dependent step of mitochondrial protein import (Pfanner *et al.*, 1988). The second observation is that the import of incompletely synthesised polypeptide chains into mitochondria requires lower levels of NTPs than the import of the corresponding completed precursor (Verner and Schatz, 1987). The likely explanation is that the incomplete polypeptide chains are only loosely folded and therefore requires less NTPs for unfolding than the completed chains.

An ATP-driven 'unfoldase' which rapidly translocates along its polypeptide substrate, unfolding the polypeptide chain, was suggested by Rothman and Kornberg (1986). As protein unfolding requires energy, the enzyme consumes ATP as it moves along the polypeptide chain. This idea is consistent with the suggestion that an ATP-dependent activity is present in the cytosol that maintains precursors in a translocation competent state (Pfanner *et al.*, 1987).

The folding and assembly of translocated proteins may well require factors additional to those listed earlier (Section 1.5.2). Studies on the assembly of the photosynthetic CO_2 fixing enzyme, ribulose biphosphate carboxylase-oxygenase (Rubisco), implicated that a chloroplast protein was involved in its assembly (Ellis, 1987; Hemmingsen et al., 1988). Rubisco consists of large (L) and small (S) subunits with the subunit structure L_8S_8. The L subunits are synthesised within the chloroplast whereas the S subunits are synthesised in the cytosol and translocated into the chloroplast. Newly synthesised large subunits are associated with an abundant chloroplast protein, termed the Rubisco subunit binding protein, which keeps the L subunits in solution. The L subunits are released from the binding protein when S subunits are available to form the correct L_8S_8 structure. The Rubisco binding protein acts to prevent the formation of incorrect structures and directs the folding and assembly of the oligomeric protein. The binding protein is not part of the final oligomer thus the idea of 'chaperone proteins' was developed. Other examples of 'chaperone proteins' are the immunoglobulin heavy-chain binding protein (BiP), heat shock protein, hsp 70, gro EL and gro ES proteins and hsp 60 which is present in the mitochondria. These examples are discussed in more detail in Chapter 7.

1.9 Aim of the work described in this thesis

The aim of this project was to study the unfolding and refolding of some translocated multi-subunit enzymes and compare the results where possible with data from corresponding non-translocated enzymes. This comparison should indicate the way in which translocation affects the folding and assembly of such proteins. In general, unfolding of enzymes by GdnHCl was studied by monitoring loss of catalytic activity and changes in structure by fluorescence, circular dichroism and exposure of reactive thiol groups. Refolding was studied by measuring the regain of catalytic activity after removal of the denaturing agent.

A separate chapter is devoted to each of the enzymes used in this study:-

Chapter 3	-	glutamate dehydrogenases from bovine liver (which is translocated), baker's yeast and *Clostridium symbiosum*.
Chapter 4	-	aspartate aminotransferase (cytoplasmic) from pig heart.
Chapter 5	-	aspartate aminotransferase (mitochondrial) from pig heart.
Chapter 6	-	citrate synthase from pig heart.

Chapter 7 is devoted to an overall discussion of the results from Chapters 3 - 6 and discusses the main conclusions in the context of present theories concerning the folding of translocated proteins.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

Guanidinium chloride Aristar grade was purchased from BDH Chemicals.

Solutions of Guanidinium chloride (GdnHCl) were made up by weight (i.e. for a 6 M solution require 0.573 g/ml). The concentrations of the solutions were checked by refractive index measurements (Nozaki, 1972).

5,5'-dithiobis-(2-nitrobenzoic acid) was purchased from Koch-Light Laboratories.

Dalton Mark VII-L (SDS-PAGE M_r Markers) was purchased from Sigma Chemical Co.

All chemicals were analar grade and were purchased from BDH Chemicals or Sigma Chemical Co.

Double-distilled water was used in all experiments.

STATEMENT OF REPRODUCIBILITY

The data presented in this thesis represent the means from at least two experiments with a variation within 10%. Note that the purification procedures shown in Tables 3.1, 4.1 and 5.1 were performed on at least three separate occasions and in each case the recovery and specific activity of the final preparation was within 10% of the values shown.

2.2 METHODS

2.2.1 Determination of protein concentration

Protein concentration was determined by the method of Sedmak and Grossberg (1977) except for the purified enzymes, when the A_{280} was measured. A protein sample was diluted with distilled water to a volume of 1 ml and to this 1 ml of Coomassie reagent was added. The Coomassie reagent consisted of 0.06% (w/v) Coomassie Brilliant Blue G250 in 3% (v/v) perchloric acid, which was filtered through Whatman No.1 filter paper. The absorbance of the protein solution was measured at 620 nm and 465 nm against an absorbance blank of distilled water. The absorbance ratio 620/465 was calculated and the absorbance ratio of a blank (1 ml distilled water + 1 ml Coomassie reagent) was subtracted.

The value obtained was used to determine the protein concentration from a calibration curve, constructed using concentrations of bovine serum albumin between 0 and 40 µg/ml. A bovine serum albumin standard was included in each set of protein determinations. A 1 mg/ml solution of bovine serum albumin gives an absorbance at 280 nm of 0.62.

2.2.2 SDS-Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed by the method of Laemmli (1970). The gels contained acrylamide at a concentration of 12% and were 1.5 mm in thickness.

The separating gel contained 10 ml separating gel buffer (1.5 M Tris-HCl, pH 8.7, 0.4% SDS), 14 ml distilled water, 16 ml acrylamide stock solution (30% acrylamide: 0.8% bis-acrylamide), 30 µl TEMED, 140 µl 10% ammonium persulphate. The stacking gel contained 5 ml stacking gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), 12.2 ml distilled water, 2.68 ml acrylamide stock solution, 20 µl TEMED, 110 µl 10% ammonium persulphate. Gels could be prepared and stored at 4°C for a few days before using.

The reservoir buffer 5 x concentrated contained 0.125 M Tris, 0.96 M Glycine, 0.5% SDS and was diluted 1 in 5 before using.

Samples were prepared by diluting 1:1 with sample buffer 2 x concentrated (30 ml 10% SDS, 12.5 ml stacking gel buffer, 10% Glycerol, pH 6.8 with HCl) containing 2 µl 2-Mercaptoethanol/ml. They were heated in a boiling water bath for 2 min then 5 µl of a 1:1 mixture of 1% bromophenol blue with 2-Mercaptoethanol was added per 100 µl of boiled sample.

Samples were applied to gels using a Hamilton syringe and the gels were run at a constant current of 60 mA until the marker dye was near the bottom of the separating gel (approx. 4 hours). Gels were stained for protein for 1 hr with Coomassie blue stain (0.1% PAGE Blue 83 in acetic acid, methanol and distilled water, 2:5:5) and destained with 10% acetic acid.

A standard protein solution (which is used for determination of the protein M_r) was prepared for each gel. Sigma Dalton Mark VII -L was made up as a 2 mg/ml solution in distilled water and stored below 0°C. Standard protein solution was diluted 1:1 with sample buffer (2 x concentrated) and treated as

for samples. The standard proteins and their M_r were:- bovine albumin (66 000), ovalbumin (45 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (25 000), trypsin inhibitor (21 000), α-lactalbumin (14 000).

2.2.3 Fluorescence studies

2.2.3.1 Fluorescence spectroscopy

Fluorescence spectra were recorded in a Perkin-Elmer MPF 3A fluorimeter at 20°C. The excitation wavelength was 290 nm and the emission spectra was recorded between 300 and 400 nm. Enzyme samples were incubated in buffer in the absence or presence of GdnHCl and spectra were recorded 15 min after addition of GdnHCl. Appropriate blanks were included containing buffer or buffer and GdnHCl and the fluorescence spectra were corrected using these blanks.

2.2.3.2 Fluorescence quenching

Quenching of protein fluorescence using acrylamide and succinimide was performed as described by Eftink and Ghiron (1984). The acrylamide and succinimide were recrystallised from ethyl acetate and ethanol respectively, before use. The measurements were performed in a Perkin-Elmer MPF 3A fluorimeter at 20°C, and the excitation and emission wavelengths were 295 nm and 325 nm respectively. The acrylamide and succinimide were made up fresh each day as 2.5 M stock solutions in the appropriate buffer.

Enzyme samples (1 ml) in the absence and presence of GdnHCl were titrated with acrylamide and succinimide up to a quencher concentration of 0.326 M. Protein fluorescence emission was recorded after each addition of quencher. The values recorded were corrected for protein dilution and 'inner filter' effects. The correction factor (c) for the inner filter effect, as described by Ward (1985), was calculated as

$$c = \frac{A_T\,(1 - 10^{-A_o})}{A_o\,(1 - 10^{-A_T})}$$

where A_o is the absorbance of the protein at 295 nm corrected for dilution

A_T is the combined absorbance of the protein and quencher at 295 nm.

Analysis of the corrected fluorescence quenching data to determine the fraction of fluorophores accessible to the quenchers was performed by a modified Stern-Volmer plot (Lehrer, 1971). Values of Fo/ ΔF were plotted against 1/Q where Fo is the corrected fluorescence value in the absence of quencher, ΔF is Fo minus the corrected fluorescence value at each quencher concentration, Q. A straight-line through the points and extrapolated back to the y-axis gives a value of 1/Fa at the point it crosses the y-axis. From this the Fa (fraction accessible) was calculated for acrylamide and succinimide at different GdnHCl concentrations.

2.2.4 Circular dichroism

Circular dichroism spectra for the glutamate dehydrogenase enzymes and the aspartate aminotransferase enzymes were recorded at 20°C in a Jobin-Yvon Dichrographe IV at Newcastle University. Spectra in the far u.v. (260-205 nm) and near u.v. (400-260 nm) were recorded in cells of path length 0.1 mm and 10 mm respectively.

Circular dichroism spectra for the citrate synthase enzyme were recorded at 20°C in a Jasco J-600 spectropolarimeter at Stirling University. Spectra in the far u.v. (250-205 nm) and near u.v. (320-260 nm) were recorded in cells of path length 1 mm and 10 mm respectively.

Samples of the enzymes in the absence and presence of GdnHCl were prepared and spectra were recorded 15 min after addition of GdnHCl. Appropriate blanks were included and measurements were corrected using these blanks.

For the glutamate dehydrogenase enzymes the circular dichroism spectra were normalised to zero ellipticity at 250 nm and the mean residual ellipticities were calculated at 5 nm intervals using the equation

$$[\Theta]_\lambda = \frac{m.\theta_\lambda}{10.d.c}$$

where m is the mean residue weight of an amino acid (112)

θ_λ is the observed ellipticity (degrees)

d is path length (cm)

c is protein concentration in g/ml

The units of $[\theta]_\lambda$ are $deg.cm^2\ dmol^{-1}$.

The $[\theta]_\lambda$ values were plotted against wavelength (nm).

For the aspartate aminotransferase and citrate synthase enzymes the data were collected and analysed on data processors attached to the instruments and the spectra were plotted as $[\theta]_\lambda$ against wavelength (nm).

2.2.5 Determination of reactive thiol groups

The number of reactive thiol groups per subunit in the enzymes was measured using 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2). Enzyme samples (0.2 mg/ml or 0.1 mg/ml) were incubated in 0.05 M sodium phosphate buffer, pH 7.4 at 20°C in the absence and presence of GdnHCl. Nbs_2 (10 mM) in 0.05 M sodium phosphate buffer, pH 7.4 (fresh each day) was added to the enzyme samples to a final concentration of 250 µM. The increase in absorbance at 412 nm on reaction with Nbs_2 was recorded and in each case the reaction was complete in 10 min. The A_{412} of a buffer blank was used to correct the A_{412} valves recorded at 10 min. The A_{412} of a 1 mM solution of thionitrophenolate, the product of the reaction, is 13.6 (Ellman, 1959). This value was used to obtain the concentration (in µM) of thiol groups in the samples from the corrected A_{412} valves. The number of reactive thiol groups/subunit was calculated using the concentration (in µM) of subunits in the samples.

2.2.6 Light-scattering

The changes in Mr of the enzymes on addition of GdnHCl were determined by light-scattering as described by Tashiro (1982). The measurements were performed at 20°C in a Perkin-Elmer MPF 3A fluorimeter. The excitation and emission wavelengths were 360 nm as used by Hand and Somero (1982). Sample solutions were filtered through a 0.2 μM pore-size filter before measuring, to ensure a dust free solution. A single fluorimeter cuvette was used to make all measurements. Enzyme samples were measured in the absence and presence of GdnHCl and control samples were measured for each GdnHCl concentration.

The data were analysed by the method of Parr and Hammes (1975). The ratio of molecular weight in the presence of GdnHCl, Mi, to that in the absence of GdnHCl, Mo, were determined from the following equation

$$\frac{Mi}{Mo} = \frac{(no)^2 (dn/dco)\mu^2 (Co) (Ii,90)}{(ni)^2 (dn/dci)\mu^2 (ci) (Io,90)}$$

where n = refractive index

$(dn/dc)\mu$ = the refractive index increment at constant chemical potential

c = enzyme concentration

I_{90} = scattered light intensity at 90°

At O M GdnHCl n = 1.333, refractive index of water. In the presence of GdnHCl ni = 1.333 + Δ Ni.

The Δ Ni values were taken from Table IV (refractive index and concentration of aqueous GdnHCl solution) by Nozaki (1972).

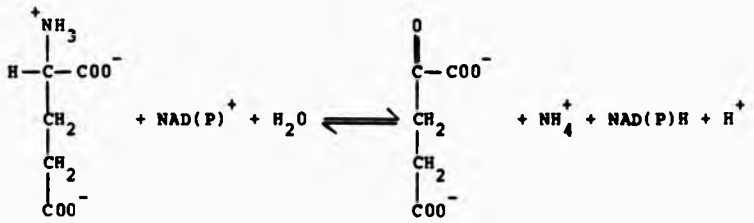
The values of $(dn/dc)\mu$ were taken from Fig.1 (refractive index increment of BSA at various GdnHCl concentrations) Noelken and Timasheff (1967).

CHAPTER THREE

THE UNFOLDING AND REFOLDING OF GLUTAMATE DEHYDROGENASES

3.1 INTRODUCTION

Glutamate dehydrogenases catalyse the interconversion of L-glutamate and 2-oxoglutarate. In the catabolic direction glutamate dehydrogenase is involved in amino acid degradation and catalyses the oxidative deamination of L-glutamate to 2-oxoglutarate, a citric acid cycle intermediate, and ammonia. In the biosynthetic direction, glutamate dehydrogenase is involved in the assimilation of ammonia into amino acids and catalyses the synthesis of L-glutamate from 2-oxoglutarate and ammonia. In the catabolic direction NAD^+ or $NADP^+$ is the oxidant whereas in the biosynthetic direction NADH or NADPH is the reductant.



L-Glutamate 2-oxoglutarate

There are three types of glutamate dehydrogenases which differ in co-enzyme specificity, those specific for NAD^+ or $NADP^+$ and those which can function with both. Many species contain both NAD^+ and $NADP^+$ specific glutamate dehydrogenases. Most glutamate dehydrogenases are allosterically regulated by purine nucleoside di- and triphosphates, the vertebrate enzymes are activated by ADP and GDP and inhibited by GTP and ATP. The enzyme is activated when the energy supply is low and inhibited when the energy supply is high. This suggests the primary role in animal tissues is to supply energy by oxidation of

glutamate allowing 2-oxoglutarate to enter the citric acid cycle despite the fact that the equilibrium for the reaction greatly favours synthesis of glutamate (Smith et al., 1975).

The three glutamate dehydrogenases studied in this work were the bovine liver enzyme which can utilise NAD^+ or $NADP^+$, the $NADP^+$-dependent enzyme from baker's yeast and the NAD^+-dependent enzyme from Clostridium symbiosum. In animal cells glutamate dehydrogenase is located in the mitochondrial matrix (de Duve et al., 1962). The enzyme is imported into mitochondria after synthesis on microsomal membranes (Godinot and Lardy, 1973). There are two distinct glutamate dehydrogenases in baker's yeast, NAD^+- and $NADP^+$-dependent, both are located in the cytoplasm (Hollenberg et al., 1970). Of the three glutamate dehydrogenases studied, only the bovine liver enzyme undergoes an import process.

Glutamate dehydrogenases from various sources have been found to be hexameric consisting of six identical subunits (Smith et al., 1975). The glutamate dehydrogenase from bovine liver consists of six identical subunits, each of M_r 56 000 giving a hexameric M_r of 336 000. The amino acid sequence of the enzyme subunit has been determined (Moon and Smith, 1973) and the sequence contained 500 amino acids. The amino acid sequence is highly conserved as there are only a few differences between the bovine liver and chicken liver enzymes (Smith et al., 1975). The subunit size of the bovine liver glutamate dehydrogenase is larger than that of most other glutamate dehydrogenases. The $NADP^+$-dependent glutamate dehydrogenase from baker's yeast was found to have a subunit M_r of 50 000 $\pm$ 2 000 (section 3.3.1) and the NAD^+-dependent glutamate dehydrogenase from C. symbiosum has a subunit M_r of 49 000, with a hexameric M_r of 294 000 $\pm$ 12 000 (Rice et al., 1985).

The low-resolution crystal structure of the NAD^+-dependent glutamate dehydrogenase from C. symbiosum has been determined by Rice et al. (1987). The hexamer was shown to consist of two sets of trimers with a cylindrical appearance. Each subunit was found to be organised into two distinct globular domains separated by a deep cleft, with one domain (A) being 50% larger than the other. The active site of the enzyme is situated between the two domains and the NAD^+ is bound to the smaller domain (B). The larger domain (A) is involved in intersubunit contacts. A schematic diagram of the overall shape of the glutamate dehydrogenase hexamer is shown in Fig. 3.1. There are no data at present for X-ray crystal structure of the bovine liver enzyme, but low-angle X-ray scattering studies (Sund et al., 1969) and electron microscopic studies (Fiskin et al., 1971) appeared to show that the hexameric enzyme was arranged as two sets of trimers. Each of the subunits in the hexameric enzyme also appeared to be divided into two domains, one of which was larger that the other. Comparisons of the data for the bovine liver and C. symbiosum enzymes suggested that they have strongly related structures.

A number of studies of the unfolding of bovine liver glutamate dehydrogenase in the presence of GdnHCl have been carried out (Müller and Jaenicke, 1980; Tashiro et al., 1982; Bell and Bell, 1984). These have shown that the hexameric enzyme dissociates in two distinct stages. In the first stage the hexamer dissociates to trimers with little or no change in secondary structure as detected by circular dichroism. The trimer is an inactive form of the enzyme but on dilution of the denaturant, enzyme activity can be restored by reassociation. In the second stage the trimer dissociates to monomers with an accompanying loss of secondary structure. The activity of the enzyme cannot be restored by dilution of the denaturant at this stage.

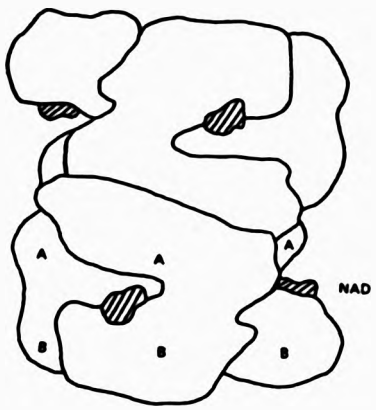


Fig. 3.1 A schematic diagram of the overall organisation of the glutamate dehydrogenase hexamer

The NAD moiety is shown as a hatched region. A and B represent the two globular domains present in each subunit.

The bovine liver glutamate dehydrogenase, as already mentioned, is a mitochondrial matrix enzyme which is imported into the mitochondria after synthesis on ribosomes. When proteins are imported into mitochondria, some undergo proteolytic processing. If this processing occurs for the bovine liver enzyme, it might explain the inability of subunits to refold and reassociate to form the active hexameric enzyme. The two other glutamate dehydrogenases studied, $NADP^+$-dependent from baker's yeast and NAD^+-dependent from C. symbiosum, do not undergo any import process.

The unfolding and refolding of these three glutamate dehydrogenases were studied in order to compare an enzyme which undergoes import into mitochondria with two enzymes which do not undergo import. If there are differences between the mitochondrial and cytoplasmic enzymes, they might arise from the processing of the mitochondrial enzyme as it is imported into the organelle. Some of the methods used to follow the unfolding and refolding of the enzymes included fluorescence, circular dichroism, light-scattering and enzyme activity.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Glutamate dehydrogenase from bovine liver was purchased as a 9 mg/ml $(NH_4)_2SO_4$ suspension from Sigma Chemical Co.

Glutamate dehydrogenase (NAD^+-dependent) from Clostridium symbiosum was generously given by Dr. P. Engel, Department of Biochemistry, University of Sheffield, Sheffield.

Glutamate dehydrogenase ($NADP^+$-dependent) from baker's yeast was purified as in section 3.2.2.1. Baker's yeast was obtained from the Distillers Company Ltd., Menstrie.

DEAE-Cellulose was obtained from Whatman Biosystems Ltd. and Sephacryl S-300 from Pharmacia Fine Chemicals.

All substrates and proteins were purchased from Sigma Chemical Co.

All other chemicals were analar grade and were purchased from BDH Chemicals or Sigma Chemical Co.

3.2.2 Methods

3.2.2.1 Extraction and purification of glutamate dehydrogenase ($NADP^+$-dependent) from baker's yeast

The first step in the isolation of glutamate dehydrogenase (GDH) from baker's yeast was lysis of the yeast. This was carried out following a method by de la Morena et al. (1968), in which 270 ml 1 M NH_4OH was added to 454g yeast and the mixture stirred overnight at 4°C. The lysed yeast was centrifuged at 10 000 rpm for 30 min to give a crude extract (Fraction I). This was subjected to two heat-treatment steps as described in the preparation of Fraction III by Grisolia et al. (1964). To each 100 ml of Fraction I, 5 g of Na_2SO_4 were added and the preparation was heated in a water bath (kept at 75°C) to 69°C for 5 min. The preparation was rapidly chilled and centrifuged at 10 000 rpm for 20 min. To each 100 ml of the supernatant (Fraction II), 35 g of $(NH_4)_2SO_4$ were added and the preparation was warmed to 37°C to dissolve the salt. The preparation was then centrifuged at 10 000 rpm for 20 min. The precipitate was dissolved in a minimum volume of 0.05 M acetate buffer, pH 5.6 and heated at 69°C for 5 min. The preparation was cooled and centrifuged at 12 000 rpm for 20 min and the supernatant kept (Fraction III).

The next stage in the purification was two ion-exchange chromatography steps on DEAE-cellulose from the method by Doherty (1970). Fraction III was dialysed against 0.01 M Tris-HCl pH 7.6 overnight. The dialysed solution was added to a DEAE-cellulose column (3.5 cm internal diameter x 13 cm) equilibrated with 0.01 M Tris-HCl, pH 7.6. The column was washed with 150 ml of the same buffer, then washed

with 150 ml of 0.05 M NaCl in buffer. The enzyme was eluted with a linear salt gradient, 0.1 M NaCl in buffer - 0.3 M NaCl in buffer, using 180 ml of each solution. The active fractions were pooled and diluted with buffer to give a final NaCl concentration of < 0.05 M, checked by conductivity measurements. The diluted pooled fraction was added to a second DEAE-cellulose column (3.5 cm internal diameter x 13 cm) equilibrated with the same buffer. The column was washed with 150 ml of 0.05 M NaCl in buffer. The enzyme was eluted with a linear salt gradient, 0.05 M NaCl in buffer - 0.25 M NaCl in buffer, using 180 ml of each solution. The active fractions were pooled and protein precipitated by addition of $(NH_4)_2SO_4$ to 90% saturation.

The final step was gel filtration on a Sephacryl S-300 column. The redissolved pellet from the $(NH_4)_2SO_4$ step was added to a Sephacryl column (2.5 cm internal diameter x 48 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The enzyme was eluted using this buffer and the active fractions were pooled. The purified protein was concentrated by precipitation with $(NH_4)_2SO_4$ at 90% saturation. The pellet was redissolved in a small volume of 0.1 M potassium phosphate, pH 7.0 and stored at $4^{\circ}C$.

3.2.2.2 Determination of protein concentration

The protein concentration of the baker's yeast GDH was determined by the method of Sedmak and Grossberg (1977), as described in section 2.2.1. The protein concentration of the bovine liver GDH was determined from the absorbance at 280 nm using a value of A_{280} of 0.93 (Egan and Dalziel, 1971). The protein concentration of the C. symbiosum GDH was determined from the absorbance at 280 nm using a value of A_{280} of 1.05 (Engel, P., personal communication).

3.2.2.3 Assays for glutamate dehydrogenase activity

Assays for glutamate dehydrogenase activity were performed at 25°C as described by Doherty (1970). The concentrations of 2-oxoglutarate and NH_4Cl in the NADH and NAD(P)H assay mixtures were 3 mM and 50 mM respectively. In the NADH assay mixture the NADH concentration was 150 µM in 0.1 M potassium phosphate buffer, pH 7.0. In the NAD(P)H assay mixture the NAD(P)H concentration was 150 µM in 0.1 M potassium phosphate buffer, pH 7.8. For each assay, 1 ml of assay mixture was used and the reaction was initiated by addition of the appropriate glutamate dehydrogenase enzyme. The oxidation of NAD(P)H was followed by measuring the rate of change at 340 nm. A 1 mM solution of NAD(P)H gives an absorbance change of 6.22 at 340 nm.

3.2.2.4 Determination of the subunit molecular weight of glutamate dehydrogenase from baker's yeast

The subunit molecular weight of baker's yeast GDH was determined from SDS/polyacrylamide gel electrophoresis. The standard proteins and their molecular weights were mentioned in section 2.2.2. A calibration curve of the mobilities of these proteins on a 12% polyacrylamide gel is shown in Fig. 3.2. The mobility of the baker's yeast GDH on the same gel can be used to obtain the subunit molecular weight from Fig. 3.2.

3.2.2.5 Determination of the native molecular weight of glutamate dehydrogenase from baker's yeast

Gel filtration on Sephacryl S-300 was used to determine the native molecular weight of baker's yeast GDH. The Sephacryl S-300 column was equilibrated with 50 mM sodium phosphate buffer, pH 7.5 and this buffer was used to elute the proteins. The void volume of the

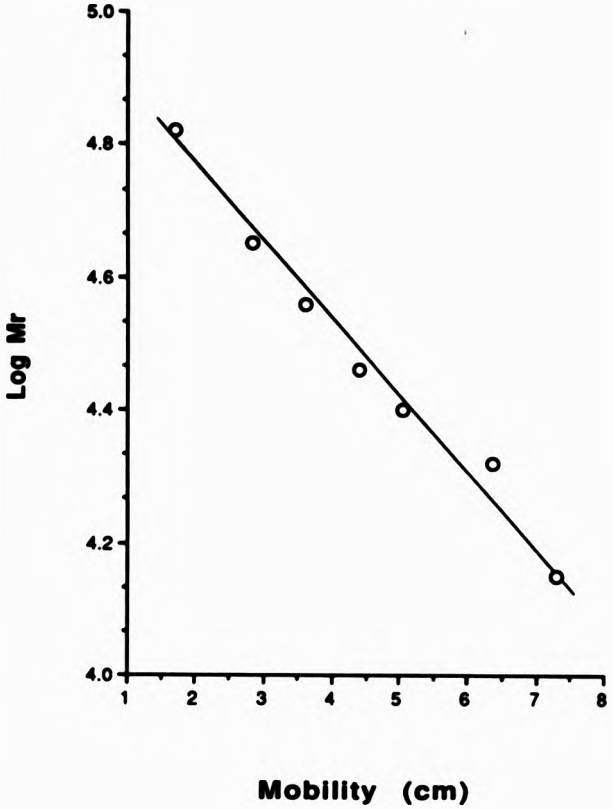


Fig. 3.2 Mobility of standard proteins on a 12% polyacrylamide gel

column was determined using Blue Dextran (M_r $2x10^6$). A 10 mg/ml solution was applied to the column and its elution volume was measured by recording the absorbance at 600 nm. The column was calibrated using proteins of known molecular weight these were: ferritin (M_r 450 000), bovine liver glutamate dehydrogenase (M_r 336 000), yeast alcohol dehydrogenase (M_r 145 000) and bovine serum albumin (M_r 66 000). A calibration curve of the Sephacryl S-300 column is shown in Fig. 3.3. The baker's yeast GDH was applied to the Sephacryl S-300 column and the elution volume was measured by recording the enzyme activity. The native molecular weight was obtained from Fig. 3.3.

3.2.2.6 Activity of glutamate dehydrogenase in the presence of GdnHCl

The effect of GdnHCl on the activities of the glutamate dehydrogenases was determined as described by Bell and Bell (1984). Glutamate dehydrogenase (25 µg/ml) was incubated in 0.1 M potassium phosphate buffer, pH 7.0 containing increasing concentrations of GdnHCl at 25°C for 30 min. Samples were diluted into an assay mixture containing the same concentrations of GdnHCl. In control experiments NaCl replaced GdnHCl.

3.2.2.7 Reactivation of glutamate dehydrogenase after incubation in GdnHCl

Glutamate dehydrogenase was incubated in 0.1 M potassium phosphate, pH 7.0 containing increasing concentrations of GdnHCl at 25°C for 30 min. Samples were diluted 100-fold into the appropriate assay mixtures and the percent reactivation was measured (Bell and Bell, 1984). The enzyme concentrations during the initial incubation were 50, 15 and 32 µg/ml for the bovine liver, baker's yeast and C. symbiosum enzymes respectively.

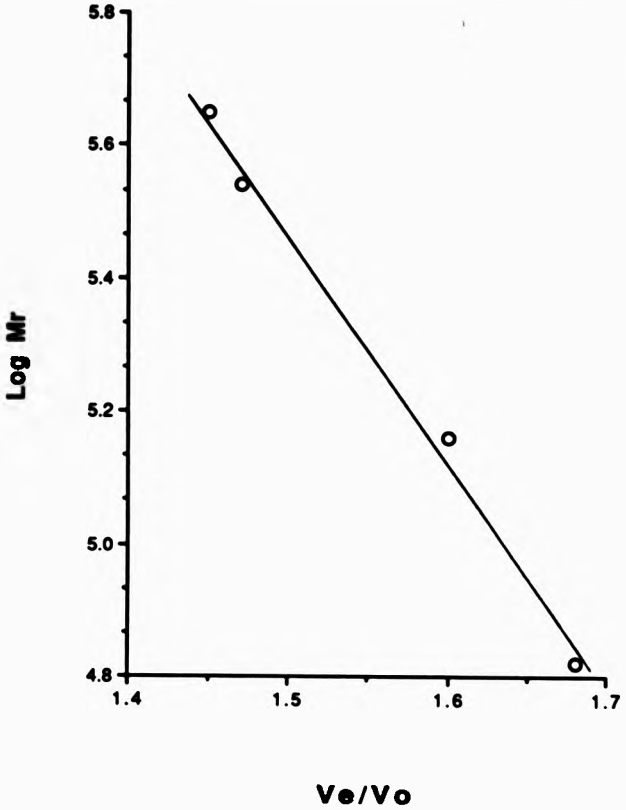


Fig. 3.3 Elution of standard proteins from a Sephacryl S-300 column

3.3. RESULTS

3.3.1 Purification and characterisation of $NADP^+$-dependent glutamate dehydrogenase from baker's yeast

The $NADP^+$-dependent glutamate dehydrogenase was purified from baker's yeast following the method in section 3.2.2.1. Using this method 18 mg of enzyme could be isolated from 500 g of baker's yeast. The purified enzyme had typically, a specific activity of about 100 µmol/min per mg. An example of a purification procedure is given in Table 3.1. The enzyme preparation was at least 95% homogenous on SDS/polyacrylamide gel electrophoresis as judged by staining with Coomassie Blue. A photograph showing the purified glutamate dehydrogenase on a 12% polyacrylamide gel is shown on Plate 3.1.

The subunit M_r of baker's yeast GDH as determined from SDS/polyacrylamide gel electrophoresis was 50 000 $\pm$ 2 000 (Section 3.2.2.4). This value is similar to a value of 48 000 obtained by Venard and Fourcade (1972). The M_r of the native enzyme as determined by gel filtration on Sephacryl S-300 was 270 000 $\pm$ 20 000 (Section 3.2.2.5). This value is comparable with previous work (Venard and Fourcade, 1972). These results for the native and subunit M_r confirm the hexameric nature of the baker's yeast GDH.

3.3.2 Unfolding of glutamate dehydrogenases in GdnHCl

3.3.2.1 Enzyme activity in the presence of GdnHCl

The three glutamate dehydrogenases were inactivated in the presence of increasing concentrations of GdnHCl. Control experiments in which NaCl replaced GdnHCl were carried out to correct for the effects of

Table 3.1 Purification of NADP$^+$-dependent glutamate dehydrogenase from baker's yeast

Stage of Purification	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)
Fraction I	6700	27025	0.25
Fraction II	5485	2465	2.2
Fraction III	6252	1084	5.8
DEAE Cellulose			
Pooled Fraction 1	3935	200	20
Pooled Fraction 2	2711	52	52
Sephacryl S-300			
Pooled Fraction 3	1436	19	76
Redissolved Pellet	1616	17	95

From 526g yeast

Fraction I - crude extract
Fraction II - after first heat step
Fraction III - after second heat step

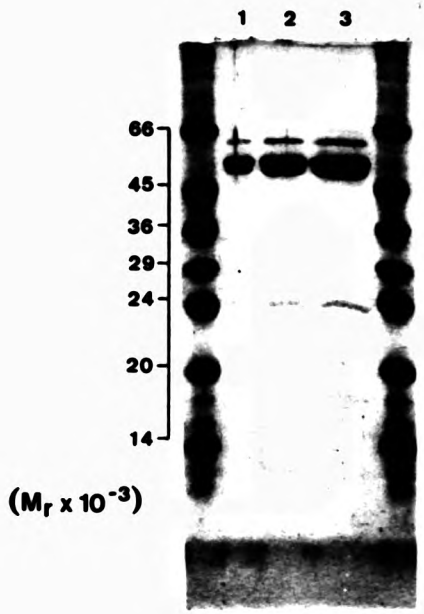


Plate 3.1 NAD^+-Glutamate dehydrogenase from baker's yeast on a 12% polyacrylamide gel

Lanes 1, 2 and 3 represent 10 µg, 20 µg and 40 µg of purified glutamate dehydrogenase from baker's yeast respectively.

ionic strength on the enzymes. The corrected activities of the enzymes at increasing concentrations of GdnHCl are shown in Fig. 3.4. There is a progressive decline in activity and all three enzymes are inactivated by incubation in 2 M GdnHCl. The baker's yeast enzyme appears to be the most sensitive, with very little activity (< 5%) remaining after incubation in 1 M GdnHCl. The GdnHCl concentrations at which 50% inactivation occur are 0.50 M, 0.70 M and 0.80 M for the enzymes from baker's yeast, C. symbiosum and bovine liver respectively.

The data for the bovine liver enzyme show the same pattern as those reported by Bell and Bell (1984) but there are significant differences in detail. The bovine liver enzyme was found to be more stable as 58% activity remained at 0.75 M GdnHCl, whereas only 14% activity remained in the work reported by Bell and Bell (1984). The differences occur in the activities of the enzyme measured in the presence of GdnHCl rather than the activities measured in the presence of NaCl (control). The differences found could be due to the quality of the GdnHCl used; Sigma Grade I was used by Bell and Bell (1984) whereas BDH (Aristar) was used in these experiments. When 6 M aqueous solutions of Sigma Grade I and BDH (Aristar) GdnHCl were measured at 230 nm absorbance values of 2.12 and 0.16 were obtained. These show that the Sigma Grade I GdnHCl contained significant amounts of material which absorbs in the u.v. The nature of such u.v. absorbing impurities and their possible effects on enzymes have been discussed previously (Fridovich, 1965; Nozaki, 1972).

3.3.2.2. Fluorescence studies

The fluorescence emission spectra of bovine liver GDH in the absence and presence of 5 M GdnHCl are shown in Fig. 3.5. The

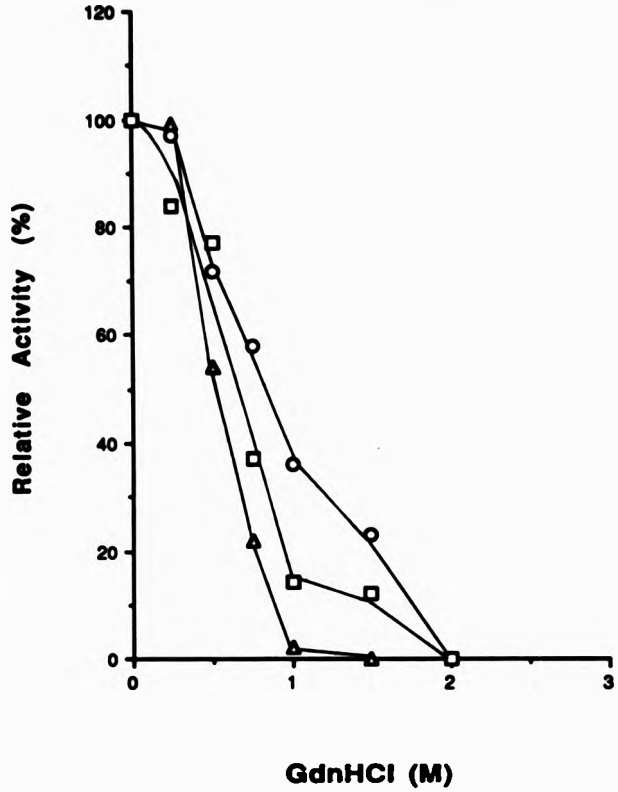


Fig. 3.4 Activity of glutamate dehydrogenase in the presence of GdnHCl

Glutamate dehydrogenase (25 µg/ml) was incubated in 0.1 M potassium phosphate buffer, pH 7.0 containing increasing concentrations of GdnHCl at 25°C for 30 min. Samples were then diluted into an assay mixture containing the same concentration of GdnHCl. The activity is expressed as a percentage of that of a control in which NaCl replaced GdnHCl. ○, △ and □represent the enzymes from bovine liver, baker's yeast and C. symbiosum respectively.

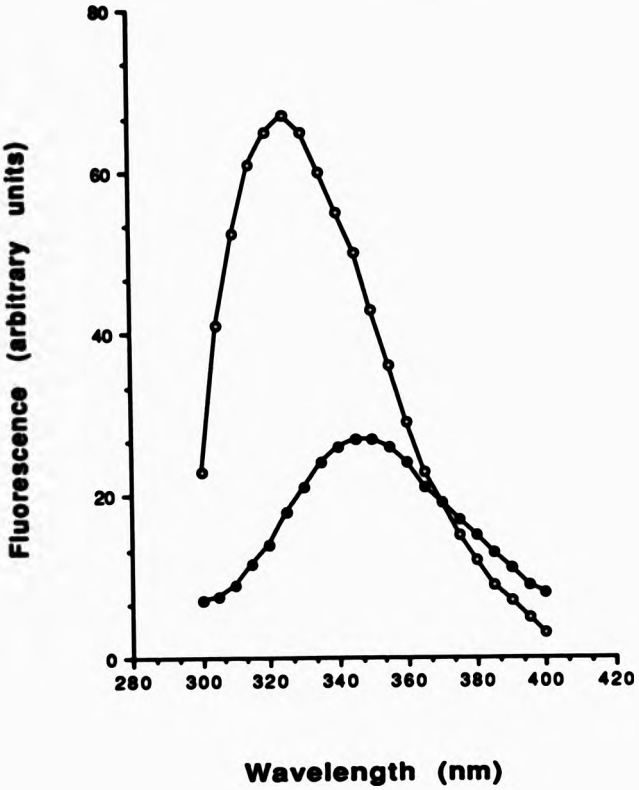


Fig. 3.5 Fluorescence emission spectra of bovine liver GDH in the absence and presence of GdnHCl

Bovine liver GDH (50 µg/ml) was incubated in 0.1 M potassium phosphate buffer, pH 7.0 at 20^{0}C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm . o and ● represent enzyme in the absence and presence of 5 M GdnHCl respectively.

fluorescence emission maximum of the native enzyme occurs at 325 nm. In the presence of 5 M GdnHCl there is a decline in the fluorescence intensity and a shift in the emission maximum to 347 nm. The fluorescence at 325 nm is decreased to approximately 25% of its value in the absence of GdnHCl. Similar spectra are obtained for the native enzymes and enzymes in the presence of 5 M GdnHCl, of baker's yeast GDH and C. symbiosum GDH. The shift in the emission maximum from 325 nm to 347 nm is representative of aromatic residues, mainly tryptophan, becoming more exposed to the aqueous solvent in the presence of GdnHCl (Teipel and Koshland, 1971). The exposure of aromatic residues in 5 M GdnHCl is the result of dissociation and unfolding of the enzymes as reported for the bovine liver GDH (Tashiro et al., 1982; Bell and Bell, 1984).

The changes in fluorescence at 325 nm for the three glutamate dehydrogenases are shown in Fig. 3.6. The changes in the fluorescence intensity at 325 nm occur at different concentrations of GdnHCl for each of the enzymes. The fluorescence intensity of the bovine liver GDH decreases between 1.5 M and 2.25 M GdnHCl. The fluorescence intensity of the C. symbiosum GDH decreases between 2 M and 3 M GdnHCl. When the GdnHCl concentration is increased to 4 M, no further change in fluorescence intensity occurs for the bovine liver or C. symbiosum enzymes. The change in fluorescence intensity of the baker's yeast GDH occurs at a higher concentration of GdnHCl. A decrease between 3.5 M and 4.5 M is found, with no change in fluorescence intensity on increasing the GdnHCl concentration to 6 M. The changes in fluorescence intensity for each of the enzymes occur at similar concentrations as the shift in the emission maximum.

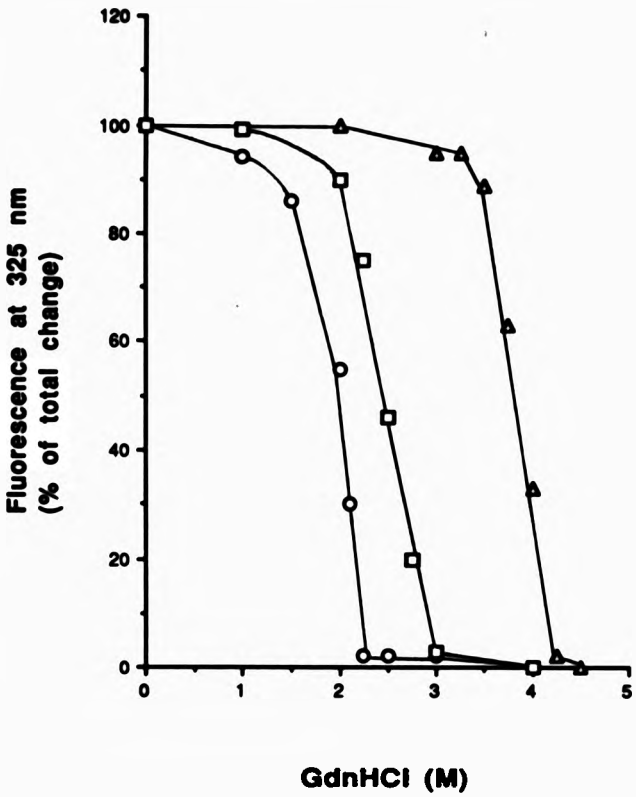


Fig. 3.6 Changes in fluorescence at 325 nm

Bovine liver GDH (50 µg/ml), baker's yeast GDH (40 µg/ml) and C. symbiosum GDH (45 µg/ml) were incubated in 0.1 M potassium phosphate buffer, pH 7.0 containing increasing concentrations of GdnHCl at 20°C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm. The changes in fluorescence intensity at 325 nm are expressed relative to the total change between 0 and 5 M GdnHCl. O, △ and □ represent the enzymes from bovine liver, baker's yeast and C. symbiosum respectively.

3.3.2.3 Circular dichroism studies

The circular dichroism spectra of the bovine liver GDH, baker's yeast GDH and C. symbiosum GDH are shown in Fig. 3.7. The spectra of the enzymes have negative ellipticity over the wavelength range 205-250 nm, which are characteristic of proteins with a significant content of α-helical structure. Using the reference values of Θ_{208} and Θ_{225} (Chen et al., 1974; Chang et al. 1978), the helical content of the native enzymes from bovine liver, baker's yeast and C. symbiosum can be calculated as 47% ± 4%, 39% ± 2% and 62% ± 3% respectively. The estimate for the C. symbiosum enzyme can be compared with a value of 55% helix obtained by X-ray crystallography (Rice et al., 1987). These values show that the C. symbiosum enzyme has a large content of helical structure, approximately 60%. The bovine liver and baker's yeast enzymes have smaller, but significant, contents of helical structure, between 40-50%.

The spectrum of baker's yeast GDH in 5 M GdnHCl is shown in Fig. 3.7. This shows a large reduction in ellipiticity (i.e. becoming less negative) which represents complete loss of secondary structure of the enzyme. Similar spectra are obtained for bovine liver GDH and C. symbiosum GDH in 5 M GdnHCl.

The changes in Θ_{225}, which reflect the changes in helical content (Chen et al., 1974), on addition of GdnHCl for the three glutamate dehydrogenases are shown in Fig. 3.8. The changes occur at different concentrations of GdnHCl for each of the enzymes. The changes in Θ_{225} of the bovine liver GDH occur between 1 M and 2.5 M GdnHCl. These changes in ellipticity occur over a very similar GdnHCl concentration range, as reported by Tashiro et al. (1982), for changes in

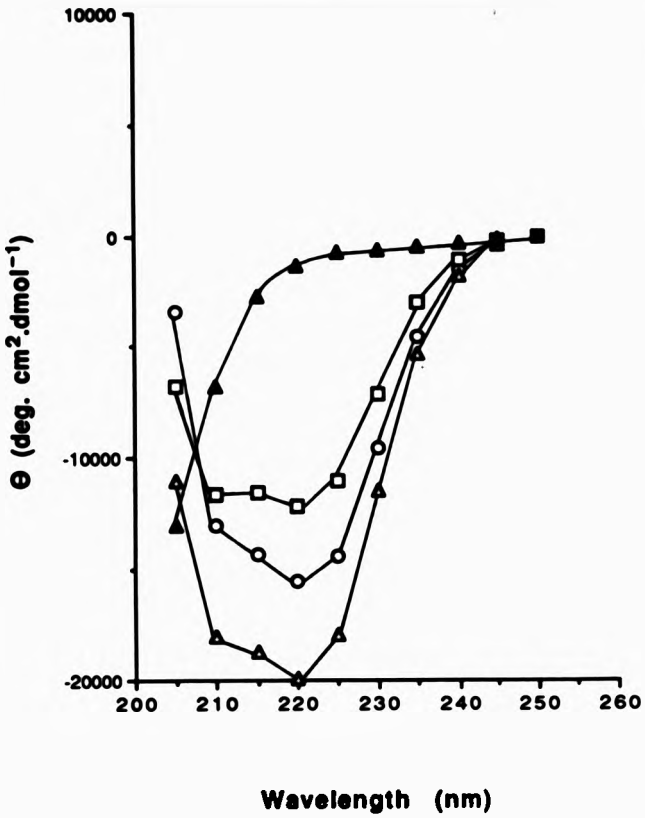


Fig. 3.7 Circular dichroism spectra of glutamate dehydrogenase

Spectra of bovine liver (○), baker's yeast (△) and C. symbiosum (□) enzymes (0.75 µg/ml) in 0.1 M potassium phosphate buffer, pH 7.0 were recorded over the wavelength range 205-250 nm at 20°C. The spectrum of the baker's yeast enzyme in 5 M GdnHCl (▲) was recorded in the same conditions.

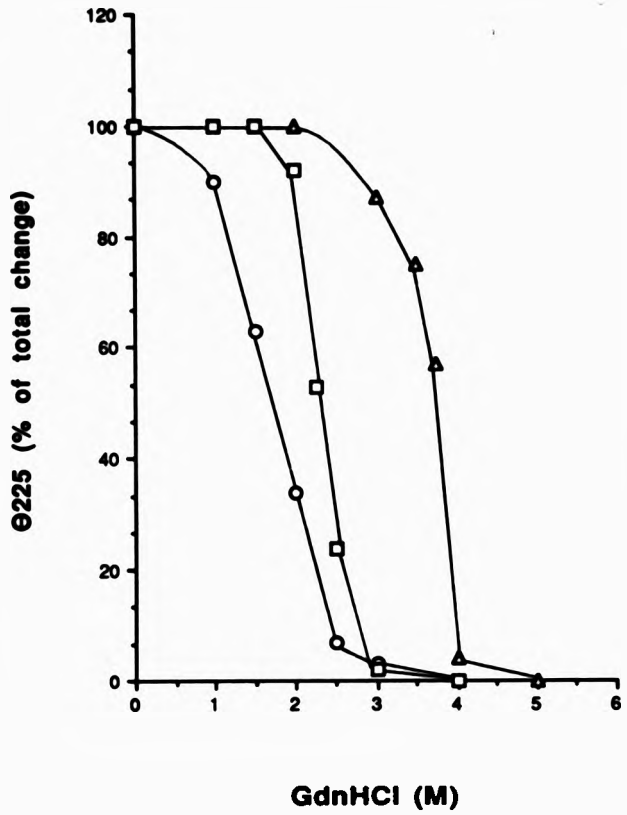


Fig. 3.8 Changes in θ_{225} in the presence of GdnHCl

Changes in θ_{225} are expressed relative to the total change between 0 and 5 M GdnHCl. O, Δ and □represent the enzymes from bovine liver, baker's yeast and C. symbiosum respectively.

bovine liver GDH at Θ_{222}. The changes in Θ_{225} of the *C. symbiosum* GDH occur between 2 M and 3 M GdnHCl. On increasing the GdnHCl concentration to 4 M there was very little change in the Θ_{225} values for the bovine liver GDH and *C. symbiosum* GDH. The changes in Θ_{225} of the baker's yeast GDH occur between 3 M and 4 M GdnHCl. These results show that the changes in circular dichroism for each of the enzymes have the same pattern as the changes in fluorescence. This confirms that the structural changes occur at a specific range of GdnHCl concentrations for each of the enzymes.

3.3.2.4 Changes in M_r on the addition of GdnHCl

The changes in M_r of the three glutamate dehydrogenases on addition of GdnHCl were determined by light-scattering as described in section 2.2.6. These changes in M_r are shown in Fig. 3.9 in which the ordinate represents the ratio of M_r in the presence of GdnHCl to the M_r in the absence of GdnHCl. The bovine liver GDH shows a decrease in the Mi/Mo value, from 1.0 to 0.5, between 0 M and 1 M GdnHCl. This decrease represents the dissociation of the hexameric native enzyme to trimers. There is a plateau region between 1 M and 1.5 M GdnHCl which suggests that the trimer exists in a stable form at these concentrations of GdnHCl. The Mi/Mo value decreases to approximately 0.19 at concentrations of GdnHCl between 1.5 M and 3 M. This represents dissociation of trimers to monomers. The data for the bovine liver GDH are similar to those reported by Tashiro *et al.* (1982), in which two transition regions occur. The first transition region represents dissociation of hexamer to trimers and the second, dissociation of trimer to monomers.

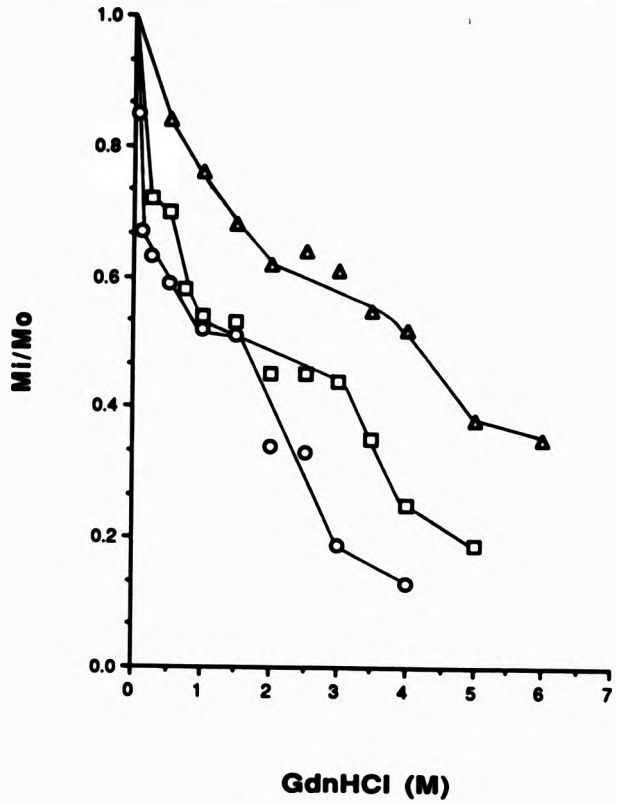


Fig. 3.9 Changes in M_r of glutamate dehydrogenase in the presence of GdnHCl

Bovine liver GDH (100 µg/ml), baker's yeast GDH (100 µg/ml) and C. symbiosum (200 µg/ml) were made up in 0.1 M potassium phosphate buffer, pH 7.0 containing increasing concentrations of GdnHCl. The excitation and emission wavelengths were 360 nm and measurements were performed at 20°C. The ordinate represents the ratio of M_r in the presence of GdnHCl to the M_r in the absence of GdnHCl. ○, △ and □ represent the enzymes from bovine liver, baker's yeast and C. symbiosum respectively.

The two transition regions are also observed for the C. symbiosum and baker's yeast enzymes. The changes in the molecular weight ratio values take place at slightly higher concentrations of GdnHCl for these two enzymes than for the bovine liver enzyme. The transitions for the C. symbiosum GDH occur between 0 M and 1.5 M GdnHCl and 2.5 M and 5 M GdnHCl. The transitions for the baker's yeast GDH occur between 0 M and 2 M GdnHCl and 3.5 M and 6 M GdnHCl. The Mi/Mo value for the baker's yeast enzyme at 6 M GdnHCl is higher than the expected value (0.17) for dissociation of hexamer to monomer. This could arise either from incomplete dissociation of the enzyme, or from the use of inappropriate values for the refractive index increments in the presence of GdnHCl. The refractive index increment values used in the calculations were taken from data for bovine serum albumin by Noelken and Timasheff (1967). These data were also used by Tashiro et al. (1982) in calculating molecular weight ratios for the bovine liver enzyme. The values for Mi/Mo for the baker's yeast enzyme between 2 M and 3.5 M GdnHCl, where a plateau is observed, were higher than the expected 0.5 value. These higher Mi/Mo values could also be due to the refractive index increment values being inappropriate.

The pattern for the dissociation from hexamer to trimer to monomer for the three glutamate dehydrogenases was shown to be similar. The data obtained for the baker's yeast enzyme shows higher concentrations of GdnHCl are required for dissociation of the enzyme than for the bovine liver and C. symbiosum enzymes. At 3 M GdnHCl the bovine liver GDH has dissociated to monomers but the baker's yeast GDH has only dissociated to trimers. This suggests that the intersubunit forces which hold the subunits together are stronger in the baker's yeast enzyme.

3.3.3 Attempted refolding of glutamate dehydrogenase after incubation in GdnHCl

The glutamate dehydrogenases were incubated in the absence and presence of GdnHCl at 25°C for 30 min and then diluted 100-fold into the appropriate assay mixtures. The concentration of GdnHCl after dilution was $\leq$ 0.06 M which had no significant effect on enzyme activity. The reactivation (%), as a function of GdnHCl concentration, is shown in Fig. 3.10. This shows the reactivation decreasing at different concentrations for each of the enzymes. The addition of dithiothreitol (1 mM) to the incubation and assay mixtures had no effect on the reactivation at high GdnHCl concentrations. Therefore the inability to regain activity at high concentrations of GdnHCl does not arise from the oxidation of thiol groups.

The bovine liver GDH shows $>$ 90% reactivation when the initial concentrations of GdnHCl are in the range 0 - 1 M. At concentrations of GdnHCl between 1 M and 2 M the reactivation decreases sharply to 18% at 2 M. The data are comparable with those obtained by Bell and Bell (1984) though there are some differences in detail. The C. symbiosum and baker's yeast enzymes show equally sharp decreases in reactivation between 1.5 M and 2.5 M GdnHCl and 3 M and 4 M GdnHCl respectively.

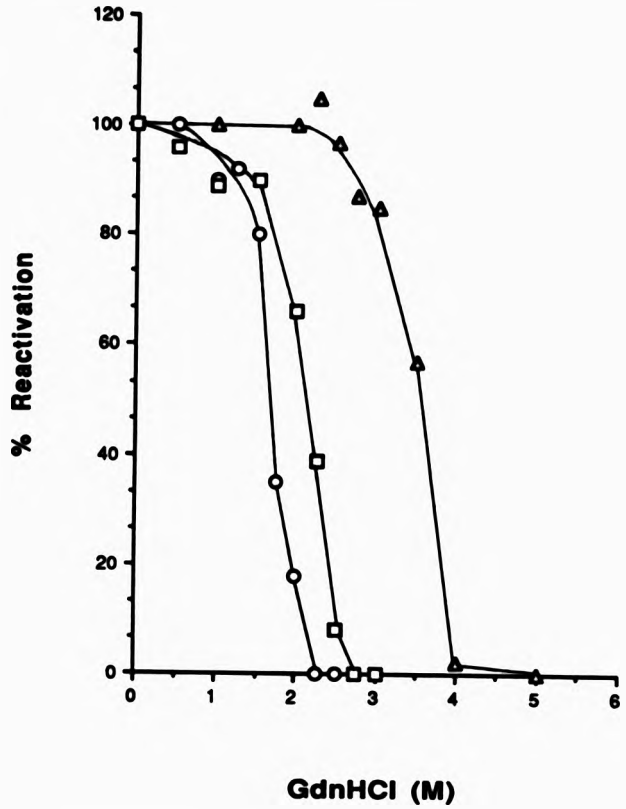


Fig. 3.10 Reactivation of glutamate dehydrogenase after unfolding in GdnHCl

Bovine liver GDH (50 µg/ml), baker's yeast GDH (15µg/ml) and C. symbiosum GDH (32 µg/ml) were incubated in 0.1M potassium phosphate buffer, pH 7.0 containing increasing concentrations of GdnHCl at 25°C for 30 min. Samples were then diluted 100 fold into assay mixtures. Activity is expressed relative to a control sample from which GdnHCl was omitted. ○, △ and □ represent the enzymes from bovine liver, baker's yeast and C. symbiosum respectively.

3.4 CONCLUSIONS AND COMPARISON OF THE GLUTAMATE DEHYDROGENASES

The results for the changes in the structure as detected by fluorescence and circular dichroism and the percentage reactivation were combined for each of the glutamate dehydrogenases as shown in Figs. 3.11a, 3.11b and 3.11c. These show the structural changes closely parallel the decrease in reactivation. The glutamate dehydrogenases are hexameric enzymes, and in the presence of GdnHCl have been shown to dissociate initially to trimers then to monomers, as shown in Fig. 3.9 and as found previously by Tashiro *et al.* (1882) and Inoue *et al.* (1984).

When the data for the changes in M_r (Fig. 3.9) are taken together with the structural changes and the decrease in percentage reactivation, it can be seen that at concentrations of GdnHCl where the hexamer dissociates to trimers, no changes in structure occur as detected by fluorescence and circular dichroism. The dissociation of hexamer to trimers thus occurs without the unfolding of the native structure of the subunit (Tashiro *et al.*, 1982). The loss of activity for each of the enzymes was shown to occur at low GdnHCl concentrations (Fig. 3.4). At concentrations of GdnHCl where the hexamer dissociates to trimers, loss of activity was found to be reversible, with close to 100% activity regained. The loss of activity on addition of denaturants can be caused by slight changes at the active site as discussed by Tsou (1986). It appears that the addition of low concentrations of GdnHCl causes slight changes at the active sites of the glutamate dehydrogenases leading to inactivation of the enzymes. When the concentration of GdnHCl is reduced by dilution, the changes which occurred at the active sites can be reversed, allowing reactivation of the enzymes. It has been recently shown by phosphorescence lifetime measurements, that changes in the conformation of the active sites of bovine liver GDH can be induced by low concentrations of GdnHCl (Strambini *et al.*, 1989).

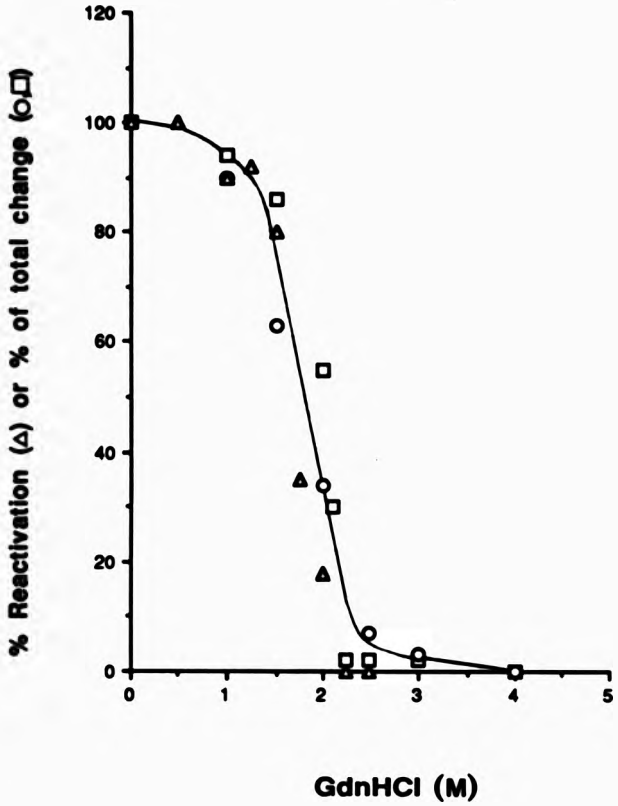


Fig. 3.11a Combined data for bovine liver glutamate dehydrogenase

□,○ and △ represent the % of total change in fluorescence at 325 nm, % of total change in θ_{225} and % reactivation respectively.

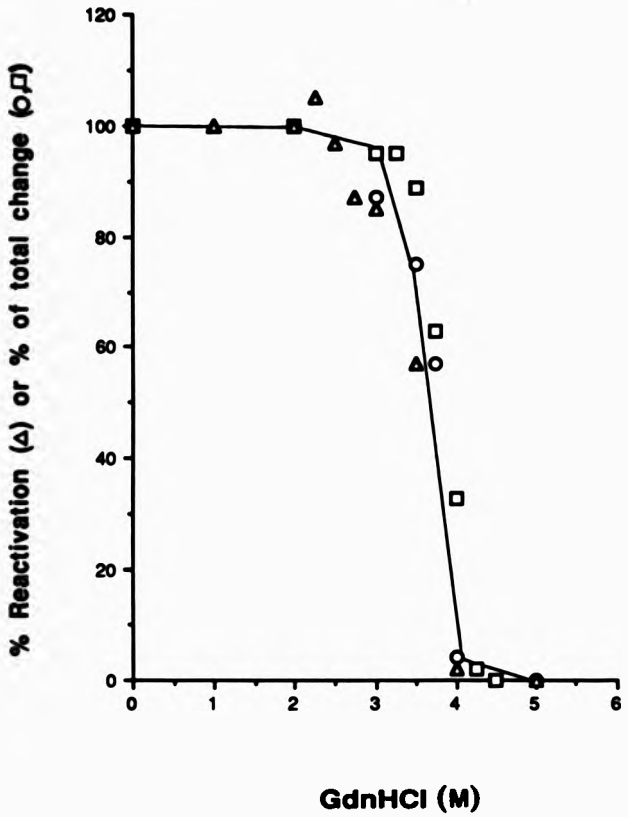


Fig. 3.11b Combined data for baker's yeast glutamate dehydrogenase

□, O and Δ represent the % of total change in fluorescence at 325 nm, % of total change in θ_{225} and % reactivation respectively.

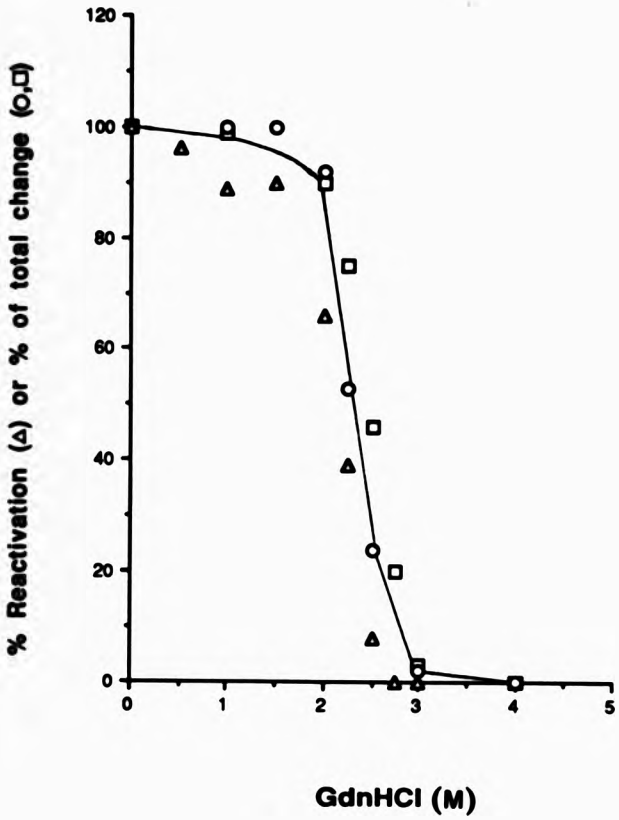


Fig. 3.11c Combined data for C. symbiosum glutamate dehydrogenase

□, O and Δ represent the % of total change in fluorescence at 325 nm, % of total change in θ_{225} and % reactivation respectively.

In concentrations of GdnHCl where the glutamate dehydrogenases form trimers, the regain of catalytic activity on dilution of the enzymes can be observed. The reactivation is the result of reassociation of the trimers to form the hexameric enzyme which has full catalytic activity. It can be concluded that the trimer form of the enzymes exists as subunits with native structure, but no catalytic activity. The trimer form of each of the enzymes is also stable over a small concentration range of GdnHCl as shown by the plateau regions in Fig. 3.9.

At the concentrations of GdnHCl where the trimer dissociates to monomers there are changes in the structure of the glutamate dehydrogenases as detected by fluorescence and circular dichroism. The fluorescence and circular dichroism measurements detect changes in the secondary structures of the enzymes. This means the dissociation of trimers to monomers involves subsequent unfolding of the polypeptide chains (Tashiro et al., 1982). Over the range of GdnHCl concentrations where the trimer dissociates to monomers the percentage reactivation decreases. This shows that the loss of activity is not reversible once the enzymes dissociate from trimers to monomers.

When the data for the three glutamate dehydrogenases are compared (Figs. 3.11a, 3.11b and 3.11c) it is seen that the changes in the baker's yeast GDH occur at a higher GdnHCl concentrations than for the bovine liver GDH and C. symbiosum GDH. This suggests that the intersubunit contacts in the baker's yeast GDH are much stronger than in the bovine liver GDH and C. symbiosum GDH. The stronger intersubunit contacts in the baker's yeast GDH implies that the nature of the contacts must be different from those in the bovine liver GDH and C. symbiosum GDH. But X-ray structural studies of the enzymes must be awaited to confirm these conclusions.

Although the intersubunit contacts appear to be stronger in the baker's yeast enzyme, the activity of the enzyme was found to be more sensitive to GdnHCl than the bovine liver and C. symbiosum enzymes (Fig. 3.4). This implies that changes in structure occur at the active sites of the baker's yeast enzyme at lower GdnHCl concentrations. There is one active site per subunit, this is found in the cleft between the two domains of the subunit, as reported for the C. symbiosum enzyme (Rice et al., 1987). The active sites in the baker's yeast GDH could be more accessible to solvent, which would allow changes to occur at slightly lower concentrations of GdnHCl.

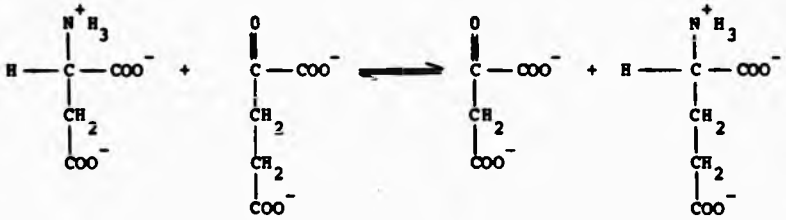
Since all three glutamate dehydrogenases show a similar pattern for structural changes and loss of reactivation, the inability of subunits of the bovine liver GDH to refold after dissociation, is unlikely to be solely due to any structural changes occurring as a result of import into mitochondria. The inability of the subunits of glutamate dehydrogenase to refold may instead be related to their size. The work of Jaenicke (1984) and Teschner et al. (1987) has highlighted the low extents of reactivation of many multidomain protein chains of >300 amino acids.

CHAPTER FOUR

UNFOLDING AND REFOLDING OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

4.1 INTRODUCTION

Aspartate aminotransferase occurs in two isoenzyme forms, one is located in the cytosol and the other is located in mitochondria. Aspartate aminotransferase catalyses the transfer of the amino group of aspartate to 2-oxoglutarate, which leads to the formation of oxaloacetate and L-glutamate. Pyridoxal 5'-phosphate is the cofactor for this enzyme.



Aspartate 2-oxoglutarate Oxaloacetate L-glutamate

The pyridoxal 5'-phosphate cofactor binds to Lysine-258 of the aspartate aminotransferase (Braunstein, 1973). The aldehyde group of pyridoxal 5'-phosphate and the amino group of Lysine-258 react to form an aldimine, which is a Schiff-base linkage. The pyridoxal 5'-phosphate is in a stable form when bound to the enzyme. During the transamination reaction, the pyridoxal phosphate cofactor is transiently converted into pyridoxamine phosphate.

The steps in the transamination reaction are shown in Fig. 4.1. First, the amino group of aspartate displaces the amino group of Lysine-258 and forms a Schiff-base linkage between the aspartate and pyridoxal 5'-phosphate, which is an aldimine. The double bond in the amino acid - pyridoxal 5'-phosphate Schiff-base linkage shifts to form a ketimine. The ketimine is then hydrolysed to form oxaloacetate and

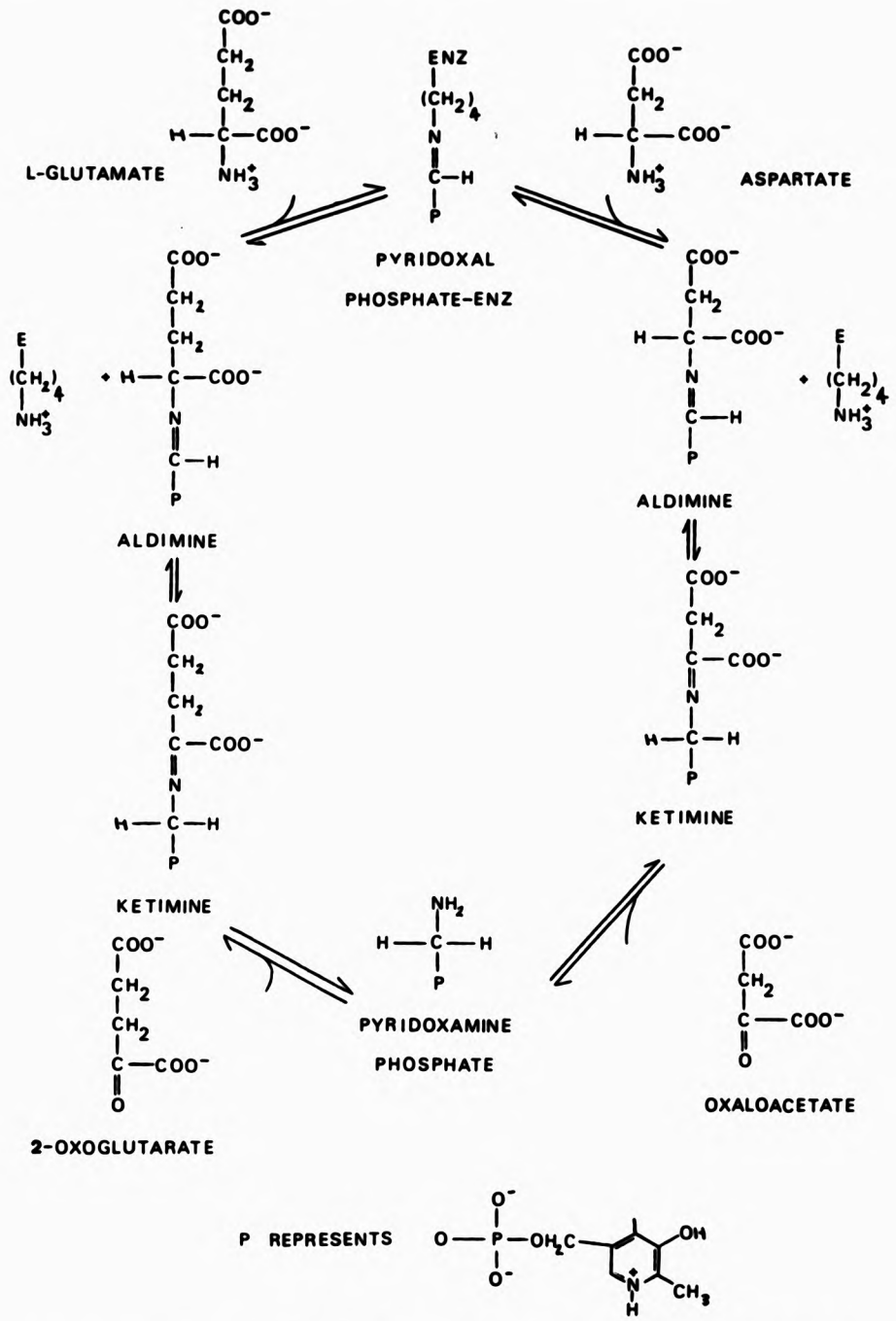


FIG. 4.1 STEPS IN TRANSAMINATION BY ASPARTATE AMINOTRANSFERASE

pyridoxamine phosphate. The oxaloacetate is free to dissociate from the enzyme. These steps constitute one half of the overall reaction, the second half is a reversal of these steps. The second substrate, 2-oxoglutarate reacts with pyridoxamine phosphate to form a ketimine. The double bond shifts forming an aldimine of glutamate and pyridoxal 5'-phosphate. The aldimine double bond shifts to form a double bond between pyridoxal 5'-phosphate and Lysine-258, releasing glutamate. The transamination reaction is reversible and occurs in both directions.

The two isoenzymes of aspartate aminotransferase participate in the 'malate-aspartate' shuttle Fig. 4.2, which is involved in the transfer of reducing equivalents across the mitochondrial membrane (Newsholme and Start, 1973). Cytoplasmic NADH is used to reduce oxaloacetate to malate by malate dehydrogenase. Malate is then transported across the mitochondrial membrane and is oxidised to oxaloacetate, with the reduction of NAD^+ by the mitochondrial isoenzyme of malate dehydrogenase. Oxaloacetate cannot return across the mitochondrial membrane but transamination of oxaloacetate forms aspartate, which can transverse the mitochondrial membrane. Aspartate aminotransferase is the enzyme which catalyses this transamination and the reverse transamination which occurs in the cytosol to regenerate oxaloacetate.

In this chapter the cytoplasmic aspartate aminotransferase from pig heart was studied and in the following chapter the mitochondrial isoenzyme was studied. Aspartate aminotransferases from various sources have been studied (Braunstein, 1973). Aspartate aminotransferase is a dimeric enzyme of two identical subunits. The cytoplasmic isoenzyme from pig heart consists of two subunits, each having a M_r of 46 344 (Barra et al., 1980), giving a dimeric M_r of 92 700. The amino acid

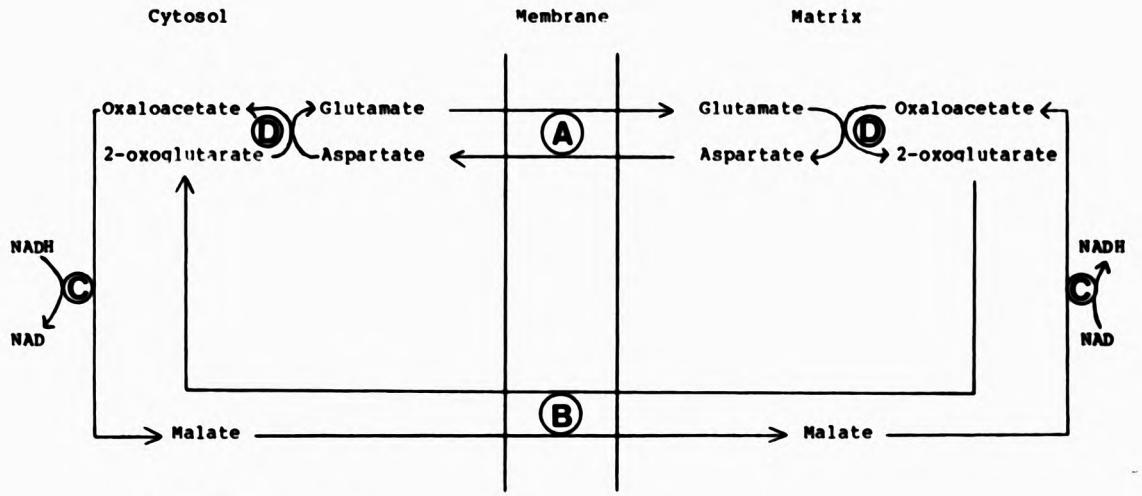


Fig. 4.2 Malate-Aspartate Shuttle

sequence of the enzyme has been determined (Doonan et al., 1970), and the sequence contains 412 amino acids.

The low resolution crystal structure of the cytoplasmic aspartate aminotransferase from pig heart has been determined by Arnone et al. (1985). The overall tertiary structure of the enzyme is similar to both the cytoplasmic and mitochondrial isoenzymes from chicken heart (Borisov et al., 1980; Ford et al., 1980). Each subunit is divided into two major parts, a large central domain constructed from residues 75-300 and a smaller peripheral domain composed of the carboxyl-terminal and amino-terminal regions. A linker region joins the two domains. The large domain consists of a twisted seven-stranded β sheet at its centre, surrounded by seven α helices. These pieces of secondary structure are sequentially ordered as αβ pairs except for one ββ pair. This types of protein structure is very common and forms one of the folding patterns observed in globular proteins. The large domain contains the cofactor binding site.

The small domain does not have the high level of secondary structure found in the large domain. Relative to the large domain it appears to be less compact and more mobile. The two domains are connected by a pair of linker peptides. The linker peptide between residues 37 and 75 only has a small amount of secondary structure, in the form of a short helix (residues 50-63). The linker peptide between residues 300 and 341 consists of two α helices with residues 300-312 forming a short helix this is perpendicular to a long helix composed of residues 313-341.

There are two active sites in each dimeric aspartate aminotransferase molecule. The active sites of the enzyme are found between the large and small domains, near the subunit interface. Most

of the residues involved at the active site are in the large domain, but there are also some residues from the opposite subunit involved.

There has been one report, on the basis of sedimentation-velocity data, that the cytoplasmic aspartate aminotransferase from pig heart does not unfold in 8 M urea or 6 M GdnHCl, since a dimeric structure was apparently retained (Banks et al., 1968a). On prolonged exposure to these agents, dissociation did occur. By contrast, Martinez-Carrion et al. (1970) reported that the pig heart enzyme was unfolded in GdnHCl, as judged by the loss of the near-u.v. circular dichroism spectrum. A report by Bertland and Kaplan (1970) found that the chicken heart enzyme was unfolded in GdnHCl as shown by loss of enzyme activity. Bertland and Kaplan (1970) reported that partial regain of activity of the cytoplasmic chicken heart could be observed, provided that pyridoxal 5'-phosphate and 2-mercaptoethanol were present during refolding.

The cytoplasmic aspartate aminotransferase from pig heart was studied to clarify some of the previous observations on the cytoplasmic enzyme. The results from the unfolding and refolding studies can then be used to compare the cytoplasmic isoenzyme with the mitochondrial isoenzyme. The results of studies on the mitochondrial isoenzyme are found in the following chapter along with a discussion on the comparison between the two isoenzymes.

The cytoplasmic aspartate aminotransferase from pig heart was initially purchased from Boehringer but a number of observations indicated that this commercial enzyme was unsatisfactory. On SDS/polyacrylamide gel electrophoresis the commercial enzyme showed significant ($\sim$10% of total staining) quantities of material with a subunit M_r 66 000 and a number of minor bands in the M_r range 55 000 - 60 000 (as shown on Plate 4.1, section 4.3.1). The number of

thiol groups per subunit reactive towards 5,5'-dithiobis-(2-nitrobenzoic acid) in the native commercial enzyme was found to be ≤ 0.1. In the presence of 6 M GdnHCl or 0.1% SDS the number of thiol groups per subunit was 2.9. These values could not be increased by prolonged incubation with 1 mM dithiothreitol followed by dialysis. These values for the number of thiol groups per subunit do not agree with data published by Birchmeier et al. (1973) and Arnone et al. (1977). The number of thiol groups per subunit found in the native enzyme was two and in the presence of 6 M GdnHCl the total number of thiol groups per subunit was five. From these results it was concluded that the commercial cytoplasmic aspartate aminotransferase had undergone some irreversible modification. As this commercial enzyme was found to be unsatisfactory, the enzyme was purified from pig heart and this preparation was used in all subsequent experiments.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Cytoplasmic aspartate aminotransferase from pig heart was purified as in Section 4.2.2.1. Pig hearts were obtained from Dunblane slaughter house.

CM cellulose and Sepharose 4B were obtained from Sigma Chemical Co., DEAE Cellulose from Whatman Biosystems Ltd., and Sephacryl S-300 from Pharmacia Fine Chemicals.

Malate dehydrogenase from pig heart (mitochondrial) 10 mg/ml in $(NH_4)_2SO_4$ was purchased from Boehringer Mannheim.

All substrates were purchased from Sigma Chemical Co.

All other chemicals were analar grade and were purchased from BDH Chemicals or Sigma Chemical Co.

4.2.2. Methods

4.2.2.1 Purification of cytoplasmic aspartate aminotransferase from pig heart

Cytoplasmic aspartate aminotransferase was purified from pig heart following the procedure (method 2) by Banks et al. (1968b) including an additional gel filtration step. The first step was to remove the connective tissue and fat from three pig hearts and then homogenise in 0.1 M succinate buffer, pH 6.0 (1L/500 g). The homogenate was heated to 65°C for 15 min and centrifuged at 3 000 rpm for 20 min to give a crude extract. The crude extract was subjected to ammonium sulphate fractionation and the protein precipitating between 55-65% saturation was collected. This protein fraction was dissolved in distilled water and dialysed against distilled water overnight. The dialysis residue was centrifuged at 15 000 rpm for 20 min and the supernatant was adjusted to pH 5.37 with 0.01 M HCl.

The next stage in the purification involved two ion-exchange chromatography steps, the first on CM cellulose and the second on DEAE cellulose. The dialysis residue was added to a CM cellulose column (3.5 cm internal diameter x 13 cm) equilibrated with 0.02 M sodium acetate buffer, pH 5.37. The enzyme was eluted using the same buffer and the active fractions were pooled. The pH of the pooled fraction was adjusted to pH 7.4 using 0.1 M NaOH. This was added to a DEAE cellulose column (3.5 cm internal diameter x 13 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.4 and the enzyme was eluted using the same buffer. The active fractions were pooled and the protein was precipitated by addition of $(NH_4)_2SO_4$ to 85% saturation.

The final step was gel filtration on Sephacryl S-300 column. The redissolved pellet from the $(NH_4)_2SO_4$ concentration step was

added to a Sephacryl column (2.5 cm internal diameter x 48 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. The enzyme was eluted from the column using this buffer and the active fractions were pooled. The purified protein was concentrated by precipitation with $(NH_4)_2SO_4$ at 90% saturation. The pellet was redissolved in a small volume of 0.1 M sodium phosphate buffer, pH 7.4 and stored at 4°C.

4.2.2.2 Determination of protein concentration

During the purification, the protein concentration was determined by the method of Sedmak and Grossberg (1977) as described in Section 2.2.1. The protein concentration of the purified cytoplasmic aspartate aminotransferase was determined from the absorbance at 280 nm using a value of $A_{1\ cm}^{0.1\%}$ of 1.51 (Birchmeier et al., 1973).

4.2.2.3 Assay for cytoplasmic aspartate aminotransferase activity

The activity of cytoplasmic aspartate aminotransferase was assayed using a coupled assay procedure. Oxaloacetate formed by the aspartate aminotransferase catalysed reaction is converted to malate by malate dehydrogenase in this coupling step NADH is converted to NAD^+. The concentrations of the substrates and coupling enzyme in 0.1 M sodium phosphate buffer, pH 7.4 were L-aspartate, 30 mM; 2-oxoglutarate, 5 mM; NADH, 0.09 mM; malate dehydrogenase, 5 µg/ml. The reaction was initiated by addition of cytoplasmic aspartate aminotransferase to an assay mix volume of 1 ml at 25°C. The rate of change in absorbance at 340 nm was recorded. One unit of enzyme activity is the amount which catalyses the disappearance of 1 µmol of NADH per minute.

4.2.2.4 Activity of cytoplasmic aspartate aminotransferase in the presence of GdnHCl

Enzyme activity in the presence of GdnHCl was determined by a discontinuous assay procedure. Cytoplasmic aspartate aminotransferase (20 μg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 in the absence and presence of GdnHCl at 0°C for 15 min. A sample (0.1 ml) was taken and added to a reaction mixture containing L-aspartate, 2-oxoglutarate and the corresponding GdnHCl concentration at 25°C. Samples (0.05 ml) were taken at 1 min and 4 min after the start of the reaction and added to 0.95 ml of solution containing NADH and malate dehydrogenase (the concentrations of the substrates and coupling enzyme were as used in the continuous assay, Section 4.2.2.3). The changes in absorbance at 340 nm were recorded for the two sampling times. These changes represent the oxaloacetate formed in the aspartate aminotransferase catalysed reaction. The rate of the cytoplasmic aspartate aminotransferase catalysed reaction was determined from the difference in the changes at A_{340} for the two sampling times.

This discontinuous method ensures that the concentration of GdnHCl carried over to the malate dehydrogenase reaction is too small ($\leqslant$ 0.1M) to interfere with the activity of the coupling enzyme. Control experiments were performed to check that the rate of the cytoplasmic aspartate aminotransferase catalysed reaction was constant over the range of times studied and proportional to the amount of enzyme added.

4.2.2.5 Absorption spectra of cytoplasmic aspartate aminotransferase

Absorption spectra of enzyme samples (0.5 mg/ml) in the absence and presence of 3 M and 6 M GdnHCl were recorded from 280-420 nm, at 5 nm intervals. Blanks for each of the GdnHCl concentrations were used as zero absorption at each wavelength. The samples were dialysed against 20 volumes 0.1 M sodium phosphate, pH 7.4 containing the same concentration of GdnHCl for 24 hr at 4°C. The absorption spectra were recorded as before except that in each case the blank was the solution remaining outside the dialysis tubing.

4.2.2.6 Activity of cytoplasmic aspartate aminotransferase after modification of the two exposed thiol groups

An enzyme sample (0.2 mg/ml) was incubated in 0.05 M sodium phosphate buffer, pH 7.4 at 20°C. Nbs_2 was added to a final concentration of 250 μM and the enzyme activity was measured at 5, 10 and 30 min after addition of Nbs_2. The enzyme activity was measured before addition of Nbs_2 and this was used as the control. Measurements of ΔA_{412} made in parallel showed that the two reactive thiol groups had been modified within 10 min.

4.2.2.7 Reactivation of cytoplasmic aspartate aminotransferase after unfolding in GdnHCl

Aspartate aminotransferase was incubated in 0.1 M sodium phosphate buffer, pH 7.4 in the absence and presence of GdnHCl for 15 min at 0°C, to allow unfolding to occur. Refolding was initiated by a 60-fold dilution into 0.1 M sodium phosphate buffer, pH 7.4 at 20°C. Refolding of the enzyme took place in the absence and presence of 0.1 mM pyridoxal 5'-phosphate (PLP) plus 1 mM dithiothreitol (DTT). The enzyme activity was assayed after 24 hr refolding.

4.2.2.8 Time dependence of reactivation of cytoplasmic aspartate aminotransferase

Aspartate aminotransferase was unfolded in 6 M GdnHCl by incubation for 15 min at 0°C. The unfolded enzyme was refolded in four different solutions. (1) no PLP or DTT, (2) only PLP, (3) only DTT, (4) PLP and DTT present. The enzyme activity regained was assayed at several time points over 24 hr.

4.2.2.9 Reactivation at different concentrations of cytoplasmic aspartate aminotransferase

Aspartate aminotransferase was unfolded in 6 M GdnHCl and then refolded in the presence of 0.1 mM PLP and 1mM DTT as in Section 4.2.2.7. The aspartate aminotransferase concentrations during refolding were 0.5, 1.0, 2.0, 4.0 and 5.6 µg/ml. Controls in which no GdnHCl was present were set up for each aspartate aminotransferase concentration. The samples and controls were assayed for activity at several time points over 24 hr.

4.2.2.10 Determination of kinetic parameters

The kinetic parameters of native and refolded aspartate aminotransferase were determined by systematically varying the concentrations of L-aspartate and 2-oxoglutarate in the assay mix. The concentrations of L-aspartate were 15, 10, 6, 4 and 2.5 mM and at each of these the concentrations of 2-oxoglutarate were 0.75, 0.25, 0.15, 0.10 and 0.075 mM. The native enzyme activity was measured in each of these assay mixes by adding enzyme to a concentration of 0.2 µg/ml. Refolded enzyme was obtained by allowing enzyme (2.6 µg/ml) to refold in the

presence of 0.1 mM PLP and 1 mM DTT for 24 hr at 20°C after 15 min denaturation in 6 M GdnHCl. The refolded enzyme activity was measured as for the native enzyme.

The data were analysed by primary (double-reciprocal) plots and secondary plots (Price and Stevens, 1982). A BBC model B computer with the computer program Microtab was used to construct the regression lines in the analysis. The primary plots were of 1/V against 1/A at each concentration of B where V = enzyme activity (units/mg), A = L-aspartate concentration (mM) and B = 2 oxoglutarate (mM). From the primary plots values were obtained for the ordinate intercept at each concentrations of B. These ordinate intercept values were plotted against 1/B in secondary plots. In the secondary plots the ordinate intercept represents the 1/V max value. The slope represents $K_B/Vmax$. In the primary plots the slope represents $K_A/Vmax$.

4.2.2.11 Gel filtration of refolded cytoplasmic aspartate aminotransferase

Gel filtration on Sepharose 4B was used to determine whether aggregation of enzyme occurred during refolding. A Sepharose 4B column (1.5 cm internal diameters x 13 cm) was equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. The void volume of the column was determined using blue dextran. The enzyme was unfolded in 6 M GdnHCl by incubation at 0°C for 15 min then refolded in the absence and presence of 0.1 mM PLP and 1 mM DTT for 24 hr at 20°C. The enzyme concentration during refolding was 5 µg/ml. The samples (volume 20 ml) were concentrated by dialysis against 20% polyethylene glycol 10 000 to a volume of 1 ml. The concentrated samples were then dialysed against 0.1 M sodium phosphate buffer, pH 7.4 before being applied to a Sepharose 4B column. Fractions of 0.97 ml were collected from the column and the absorbance at 280 nm and the enzyme activity of the fractions were measured.

4.2.2.12 Addition of pyridoxal 5'-phosphate and dithiothreitol during refolding of cytoplasmic aspartate aminotransferase

Four identical samples were incubated in 6 M GdnHCl at 0°C for 15 min. Refolding was then initiated by 60-fold dilution of the samples and incubation at 20°C. To the first sample, 0.1 mM PLP and 1 mM DTT were added at zero time. The second and third samples were incubated for 1 hr and 24 hr, respectively before addition of PLP and DTT. The fourth sample had no PLP or DTT added. Controls in which no GdnHCl was present were set up for each of the samples. The activites of the samples and controls were determined at several time points over a 48 hr period.

4.3. RESULTS

4.3.1. Purification and characterisation of cytoplasmic aspartate aminotransferase from pig heart

Cytoplasmic aspartate aminotransferase (c. AAT) was purified from pig heart following the method in Section 4.2.2.1. Using this method 16 mg of enzyme could be isolated from 400 g of pig heart. The purified enzyme had typically, a specific activity of 154 μmol/min per mg. This value can be compared with a value of 138 μmol/min per mg obtained by Banks et al. (1968b) under slightly different assay conditions. An example of a purification procedure is given in Table 4.1. The enzyme preparation was >95% homogeneous on SDS/polyacrylamide gel electrophoresis as judged by staining with Coomassie Blue. A photograph showing the c. AAT from pig heart and the commercial enzyme on a 12% polyacrylamide gel is shown on Plate 4.1. This shows that the commercial enzyme contains significant higher M_r material as mentioned in Section 4.1.

The subunit M_r of the c. AAT as determined from SDS/polyacrylamide gel electrophoresis was estimated to be 45 000 ± 2000. This value can be compared with a value of 46 344 calculated from the amino acid sequence (Barra et al., 1980).

4.3.2. Unfolding of cytoplasmic aspartate aminotransferase in GdnHCl

4.3.2.1 Enzyme activity in the presence of GdnHCl

Cytoplasmic aspartate aminotransferase was incubated in the presence of increasing concentrations of GdnHCl and then assayed in the presence of the same concentration of GdnHCl by the discontinuous assay

Table 4.1 Purification of cytoplasmic aspartate aminotransferase

Stage of purification	Total Activity(units)	Total Protein(mg)	Specific Activity (units/mg)
Fraction I	15640	3956	4
$(NH_4)_2SO_4$ Fractionation			
Fraction II	12985	2241	6
Fraction III	10289	490	21
CM cellulose			
Pooled Fraction 1	6322	70	90
DEAE Cellulose			
Pooled Fraction 2	4476	32	140
Sephacryl S-300			
Pooled Fraction 3	2478	16	155
Redissolved Pellet	2606	16	163

From 408 g pig heart

Fraction I - crude extract
Fraction II - 55% supernatant
Fraction III - 65% pellet

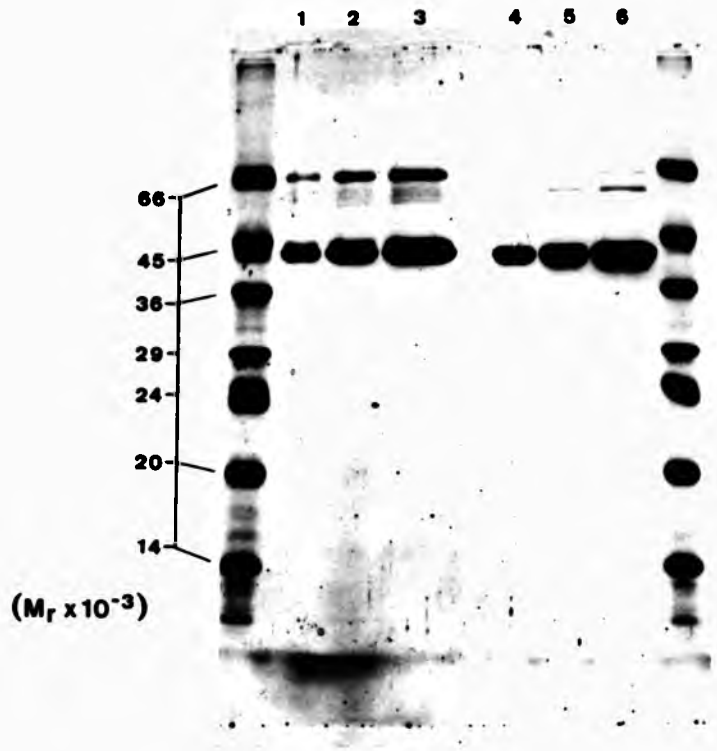


Plate 4.1 Cytoplasmic aspartate aminotransferase from pig heart on a 12 % polyacrylamide gel

Lanes 1, 2 and 3 represent 10 µg, 20 µg and 40 µg of the commercial cytoplasmic aspartate aminotransferase respectively. Lanes 4, 5 and 6 represent 10 µg, 20 µg and 40 µg of the purified cytoplasmic aspartate aminotransferase from pig heart respectively.

procedure, described in Section 4.2.2.4. The activities at different GdnHCl concentrations are expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 4.3.

There is a progressive loss of activity with increasing concentration of GdnHCl and by 2 M GdnHCl there is $< 10\%$ activity remaining. The GdnHCl concentration at which 50% loss of activity occurs is 0.7 M. These findings are consistent with the observation by Bertland and Kaplan (1970) that the c. AAT from chicken heart completely loses activity on incubation in 6 M GdnHCl.

4.3.2.2 Fluorescence studies

The fluorescence emission spectra of c. AAT in the absence of GdnHCl and in the presence of 3 M and 6 M GdnHCl are shown in Fig. 4.4, represented by the continuous curves (0), (3) and (6) respectively. The fluorescence emission maximum of the native enzyme, when excited at 290 nm, occurs at 330 nm. This is characteristic of tryphophan side chains partially shielded from the aqueous solvent (Teipel and Koshland, 1971). In the presence of GdnHCl, various changes occur in the fluorescence spectra. In the concentration range up to 3 M GdnHCl the fluorescence intensity decreases and there is a shift in the emission maximum to 340 nm. As the concentration of GdnHCl is increased up to 6 M GdnHCl the fluorescence intensity increases and the emission maximum shifts to 350 nm. This emission maximum value is characteristic of tryptophan side chains exposed to the aqueous solvent (Teipel and Koshland, 1971). The exposure of the tryptophan side chains is due to the dissociation and unfolding of the enzyme. The changes in fluoresence at 350 nm are shown in Fig. 4.5.

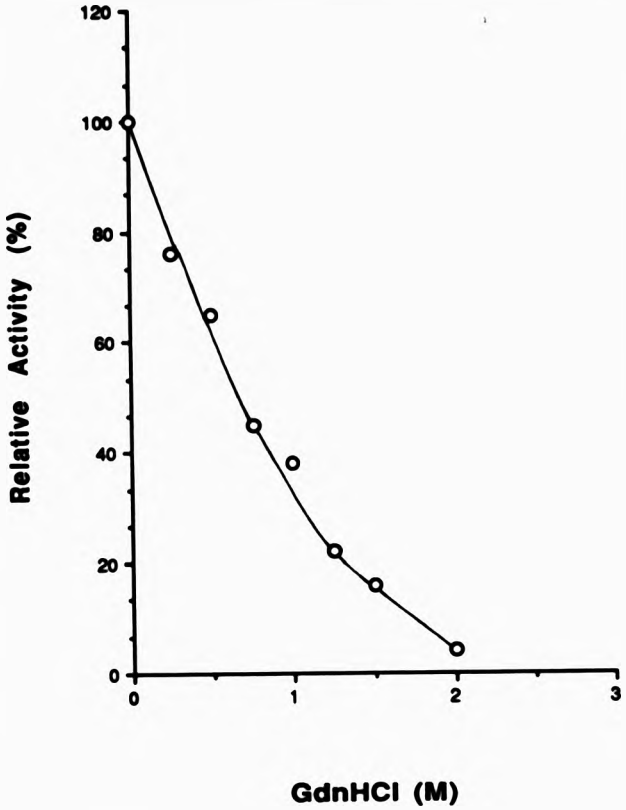


Fig. 4.3 Activity of cytoplasmic aspartate aminotransferase in the presence of GdnHCl

Enzyme (20 µg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C for 15 min. Samples were then assayed by the discontinuous procedure (Section 4.2.2.3).Activities are expressed relative to a control sample from which GdnHCl was omitted.

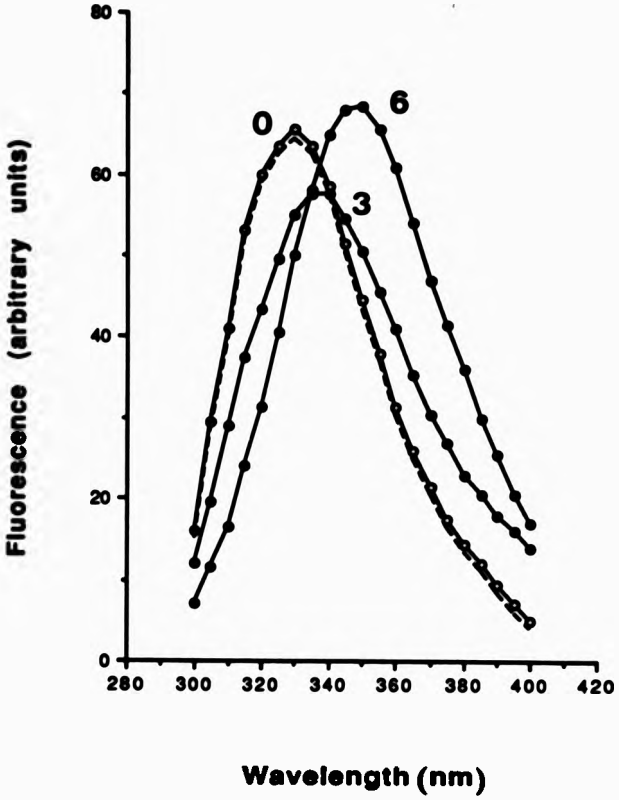


Fig. 4.4 Fluorescence emission spectra of cytoplasmic aspartate aminotransferase in the absence and presence of GdnHCl

Enzyme (20 μg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4, in the absence and presence of GdnHCl, at 20°C. Spectra were recorded 15 min after the addition of GdnHCl. The excitation wavelength was 290 nm. The curves 0, 3 and 6 represent enzyme in the absence of GdnHCl and in the presence of 3 M and 6 M GdnHCl respectively. The dashed curve represents enzyme refolded in the presence of 0.1 mM PLP and 1 mM DTT for 24 hr, after unfolding in 6 M GdnHCl.

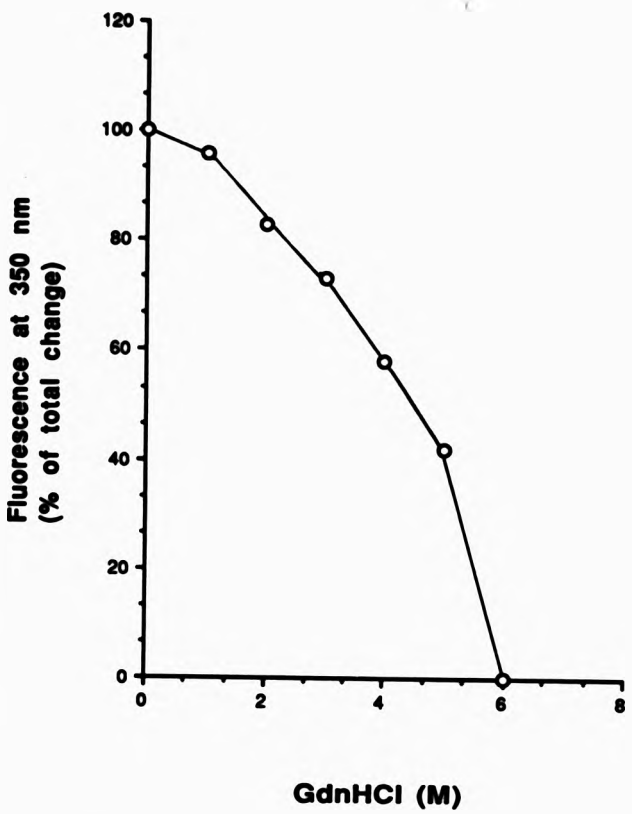


Fig. 4.5 Changes in fluorescence at 350 nm

Cytoplasmic aspartate aminotransferase (20 μg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm. The changes in fluorescence intensity at 350 nm are expressed relative to the total change between 0 and 6 M GdnHCl.

The increased exposure of tryptophan side chains on addition of GdnHCl was confirmed by measuring the fraction accessible (*fa*) to quenching by succinimide. The fraction of fluorophores accessible to quenching by succinimide at different concentrations of GdnHCl is shown in Fig. 4.6. The fraction accessible was determined by the method of Lehrer (1971) to be 0.52 for the native c. AAT. In the GdnHCl concentration range between 0 M and 2 M there was no significant change in the *fa* value. As the concentration of GdnHCl was increased beyond 2 M to 5 M the value of *fa* rose towards 1.0. The maximum value for *fa* is 1.0, which corresponds to quenching of all tryptophan residues present in the protein. These results show that at 5 M GdnHCl all the tryptophan residues in c. AAT are quenched by succinimide. This is due to the dissociation and unfolding of the enzyme at this concentration of GdnHCl, which exposes all the tryptophan residues to aqueous solvent.

Measurements were also made for the fraction accessible to quenching by acrylamide, which is known to be a very efficient quencher of tryptophan fluorescence (Eftink and Ghiron, 1984). Acrylamide is a more efficient quencher of protein fluorescence, as it is smaller than succinimide and can penetrate proteins to quench the buried tryptophan residues, which succinimide cannot quench. The *fa* value for the native c. AAT was found to be 1.0 and this value did not change appreciably as the concentration of GdnHCl was increased.

4.3.2.3 Circular dichroism studies

The far-u.v. circular dichroism (c.d.) spectrum of the native c. AAT is shown in Fig. 4.7. The spectrum is similar to that reported by Martinez-Carrion *et al.* (1970). The negative ellipticity values over the wavelength range 205-250 nm are characteristic of a protein with a

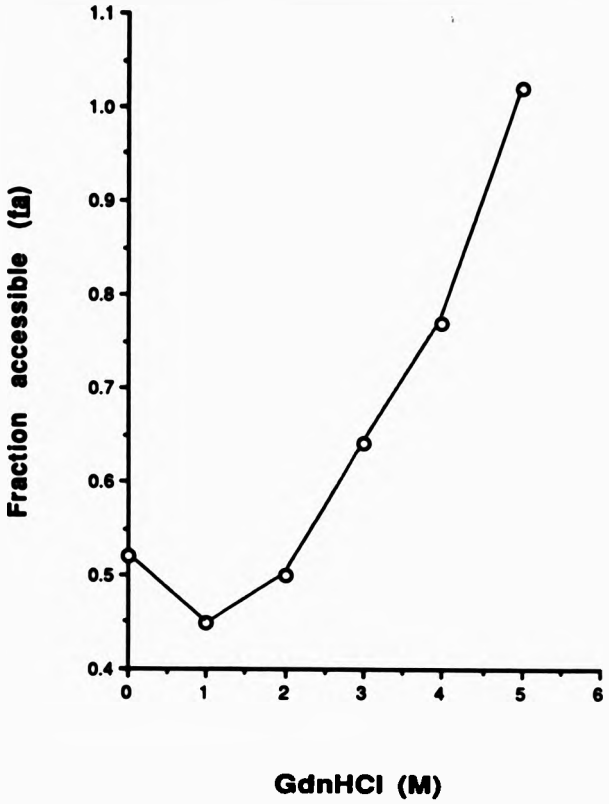


Fig. 4.6 Fraction of fluorophores accessible to quenching by succinimide

Enzyme (40 µg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20^{o}C. The emission wavelength was 325 nm.

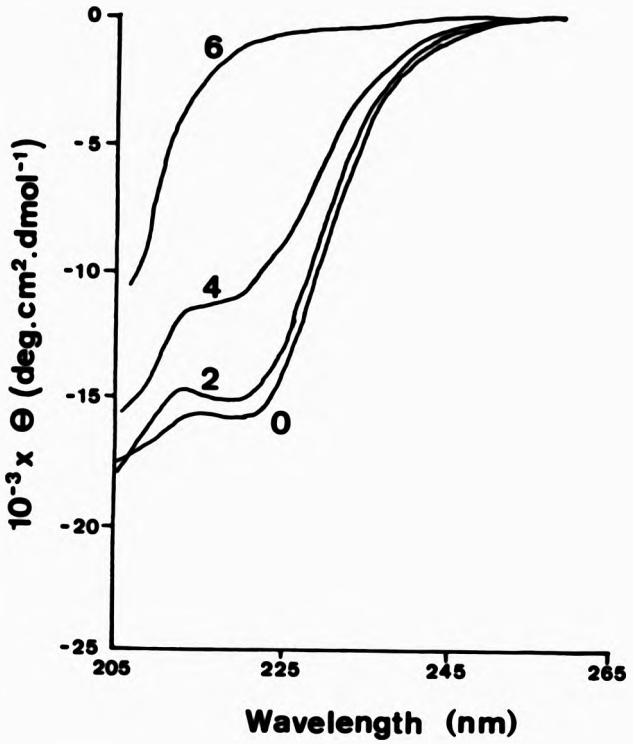


Fig. 4.7 Far-u.v. circular dichroism spectra

Spectra of cytoplasmic aspartate aminotransferase (0.8 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4 were recorded in the absence of GdnHCl and in the presence of 2 M, 4 M and 6 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample.

significant content of α-helical structure. Using the reference value of θ_{225} (Chen et al., 1974; Chang et al., 1978) the α-helical content can be calculated to be 49%. This agrees well with the value of 48% determined by X-ray crystallography of both cytoplasmic and mitochondrial forms of the chicken enzyme (Borisov et al., 1980, 1985).

The far-u.v. c.d. spectra of c. AAT in the presence of 2 M, 4 M and 6 M GdnHCl are shown in Fig. 4.7. In the presence of 2 M GdnHCl there is relatively little change in the c.d. spectrum. In 4 M GdnHCl there are larger changes in the c.d. spectrum with the ellipticity values becoming less negative. In 6 M GdnHCl there is a large reduction in ellipticity which represents complete loss of secondary structure of the enzyme. The θ_{225} value for the c. AAT in 6 M GdnHCl is 63% of that of the native enzyme. The changes in θ_{225}, which reflect the changes in helical content (Chen et al., 1974) occur between 2 M and 6 M GdnHCl.

The near-u.v. c.d. spectrum of the native c. AAT is shown in Fig. 4.8, represented by the continuous line. The spectrum shows weak bands at 280 nm (positive), 300 nm (negative) and 365 nm (positive). The first two bands arise from tyrosine and tryptophan side chains respectively (Alder et al., 1973). The third band is due to the pyridoxal 5'-phosphate cofactor.

The near-u.v. c.d. spectra of c. AAT in the presence of 2 M and 4 M GdnHCl are also shown in Fig. 4.8. The near-u.v. c.d. spectrum in the presence of 6 M GdnHCl showed no signal. Addition of GdnHCl causes substantial changes in the spectra. There are changes in the near-u.v. c.d. spectrum at 2 M GdnHCl, corresponding to tyrosine and tryptophan. These changes occur at lower concentrations of GdnHCl than the changes in the far-u.v. This is consistent with a general observation that the tertiary structure of proteins is more readily disrupted by GdnHCl than

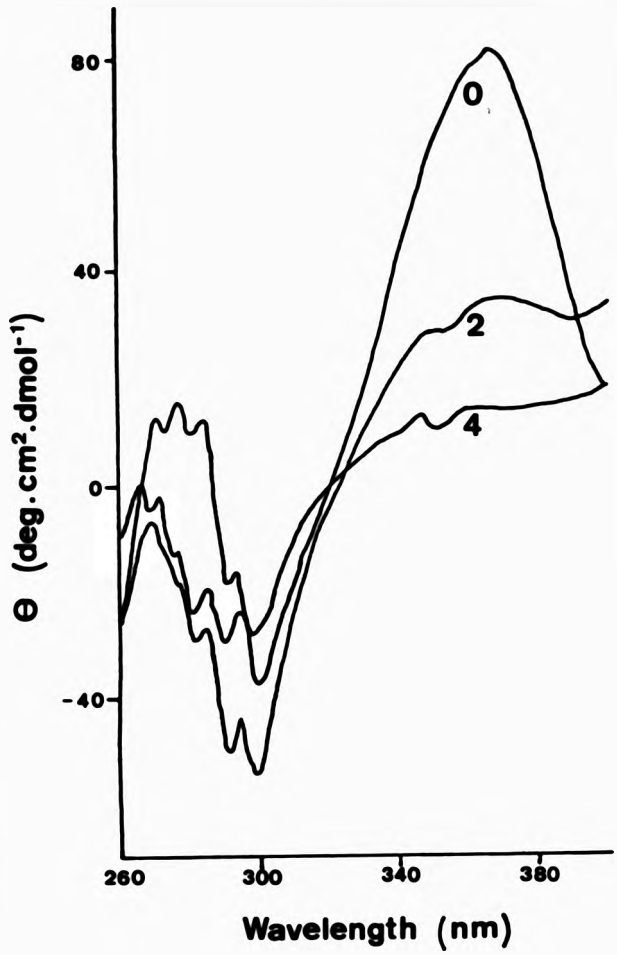


Fig. 4.8 Near-u.v. circular dichroism spectra

Spectra of cytoplasmic aspartate aminotransferase (0.8 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4 were recorded in the absence of GdnHCl and in the presence of 2 M and 4 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample. The spectrum of the enzyme in the presence of 6 M showed no signal.

the secondary structure (Creighton, 1978; Tsou, 1986). The decrease in the value of θ_{365} at high concentrations of GdnHCl is associated with the dissociation of cofactor from the enzyme. This is consistent with results from an experiment in which the binding of cofactor was studied (Section 4.3.2.4).

4.3.2.4 Binding of cofactor

The absorption spectra of c. AAT in the absence of GdnHCl and in the presence of 3 M and 6 M GdnHCl were recorded at 5 nm intervals from 280 nm to 420 nm, as in Section 4.2.2.5. The spectrum of the native c. AAT showed a small peak at 360 nm, corresponding to the bound pyridoxal 5'-phosphate cofactor as shown in Fig. 4.9. This peak did not change if the enzyme was dialysed for 24 hr at 4°C against 20 vol. of buffer (0.1 M sodium phosphate, pH 7.4). In the presence of 3 M GdnHCl, the spectrum showed the peak had shifted to 330 nm and had a slightly lower intensity (Fig. 4.9). After dialysis against buffer plus 3 M GdnHCl there was little change in the absorption spectrum showing that the cofactor remained bound to the enzyme. In the presence of 6 M GdnHCl, the spectrum showed two small peaks at 390 nm and 330 nm which were of approximately equal intensity (Fig. 4.9). These wavelengths correspond to the maximum and shoulder respectively, of the absorption spectrum of the pyridoxal 5'-phosphate. After dialysis against buffer plus 6 M GdnHCl, the peaks at 390 nm and 330 nm were eliminated (Fig. 4.9). This showed that the bound pyridoxal 5'-phosphate cofactor had become dissociated from c. AAT, which confirms the changes seen in the near-u.v. c.d. spectrum.

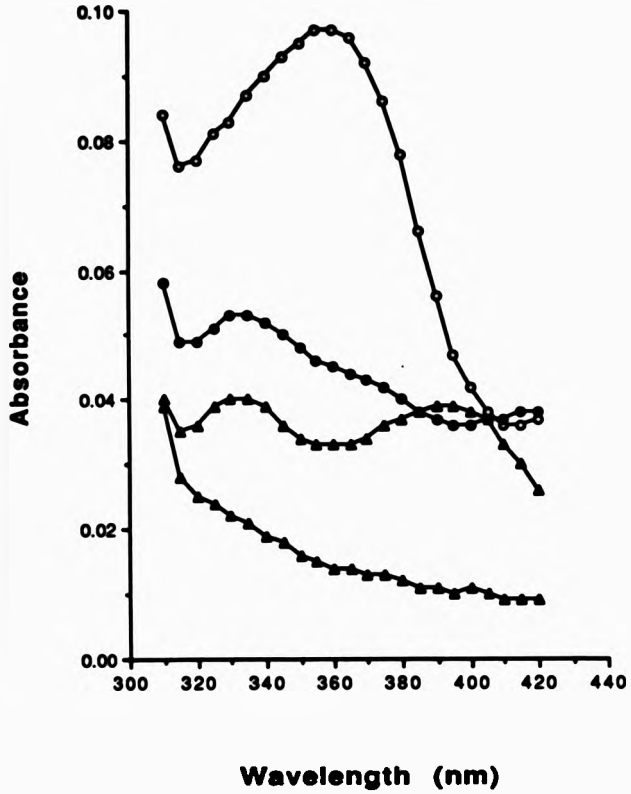


Fig. 4.9 Absorption spectra of cytoplasmic aspartate aminotransferase

Absorption spectra of enzyme (0.5 mg/ml) in the absence of GdnHCl (○) and in the presence of 3 M (●) and 6 M (△) GdnHCl were recorded from 280-420 nm, at 5 nm intervals. The spectra from 310-420 nm are shown. (▲) represents the absorption spectrum of enzyme in the presence of 6 M GdnHCl after dialysis.

4.3.2.5 Determination of reactive thiol groups

The c. AAT was incubated in different concentrations of GdnHCl, and the increase in absorbance at 412 nm on reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) was used to calculate the number of reactive thiol groups per subunit. The number of reactive thiol groups per subunit at increasing concentrations of GdnHCl are shown in Fig. 4.10. The native c. AAT possessed 2.0 thiol groups per subunit which reacted rapidly with Nbs_2. As the concentration of GdnHCl was increased to 3 M, there was little change in the number of reactive thiol groups. On increasing the GdnHCl concentration to 6 M, the number of reactive thiol groups per subunits increase to 5.1. This value corresponds to the known content of cysteine residues in each subunit (Barra et al., 1980).

These results which show two exposed thiol groups in the native enzyme and five thiol groups exposed in the unfolded form of the enzyme agree with data published by Birchmeier et al. (1973) and Arnone et al. (1977). The two thiol groups which are exposed in the native enzyme have been identified as the side chains of Cys-45 and Cys-82 (Arnone et al., 1977). The other three thiol groups which are exposed by the unfolding of the enzyme at high concentrations of GdnHCl are Cys-191, Cys-252 and Cys-390.

The activity of the c. AAT after modification of the two exposed thiol groups with Nbs_2 was measured as in section 4.2.2.6. There was little ($< 10\%$) loss of activity which agrees with data reported by Birchmeier et al. (1973).

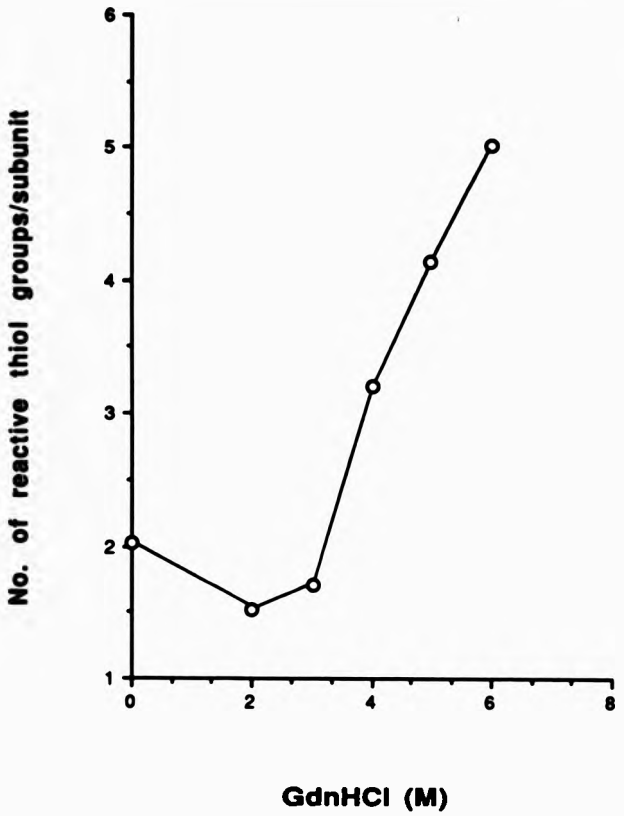


Fig. 4.10 Reactive thiol groups of cytoplasmic aspartate aminotransferase

Enzyme (0.2 mg/ml) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C. The increase in A_{412} on reaction with Nbs_2 (250 μM) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

4.3.3 Refolding of cytoplasmic aspartate aminotransferase after unfolding in GdnHCl

4.3.3.1 Reactivation of cytoplasmic aspartate aminotransferase

Cytoplasmic aspartate aminotransferase was incubated in the presence of GdnHCl to allow unfolding to occur. To initiate refolding of the enzyme, the concentration of GdnHCl was lowered by a 60-fold dilution into 0.1 M potassium phosphate buffer, pH 7.4 at 20°C. The concentration of the GdnHCl after dilution was $\leq$ 0.1 M which had no significant effect on enzyme activity. The enzyme activity was assayed 24 hr after dilution, and the regain of activity was expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 4.11. The extent of the reactivation depended on the initial concentration of GdnHCl.

Enzyme samples were allowed to refold either in the absence or presence of 0.1mM pyridoxal 5'-phosphate (PLP) and 1 mM dithiothreitol (DTT). The data show that there is a marked difference in the enzyme samples which are allowed to refold in the presence of PLP and DTT, to those which are allowed to refold in the absence of PLP and DTT, at concentrations of GdnHCl above 3 M. After incubation of c. AAT in 6 M GdnHCl 65% activity was regained after 24 hr in the presence of PLP and DTT, whereas only 2% activity was regained in the absence of PLP and DTT. Separate experiments showed that there was no significant increase in the regain of activity on increasing the PLP concentration to 1 mM during refolding. The addition of PLP or DTT separately led to much lower reactivation, approximately 10% in each case.

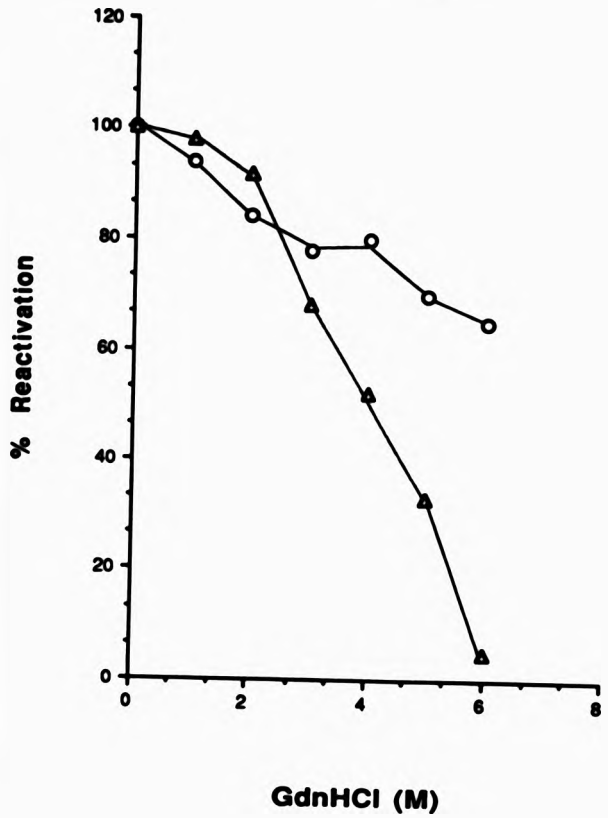


Fig. 4.11 Reactivation of cytoplasmic aspartate aminotransferase after unfolding in GdnHCl

The unfolding and refolding of the enzyme were performed as described in Section 4.2.2.7. The enzyme concentration during refolding was 2.5 µg/ml. The activity was assayed after 24 hr refolding and expressed relative to a control sample from which GdnHCl had been omitted. ○ enzyme refolded in the presence of 0.1 mM PLP and 1 mM DTT; △ enzyme refolded in the absence of these ligands.

The time dependence of the reactivation of c. AAT was studied as described in Section 4.2.2.8. This showed that the reactivation of the enzyme was time dependent and by 24 hr the activity regained was at a maximum (Fig. 4.12). An enzyme sample denatured by incubation in 6 M GdnHCl and allowed to refold in the presence of PLP and DTT had only 3% activity at 30 sec. After 30 min the activity regained had increased to 30%, by 1 hr the reactivation value increased to 42% and after 24 hr, 65% activity had been regained.

4.3.3.2 Dependence of regain of activity on concentration of cytoplasmic aspartate aminotransferase

The rate of regain of enzyme activity after unfolding in 6 M GdnHCl was studied as a function of the c. AAT concentration. Both pyridoxal 5'-phosphate and dithiothreitol were included during refolding. The regain of activity at the different concentrations of c. AAT was measured at several time points over 24 hr and the results are shown in Fig. 4.13. If the results from the samples of enzyme concentrations 1 μg/ml and 4 μg/ml are compared, the rate of reactivation is shown to be much faster in the 4 μg/ml sample. After 1 hr refolding time the 4 μg/ml sample regained 38% activity, whereas the 1 μg/ml sample has only regained 12% activity. After 24 hr refolding time the percentage reactivation was 64% and 45% for the 4 μg/ml and 1 μg/ml respectively. These values represent the limiting extents of reactivation for each of the samples, which shows the 4 μg/ml sample to have a higher value. These results show that both the rate and extent of reactivation increase with concentration of enzyme. The changes in the rate of reactivation with concentration of enzyme imply that an association step is rate-determining in the formation of active enzyme.

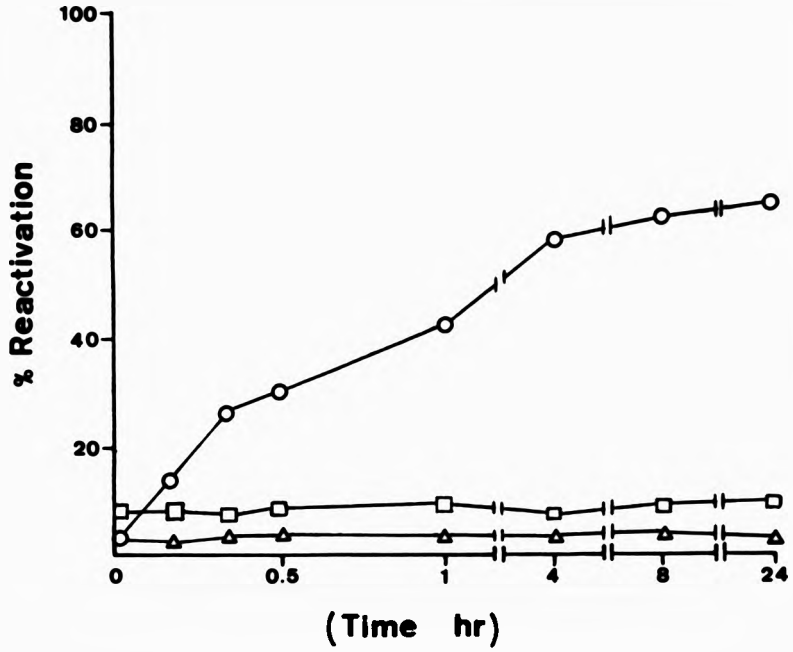


Fig. 4.12 Time-dependence of reactivation

Enzyme was unfolded in 6 M GdnHCl and refolded as described in Section 4.2.2.8. Activity was assayed at several time points over 24 hr and expressed relative to a control sample from which GdnHCl had been omitted. O, □ and △ represent enzyme refolded in the presence of PLP (0.1 mM) and DTT (1 mM), enzyme refolded in the presence of PLP or DTT and enzyme refolded in the absence of these ligands.

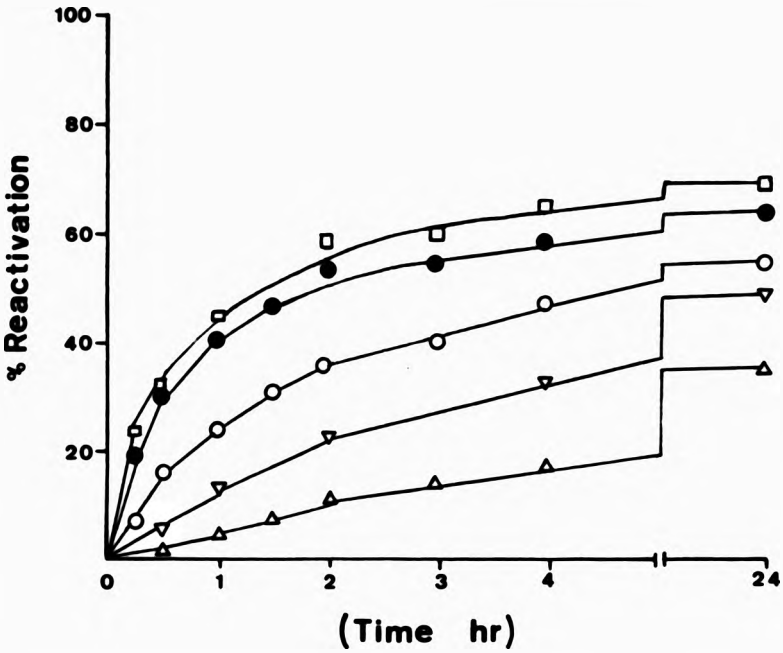


Fig. 4.13 Rate of refolding of cytoplasmic aspartate aminotransferase after unfolding in GdnHCl

Enzyme was unfolded and refolded in the presence of 0.1 mM PLP and 1 mM DTT as described in Section 4.2.2.9. △, ▽, ○, ● and □ represent enzyme concentrations of 0.5, 1.0, 2.0, 4.0 and 5.6 μg/ml respectively. Activity is expressed relative to control samples at each concentration from which GdnHCl was omitted.

The data shown in Fig. 4.13 can be fitted by a second order progress curve, with a rate constant, $k = 5.4 \times 10^3\ M^{-1}\ s^{-1}$. The assumed limiting values of reactivation measured after 48 hr refolding were 35, 48, 54, 64 and 69% repectively.

4.3.3.3. Characterisation of cytoplasmic aspartate aminotransferase after refolding

When the c. AAT was allowed to refold in the presence of pyridoxal 5'-phosphate and dithiothreitol, it was found to regain substantial activity, as shown in Figs. 4.11 and 4.13. The refolded enzyme was studied by fluorescence and kinetic measurements to see how comparable it was to the native enzyme.

To record the fluorescence of the re-activated enzyme, the enzyme sample was concentrated by dialysis against polyethylene glycol 10 000. The concentrated enzyme sample was then dialysed against 0.1 M sodium phosphate buffer, pH 7.4. The fluorescence spectrum was recorded as in Section 2.2.3.1. The fluorescence spectrum for the refolded enzyme is shown in Fig. 4.4. This shows that the refolded enzyme resembles the native enzyme as the fluorescence intensity and wavelength maximum are almost identical.

The kinetic parameters of the native and refolded enzyme were determined from primary and secondary plots as described in Section 4.2.2.10. The kinetic parameters for the native and refolded enzyme are shown in Table 4.2. The parallel lines in the primary plots show both the native and refolded enzymes obey the characteristic 'enzyme substitution' mechanism. The data in Table 4.2 show that the Michaelis constants for the substrates are largely unchanged in the refolded enzyme, as compared to the native enzyme. This indicates that the

Table 4.2 Kinetic parameters for native and refolded cytoplasmic aspartate aminotransferase

Enzyme	V (μmol/min per mg)	K_A (mM)	K_B (mM)
Native	170 ± 10	3.0 ± 0.3	0.10 ± 0.01
Refolded	138 ± 14	3.0 ± 0.3	0.13 ± 0.015

Enzyme (2.6 μg/ml) was refolded in the presence of 0.1 mM pyridoxal 5'-phosphate and 1 mM dithiothreitol for 24hr, by which time 65% activity had been regained.

refolded enzyme has similar kinetic properties to the native enzyme. In Table 4.2 the value of V for the refolded enzyme is found to be lower than for the native enzyme. The result is consistent with the incomplete regain of activity found on refolding.

4.3.3.4 Aggregation of cytoplasmic aspartate aminotransferase during refolding

After unfolding in 6 M GdnHCl, little activity (< 2%) is regained in the absence of pyridoxal 5'-phosphate and dithiothreitol as shown in Fig. 4.11. To determine whether aggregation of the enzyme occurs during refolding, gel filtration on a Sepharose 4B column was carried out. Initially a sample of the native enzyme was applied to the column. The enzyme was eluted as a single peak, as measured by absorbance at 280 nm and enzyme activity, with a maximum at fraction 18. The void volume of the column corresponded to fraction 6.

Enzyme samples incubated in 6 M GdnHCl were refolded in the absence and presence of pyridoxal 5'-phosphate and dithiothreitol for 24 hr. The reactivated and inactive samples were concentrated by dialysis against polyethylene glycol 10 000 and then dialysed against 0.1 M potassium phosphate buffer, pH 7.4. The samples were applied separately to a Sepharose 4B column and fractions collected as for the native enzyme. As shown in Fig. 4.14 most of the protein in the reactivated sample emerged at fraction 18, the elution volume of the native enzyme with only a small amount eluting at the void volume. This confirms the results in Section 4.3.3.3 that the reactivated enzyme resembles the native enzyme.

The inactive enzyme sample had a different elution profile as shown in Fig. 4.14. The majority of the protein emerged at the void

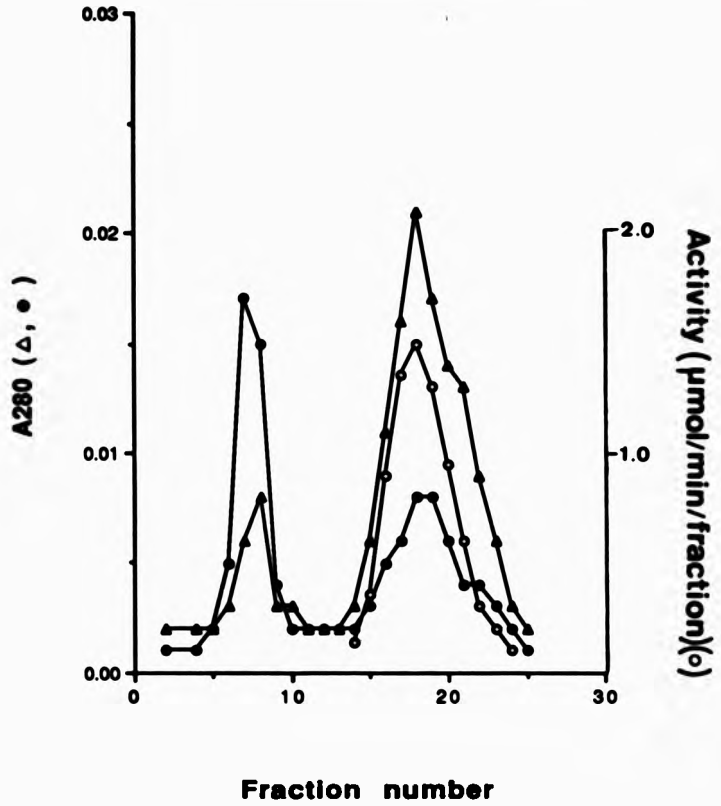


Fig. 4.14 Gel filtration of refolded cytoplasmic aspartate aminotransferase

Enzyme was unfolded in 6 M GdnHCl and refolded in the presence or absence of added 0.1 mM PLP and 1 mM DTT. After 24 hr,60 % and 1 % activity was regained in the presence and absence of PLP and DTT respectively. The enzyme samples were concentrated, dialysed and applied to a column of Sepharose 4B as described in Section 4.2.2.11. Fractions (0.97 ml) were collected and the absorbance at A_{280} was measured for enzyme in the presence (Δ) and absence (●) of PLP and DTT. Activity (○) was measured for enzyme in the presence of PLP and DTT. Fraction 6 corresponds to the void volume of the column and fraction 18 represents the elution volume of the native enzyme.

volume, but a significant amount was eluted at the position corresponding to the elution volume of the native enzyme. These results show that in the absence of PLP and DTT, in the refolding solution, the unfolded c. AAT refolded to yield a substantial amount of inactive aggregated material.

The fluorescence emission spectrum was recorded for the inactive enzyme sample, as shown in Fig. 4.4. The fluorescence spectrum shows an emission maximum at 335 nm. This differs from the native enzyme and enzyme refolded in the presence of PLP and DTT, which have emission maxima of 330 nm. This suggests that the inactive enzyme sample was more loosely folded as the tryptophan side chains are more exposed to the aqueous solvent.

As this aggregated inactive material occurs in the enzyme sample refolded in the absence of PLP and DTT, an experiment was undertaken to check whether this aggregated and/or other inactive material could be induced to regain activity by addition of PLP and DTT, as described in Section 4.2.2.12. Three samples had PLP and DTT added at different time points during refolding - 0 hr, 1 hr and 24 hr. A control sample was included which had no PLP and DTT added. The regain of enzyme activity as expressed relative to a control sample for each of the samples is shown in Fig. 4.15. The first sample to which PLP and DTT were added at 0 hr, shows 60% reactivation has been reached by 24 hr, which corresponds to the results shown in Fig. 4.11. If the sample is left for 48 hr, no further increase in the reactivation value occurs. The second sample showed only 2% reactivation after 1 hr incubation at 20^{o}C. PLP and DTT were then added to the sample and after 24 hr 43% reactivation occurred. This reactivation value did not increase further if left until 48 hr. This shows reactivation of the enzyme occurs to a smaller

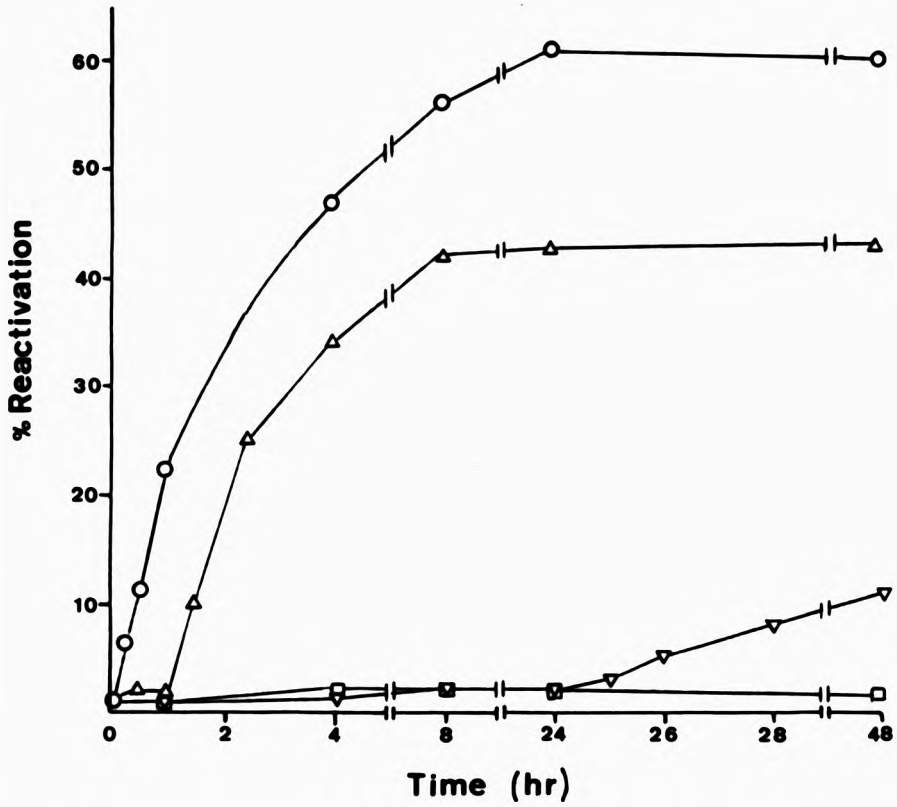


Fig. 4.15 Reactivation over a time course

Enzyme was unfolded in 6 M GdnHCl and refolded as described in Section 4.2.2.12. ○, △, ▽ represent addition of 0.1 mM PLP and 1 mM DTT at 0 hr, 1 hr and 24 hr during refolding and □ represents a sample which had no PLP or DTT added. Activity was measured at several time points over 48 hr. Activity is expressed relative to a control, for each of the samples, which had no GdnHCl present.

extent if PLP and DTT are added later. The third sample showed only 2% reactivation after 24 hr. PLP and DTT were then added and after a further 24 hr only 10% reactivation was found. This reactivation value is much smaller than those in the other two samples. The control sample to which no PLP and DTT were added, only showed 2% reactivation after 48 hr.

These results show that when refolding of the enzyme occurs in the absence of PLP and DTT, there is a progressive decrease in the potential degree of reactivation of the enzyme by subsequent addition of these ligands. This decrease is probably associated with the formation of aggregates. In the refolding solution the proportion of aggregated material will increase with time and this will leave less enzyme available for reactivation.

4.4. CONCLUSIONS

The results of the experiments described in this chapter show that cytoplasmic aspartate aminotransferase is unfolded by incubation in GdnHCl. The results for the changes in structure as detected by fluorescence, circular dichroism, fluorescence quenching and exposure of thiol groups were combined, as shown in Fig. 4.16. The changes in enzyme activity (Fig. 4.3) occur at lower concentrations of GdnHCl than those in fluorescence, circular dichroism, fluorescence quenching and exposure of thiol groups, as has been found for a number of enzymes (Tsou, 1986).

The loss of enzyme activity which occurs at low concentrations of GdnHCl ($\leq$ 2 M) can be caused by slight changes at the active sites as discussed by Tsou (1986). At concentrations of GdnHCl < 3 M, the changes which are seen in fluorescence, circular dichroism, fluorescence quenching and exposure of thiol groups (Fig. 4.16) are very small. Over this GdnHCl concentration range there is a high degree of reactivation of c. AAT, both in the absence and presence of pyridoxal 5'-phosphate and dithiothreitol (Fig. 4.14). These results show that at concentrations of GdnHCl between 0-3 M there are small changes at the active site of the enzyme. The changes lead to a loss of enzyme activity, but there are no changes in structure as detected by fluorescence and circular dichroism. The results obtained for fluorescence quenching and exposure of thiol groups show no increase in exposure of tryptophan side chains and thiol groups respectively. The regain of enzyme activity occurs when the GdnHCl is diluted, allowing reversal of the small changes at the active site which were caused by the GdnHCl.

At GdnHCl concentrations above 3 M there are changes in the structure of the c. AAT as detected by fluorescence and circular

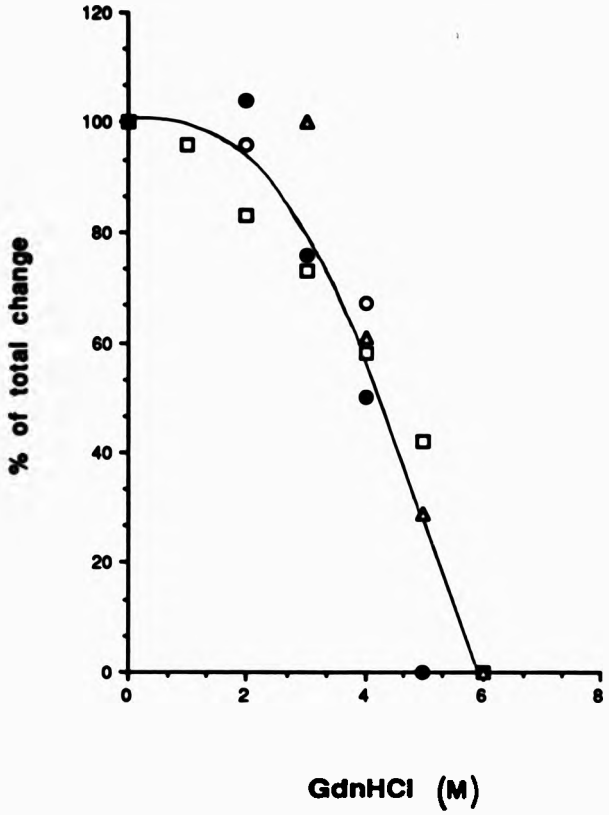


Fig. 4.16 Combined data for cytoplasmic aspartate aminotransferase

The changes in fluoresence intensity at 350 nm (□), θ_{225} (○) and the number of reactive thiol groups/subunit (△) are expressed relative to the total change between 0 and 6 M GdnHCl. The changes in the fraction of fluorophores accessible to quenching by succinimide (●) are expressed relative to the total change between 0 and 5 M GdnHCl.

dichroism. The fluorescence and circular dichroism measurements detect changes in the secondary structure of the enzyme. This shows that dissociation and unfolding of the enzyme occurs at concentrations of GdnHCl above 3 M. Confirmation of the unfolding of the enzyme at concentrations of GdnHCl above 3 M is provided by the increased exposure of tryptophan side chains and reactive thiol groups (Figs. 4.6 and 4.10).

The reactivation of c. AAT at higher concentrations of GdnHCl is dependent on the presence of pyridoxal 5'-phosphate and dithiothreitol (Fig. 4.11). The requirement for pyridoxal 5'-phosphate can be explained by the observation that the cofactor dissociates from the enzyme at high concentrations of GdnHCl. Addition of pyridoxal 5'-phosphate to the refolding solution increases the concentration of pyridoxal 5'-phosphate available to the enzyme and in turn increases the amount of enzyme which can regain activity by binding pyridoxal 5'-phosphate. The requirement for dithiothreitol could be explained by the need to maintain the cysteine side chains in a reduced state when exposed to high concentrations of GdnHCl.

The regain of enzyme activity follows second-order kinetics (Fig. 4.13), with a rate constant (5.4×10^3 $M^{-1}.s^{-1}$) typical of that for association of subunits or groups of subunits during reactivation of oligomeric enzymes (Jaenicke, 1987). The value for the rate constant is lower than the value of $10^7 - 10^8$ $M^{-1}.s^{-1}$ expected for diffusion-controlled association of subunits (Koren and Hammes, 1976), implying a large steric restriction factor consistent with highly specific interactions between subunits at preformed contact sites (Jaenicke, 1987). The kinetics of reactivation are consistent with a mechanism in which association between subunits is required for the expression of catalytic activity. X-ray crystallography data have shown

that each active site in the dimeric enzyme contains contributions from both subunits at the subunit interface (Borisov et al., 1985).

The decline in degree of reactivation at low concentrations of enzyme (Fig.413) is associated with long-term instability of intermediates in the refolding and assembly process (Jaenicke, 1987). During refolding of the enzyme the formation of aggregates was only found to a small extent when pyridoxal 5'-phosphate and dithiothreitol were present. In the absence of pyridoxal 5'-phosphate and dithiothreitol there was a large amount of aggregated material formed (Fig. 4.14). The presence of pyridoxal 5'-phosphate may help to stabilize a folded intermediate which has the correct binding site for subunit association to form the native dimeric enzyme. This type of effect has been suggested for a number of ligands during refolding of proteins (Jaenicke, 1987).

CHAPTER FIVE

UNFOLDING AND REFOLDING OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE

5.1 INTRODUCTION

Aspartate aminotransferase occurs in two isoenzyme forms; one is located in the cytosol and the other is located in the mitochondrion. The cytoplasmic isoenzyme from pig heart was studied in the preceding chapter and the transamination reaction catalysed by aspartate aminotransferase was described in section 4.1. The mitochondrial aspartate aminotransferase from pig heart is a dimeric enzyme of identical subunits, which each have a M_r of 44 666 (Barra et al., 1980) giving a dimeric M_r of 89 332. The amino acid sequence of the enzyme has been determined (Barra et al., 1980) and the sequence contains 401 amino acid residues, 11 residues less than the cytoplasmic isoenzyme.

The two aspartate aminotransferase isoenzymes have been studied from various sources (Christen and Metzler, 1985). These studies show that there is a high degree of similarity between the two isoenzymes. The amino acid sequences of the two isoenzymes from pig heart are 48% identical (Barra et al., 1980). If the amino acid sequences of the aspartate aminotransferases from pig heart and chicken heart are compared an identity of 82% is found between the cytoplasmic isoenzymes and an identity of 85% between the mitochondrial isoenzymes (Ford et al., 1980).

The X-ray crystallographic structures of the two isoenzymes from chicken heart have been determined and they show that the tertiary structures are very similar (Borisov et al., 1985). The chicken heart mitochondrial aspartate aminotransferase subunits consist of a large coenzyme binding domain consisting of residues 48-325 and a small domain composed of the carboxyl-terminal and amino-terminal regions (Jansonius et al., 1984). There is a high content of secondary structure in the enzyme molecule. The coenzyme binding domain contains a seven-strand β-pleated sheet surrounded by α-helices to form a typical α/β supersecondary structure. This structure makes the coenzyme binding

domain a very stable and rigid structure. The small domain contains less secondary structure, two β-ribbons, one parallel and the other antiparallel. There are two active sites in each dimeric aspartate aminotransferase enzyme. These are found near the subunit interface, with some important residues being contributed by the neighbouring subunit (i.e. Tyr 70). The coenzyme binds to Lys-258.

The mitochondrial isoenzyme is synthesised in the cytosol as a higher molecular weight precursor. The enzyme is then translocated across the mitochondrial membrane into the matrix with proteolytic cleavage of the N-terminal signal sequence. In the chicken and pig enzymes the pre-sequences consist of 22 and 29 amino acids respectively (Jaussi et al., 1985; Nishi et al., 1989). The mitochondrial isoenzyme is probably translocated in a monomeric form (O'Donovon et al., 1984) with assembly of the native dimeric enzyme taking place inside the mitochondrion.

Expression of the gene corresponding to the precursor of the chicken mitochondrial isoenzyme in E. coli leads to the formation of aggregates of high M_r, whereas expression of the gene corresponding to the mature enzyme leads to formation of active dimeric enzyme (Jaussi et al., 1987). The mature protein acts as an independent folding unit which can fold to the correct conformation in the absence of the pre-sequence, translocation across the mitochondrial membrane and proteolytic processing. A more recent report has shown that the gene corresponding to the precursor of the rat liver enzyme in E. coli leads to the formation of soluble active enzyme (Altieri et al., 1989). This indicates that the pre-sequence peptide does not necessarily interfere with the folding and basic structural properties of the mature protein.

The mitochondrial aspartate aminotransferase from pig heart was studied in order to compare the unfolding and refolding behaviour of the mitochondrial isoenzyme with that of the cytoplasmic isoenzyme. In the preceding chapter the cytoplasmic isoenzyme could be refolded in reasonable yield after denaturation in 6 M GdnHCl, provided pyridoxal 5'-phosphate and dithiothreitol were present. In the absence of these ligands, formation of high M_r aggregates occurred. A comparison of the unfolding and refolding behaviour of the two isoenzymes should give some information on the steps involved in the formation of the mature mitochondrial isoenzyme. It has been noted that in preliminary renaturation experiments the isoenzyme from chicken liver could not be refolded after denaturation, though no details were given (Jaussi *et al.*, 1987).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Mitochondrial aspartate aminotransferase from pig heart was purified as in section 5.2.2.1. Pig hearts were obtained from Dunblane slaughter house.

CM Cellulose, CM Sephadex and QAE Sephadex were purchased from Sigma Chemical Co. and Sephacryl S-300 from Pharmacia Fine Chemicals.

Malate dehydrogenase from pig heart (mitochondrial) 10 mg/ml in $(NH_4)_2SO_4$ was purchased from Boehringer Mannheim.

All substrates were purchased from Sigma Chemical Co.

All other chemicals were analar grade and were purchased from BDH Chemicals or Sigma Chemical Co.

5.2.2. Methods

5.2.2.1 Purification of mitochondrial aspartate aminotransferase from pig heart

Mitochondrial aspartate aminotransferase was purified from pig heart following the method by Barra et al., (1976) including an additional gel filtration step. The first step was to remove the connective tissue and fat from three pig hearts and then homogenise in 400 ml 10 mM potassium phosphate buffer, pH 6.8 containing 5 mM 2-oxoglutarate and 0.02 μM pyridoxal 5'-phosphate. The insoluble material was collected by centrifugation at 12 000 rpm for 30 min and re-extracted with a further 300 ml of the same buffer. The combined supernatants (crude extract) were dialysed against 4 L of the same buffer overnight. The dialysed solution was clarified by centrifugation at 12 000 rpm for 20 min, to give Fraction I.

The next stage in the purification involved an ion-exchange chromatography step on CM Cellulose. Fraction I was added to a CM Cellulose column (3.5 cm internal diameter x 13 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The mitochondrial aspartate aminotransferase was eluted using 350 mM NaCl in buffer and fractions were collected overnight. The active fractions were pooled and dialysed against 2.5 L 20 mM potassium phosphate buffer, pH 6.8 overnight. The dialysed solution was centrifuged at 12 000 rpm for 20 min to give Fraction II (Supernatant).

Fraction II was applied to a CM Sephadex column (3.5 cm internal diameter x 13 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.8. The mitochondrial aspartate aminotransferase was eluted using a linear salt gradient 0 - 300 mM NaCl in buffer (180 ml of each

solution). The active fractions were pooled and protein was precipitated by addition of $(NH_4)_2SO_4$ to 85% saturation. The precipitated protein was redissolved in 20 mM potassium phosphate buffer, pH 6.8.

The next step was to dialyse the redissolved pellet against 10 mM 2-amino-2-methyl-propan-1,3-diol HCl buffer, pH 9.4 overnight. The dialysed solution was clarified by centrifugation in a bench centrifuge at 2 000 rpm for 5 min. The dialysed solution was applied to a QAE Sephadex column (2 cm internal diameter x 15 cm) equilibrated with 10 mM 2-amino-2-methyl-propan-1,3-diol HCl buffer, pH 9.4. The mitochondrial aspartate aminotransferase was eluted using the same buffer. The active fractions were pooled and the protein was concentrated by precipitation with $(NH_4)_2SO_4$ at 85% saturation. The precipitated protein was redissolved in 50 mM potassium phosphate buffer, pH 6.0.

The final step was gel filtration on a Sephacryl S-300 column (2.5 cm internal diameter x 48 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.0. The redissolved pellet was applied to the Sephacryl column and the mitochondrial aspartate aminotransferase was eluted from the column using the same buffer. The active fractions were pooled and the purified protein was concentrated by precipitation with $(NH_4)_2SO_4$ at 90% saturation. The precipitated protein was redissolved in a small volume of 50 mM potassium phosphate buffer, pH 6.0. The purified enzyme was stored at $4^{\circ}C$ in this buffer, to which 0.1 mM pyridoxal 5'-phosphate was added.

5.2.2.2 Determination of protein concentration

During the purification, the protein concentration was determined by the method of Sedmak and Grossberg (1977) as described in section 2.2.1. The protein concentration of the purified mitochondrial aspartate aminotransferase was determined from the absorbance at 280 nm using a value of $A^{0.1\%}_{1\ cm}$ of 1.40 (Barra et al., 1976).

5.2.2.3 Assay for mitochondrial aspartate aminotransferase activity

The activity of mitochondrial aspartate aminotransferase was assayed using a coupled assay procedure involving malate dehydrogenase, as described for the cytoplasmic enzyme in section 4.2.2.3. The concentrations of the substrates and coupling enzyme in 0.05 M potassium phosphate buffer, pH 6.0 were L-aspartate, 3.3 mM; 2-oxoglutarate, 3.3 mM; NADH, 80 μM; malate dehydrogenase, 5 μg/ml. The reaction was initiated by addition of mitochondrial aspartate aminotransferase to an assay mix volume of 1 ml at 25°C. The rate of change in absorbance at 340 nm was recorded. One unit of enzyme activity is the amount which catalyses the disappearance of 1 μmol of NADH per minute.

5.2.2.4 Activity of mitochondrial aspartate aminotransferase in the presence of GdnHCl

Enzyme activity in the presence of GdnHCl was determined by a discontinuous assay procedure as in section 4.2.2.4 for cytoplasmic aspartate aminotransferase. Mitochondrial aspartate aminotransferase (40 μg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4 in the absence and presence of GdnHCl at 0°C for 15 min. A sample (0.2 ml) was taken and added to a reaction mixture containing

L-aspartate, 2-oxoglutarate and the corresponding GdnHCl concentration at 25°C. Samples (0.05 ml) were taken at 1 min and 4 min after the start of the reaction and added to 0.95 ml of solution containing NADH and malate dehydrogenase (the concentrations of the substrates and coupling enzyme were as used in the continuous assay section 5.2.2.3). The rate of the mitochondrial aspartate aminotransferase catalysed reaction was determined from the difference in the changes at A_{340} for the two sampling times.

Control experiments were performed to check that the rate of the mitochondrial aspartate aminotransferase catalysed reaction was constant over the range of times studied and proportional to the amount of enzyme added.

5.2.2.5 Absorption spectra of mitochondrial aspartate aminotransferase

Absorption spectra of enzyme samples (0.5 mg/ml) in the absence and presence of 1 M and 2 M GdnHCl were recorded from 280-420 nm, at 5 nm intervals. Blanks for each of the GdnHCl concentrations were used as zero absorption at each wavelength. The samples were dialysed against 20 volumes 0.05 M potassium phosphate buffer, pH 7.4 containing the same concentration of GdnHCl for 24 hr at 4°C. The absorption spectra were recorded as before except that in each case the blank was the solution remaining outside the dialysis tubing.

5.2.2.6 Reactivation of mitochondrial aspartate aminotransferase after unfolding in GdnHCl

Mitochondrial aspartate aminotransferase was incubated in 0.05 M potassium phosphate buffer, pH 7.4 in the absence or presence of GdnHCl for 15 min at 0°C. Refolding was initiated by a 60-fold dilution into 0.05 M potassium phosphate buffer, pH 7.4 at 20°C.

5.2.2.7 Time dependence of reactivation of mitochondrial aspartate aminotransferase

Aspartate aminotransferase was incubated in GdnHCl concentrations of 1.25 M, 1.5 M and 1.75 M for 15 min at 0°C. The samples were diluted 60-fold into 0.05 M potassium phosphate buffer, pH 7.4 at 20°C. The activities of the enzyme samples were assayed at several time points over 24 hr. A control sample was incubated from which GdnHCl was omitted.

RESULTS

5.3.1 Purification and characterisation of mitochondrial aspartate aminotransferase from pig heart

Mitochondrial aspartate aminotransferase (m. AAT) was purified from pig heart following the method in section 5.2.2.1. Using this method 18 mg of enzyme could be isolated from 450 g of pig heart. The enzyme was found to have a lower specific activity (100 μmol/min per mg) if it was stored in the absence of added pyridoxal 5'-phosphate (0.1 mM). In the presence of pyridoxal 5'-phosphate the specific activity was increased to a value of 170 μmol/min per mg. This value is identical to the value reported by Barra *et al*. (1976). The results suggest that previous reports of lower specific activities of preparations of the m. AAT (Wada and Morino, 1964; Michuda and Martinez-Carrion, 1969) could arise by partial loss of cofactor from the preparations. An example of a purification procedure is given in Table 5.1

The enzyme preparation was >95% homogeneous on SDS/polyacrylamide gel electrophoresis as judged by staining with Coomassie blue. A photograph showing the mitochondrial aspartate aminotransferase from pig heart on a 12% polyacrylamide gel is shown on Plate 5.1. The subunit M_r of the m. AAT as determined from SDS/polyacrylamide gel electrophoresis was estimated to be 43 000 ± 2 000. This can be compared with a value of 44 657 calculated from the amino acid sequence (Barra *et al*., 1980).

Table 5.1 Purification of mitochondrial aspartate aminotransferase

Stage of Purification	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)
Crude extract	13900	18591	0.75
Fraction I	13395	16920	0.79
CM cellulose			
Pooled Fraction 1	9060	2262	4.0
Fraction II	8294	1956	4.2
CM Sephadex			
Pooled Fraction 2	7047	1154	6.1
QAE Sephadex			
Pooled Fraction 3	3520	25	141
Sephacryl S-300			
Pooled Fraction 4	3060	18	170

From 455g pig heart

Fraction I - crude extract after dialysis
Fraction II - pooled fraction 1 after dialysis

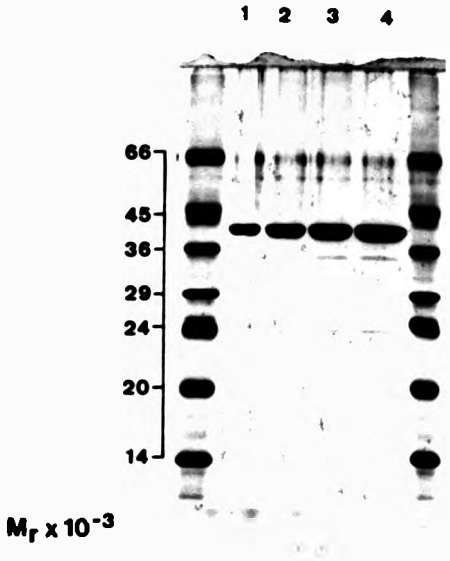


Plate 5.1 Mitochondrial aspartate aminotransferase from pig heart on a 12 % polyacrylamide gel

Lanes 1, 2, 3 and 4 represent 10 µg, 20 µg, 30 µg and 40 ug of purified aspartate aminotransferase from pig heart respectively.

5.3.2 Unfolding of mitochondrial aspartate aminotransferase in GdnHCl

5.3.2.1 Enzyme activity in the presence of GdnHCl

Mitochondrial aspartate aminotransferase was incubated in the presence of increasing concentrations of GdnHCl and then assayed in the presence of the same concentration of GdnHCl by the discontinuous assayprocedure, as described in section 5.2.2.4. The activities at different GdnHCl concentrations are expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 5.1.

There is a progressive loss of activity with increasing concentrations of GdnHCl and by 1.25 M there is $< 5\%$ activity remaining. The GdnHCl concentration at which 50% loss of activity occurs is 0.5 M. These data when compared with the data obtained for the cytoplasmic AAT (as shown in Fig. 5.2) show that the mitochondrial isoenzyme is more sensitive to GdnHCl.

5.3.2.2 Fluorescence studies

The fluorescence emission spectra of m. AAT in the absence of GdnHCl and in the presence of 1 M, 1.25 M and 2 M GdnHCl are shown in Fig. 5.3, represented by curves 1, 2, 3 and 4, respectively. The fluorescence emission maximum of the native enzyme when excited at 290 nm, occurs at 335 nm, which is characteristic of tryptophan side chains partially shielded from aqueous solvent (Teipel and Koshland, 1971). On the addition of GdnHCl there is a decrease in the fluorescence intensity, which occurs mainly between 1 M and 2 M GdnHCl. There is also a shift in the emission maximum to 345 nm at 2 M GdnHCl. If the GdnHCl concentration is increased to 4 M there is no further decrease in fluorescence intensity, but there is a small shift in the

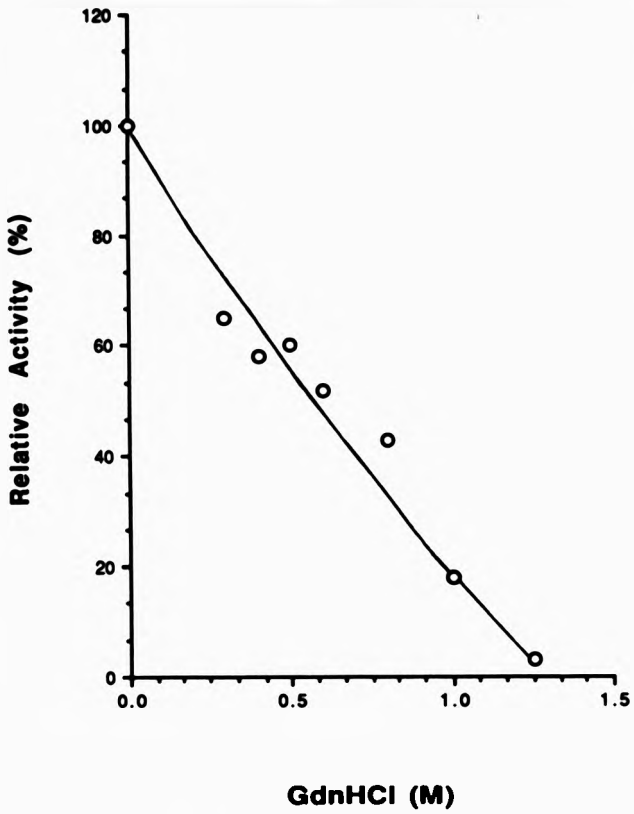


Fig. 5.1 Activity of mitochondrial aspartate aminotransferase in the presence of GdnHCl

Enzyme (40 µg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20^{o}C for 15 min. Samples were then assayed by the discontinuous procedure (Section 5.2.2.3). Activities are expressed relative to a control sample from which GdnHCl was omitted.

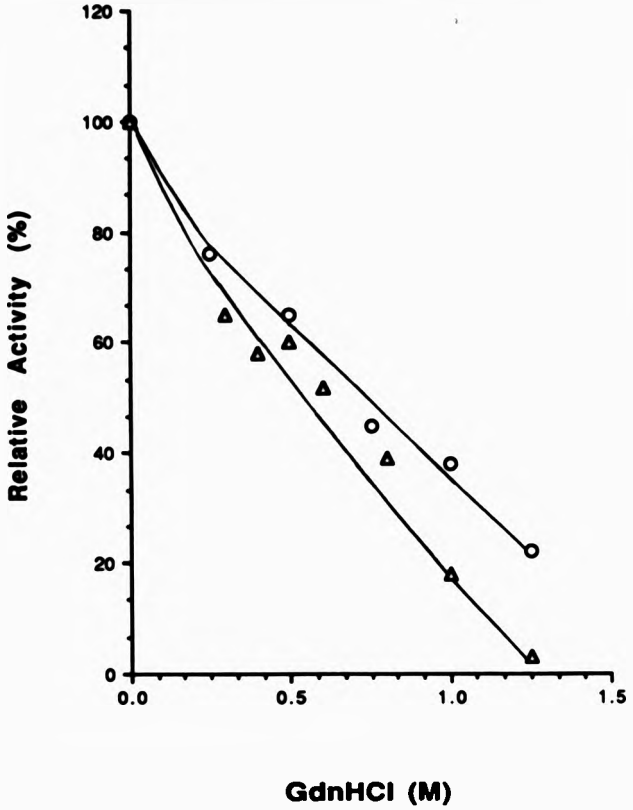


Fig. 5.2 Activity of cytoplasmic and mitochondrial aspartate aminotransferase in the presence of GdnHCl

O represents the data for the cytoplasmic isoenzyme (Fig. 4.3); Δ represents the data for the mitochondrial isoenzyme (Fig. 5.1).

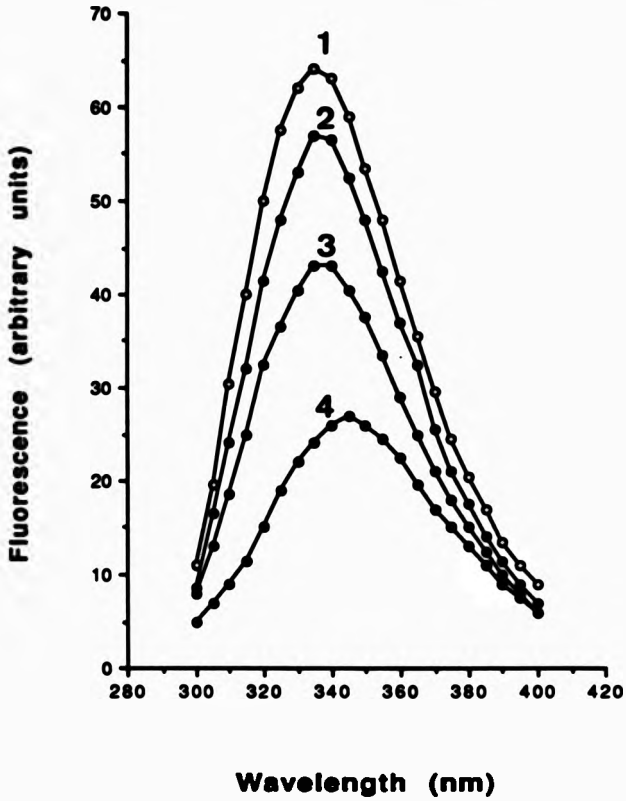


Fig. 5.3 Fluorescence emission spectra of mitochondrial aspartate aminotransferase in the absence and presence of GdnHCl

Enzyme (20 μg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4, in the absence and presence of GdnHCl at 20°C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm. The curves 1, 2, 3 and 4 represent enzyme in the absence of GdnHCl and in the presence of 1 M, 1.25 M and 2 M GdnHCl respectively.

emission maximum to 350 nm. This emission maximum value is characteristic of tryptophan side chains exposed to the aqueous solvent (Teipel and Koshland, 1971). The exposure of the tryptophan side chains is due to the dissociation and unfolding of the enzyme. The changes in fluorescence at 340 nm are shown in Fig. 5.4. This shows the changes occurring between 1 M and 2 M GdnHCl.

The emission maximum of the mitochondrial isoenzyme (335 nm) is higher than that for the cytoplasmic isoenzyme, which gave a value of 330 nm. This suggests that the tryptophan side chains are more exposed to solvent in the mitochondrial isoenzyme.

In fluorescence quenching studies of the m. AAT, the fraction of tryptophan side chains accessible to quenching by succinimide was measured. The fraction of fluorophores accessible to quenching by succinimide at different concentrations of GdnHCl is shown in Fig. 5.5. The fraction accessible (fa) for the native m. AAT was determined to be 0.70. In the GdnHCl concentration range between 0 M and 2 M there was no significant change in the fa value. As the concentration of GdnHCl was increased to 3 M the fa value rose towards 1.0. This value did not change significantly as the GdnHCl concentration was raised to 4 M. The maximum value for fa is 1.0, which represents the quenching of all the tryptophan resides present in the protein. These results show that all the tryptophan residues are quenched by succinimide at 3 M GdnHCl.

The fraction accessible to quenching by acrylamide, which is known to be an efficient quencher of tryptophan fluorescence (Eftink and Ghiron, 1984), was found to give a value of 1.0 in the absence of GdnHCl. This fa value did not change appreciably on increasing the GdnHCl concentration.

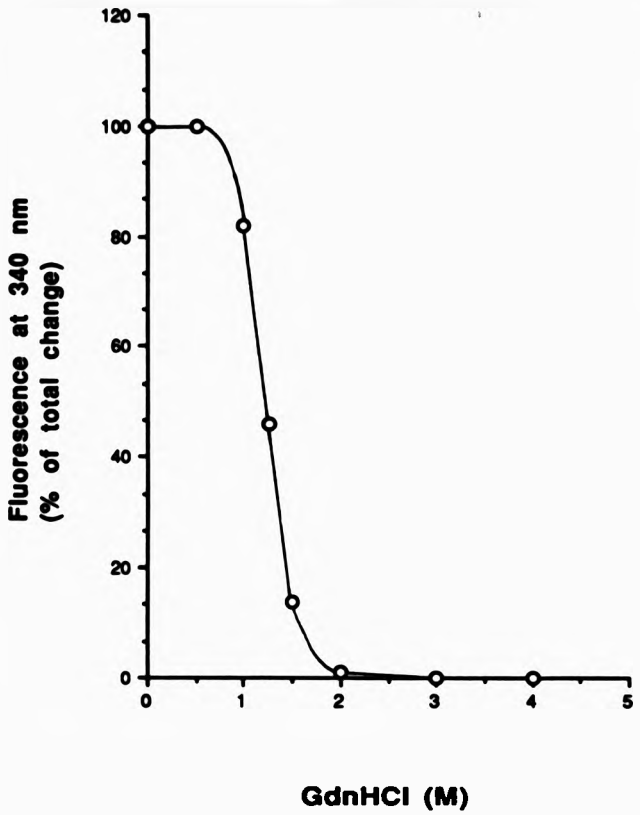


Fig. 5.4 Changes in fluorescence at 340 nm

Mitochondrial aspartate aminotransferase (20 μg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C. Spectra were recorded 15 min. after addition of GdnHCl. The excitation wavelength was 290 nm. The changes in fluorescence intensity at 340 nm are expressed relative to the total change between 0 and 4 M GdnHCl.

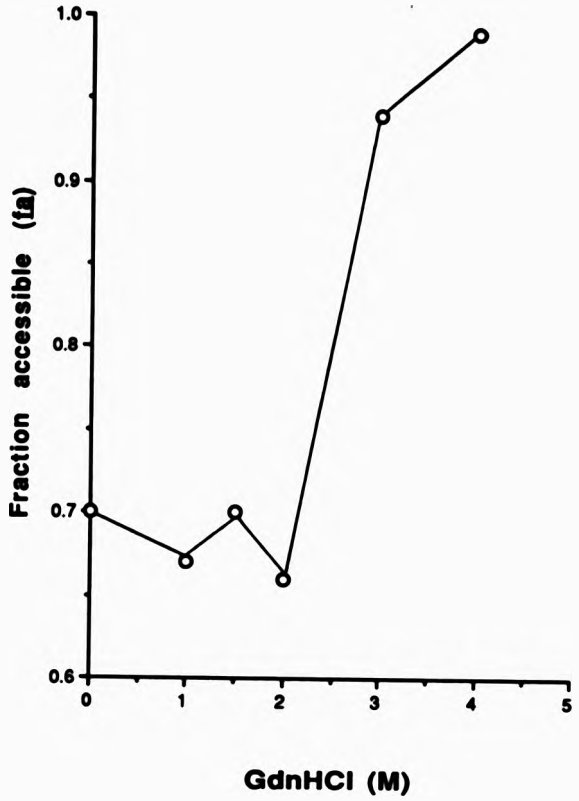


Fig. 5.5 Fraction of fluorophores accessible to quenching by succinimide

Enzyme (40 µg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C. The emission wavelength was 325 nm.

The *fa* value for the native mitochondrial isoenzyme was found to be 0.70. This value is higher than the *fa* found for the native cytoplasmic isoenzyme, which was 0.52. These results show that the tryptophan side chains are more exposed to solvent in the mitochondrial isoenzyme. This is consistent with the results obtained for the fluorescence emission maximum of the two isoenzymes.

5.3.2.3 *Circular dichroism studies*

The far-u.v. circular dichroism (c.d.) spectrum of the native m. AAT is shown in Fig. 5.6. The spectrum is similar to that reported by Martinez-Carrion *et al.* (1970). The spectrum has negative ellipticity values over the wavelength range 205-250 nm with minima at 208 nm and 220 nm, characteristic of a protein with a significant content of α-helical structure. The α-helical content can be calculated from the Θ_{225} value (Chen *et al.*, 1974; Chang *et al.*, 1978) and gave a value of 65%. This value is higher than the value (50%) determined by X-ray crystallography for the mitochondrial isoenzyme from chicken (Ford *et al.*, 1980). The reason for this difference is not clear, but the X-ray work shows that several of the helices are irregular, with interruptions to the hydrogen bonding pattern.

The far-u.v. c.d. spectra of m. AAT in the presence of 1 M, 1.5 M and 2 M GdnHCl are shown in Fig. 5.6. In the presence of 1 M GdnHCl there is relatively little change in the c.d. spectrum. In 1.5 M GdnHCl there are large changes in the c.d. spectrum with the ellipticity values becoming less negative. In 2 M GdnHCl there is very little secondary structure remaining. These results show the changes in secondary structure occur between 1 M and 2 M GdnHCl.

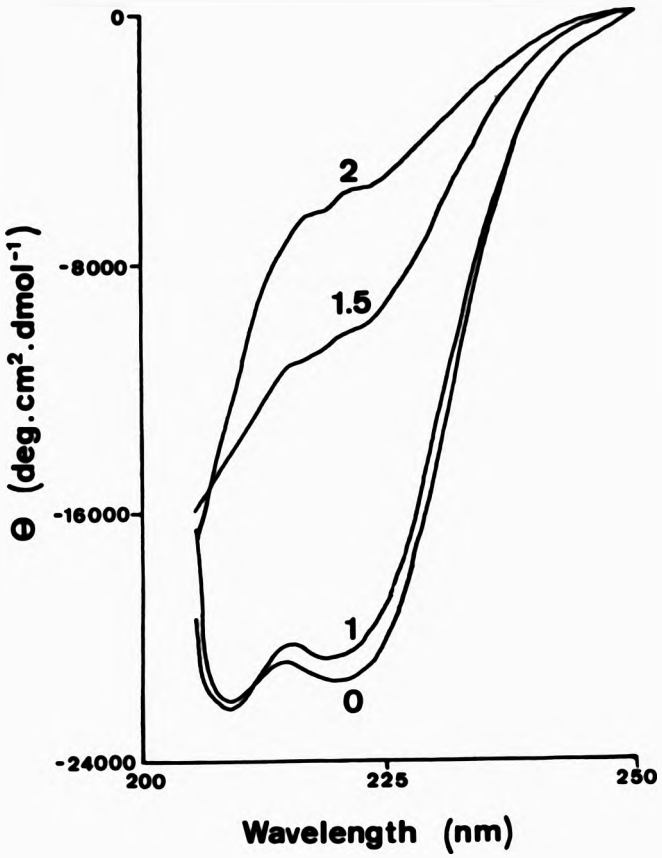


Fig. 5.6 Far-u.v. circular dichroism spectra

Spectra of mitochondrial aspartate aminotransferase (0.8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4 were recorded in the absence of GdnHCl and in the presence of 1 M, 1.5 M and 2 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample.

The near-u.v. c.d. spectrum of the native m. AAT is shown in Fig. 5.7. The spectrum shows at 275 nm (positive) and 290 nm (negative) which arise from tyrosine and tryptophan side chains respectively (Alder et al., 1973). The third band at 355 nm (positive) is due to the pyridoxal 5'-phosphate cofactor. The near-u.v. c.d. spectra of m. AAT in the presence of 1 M and 2 M GdnHCl are also shown in Fig. 5.7. On addition of GdnHCl there are substantial changes in the near-u.v. c.d. spectra. In the presence of 1 M GdnHCl the bands corresponding to the tyrosine and tryptophan side chains and the pyridoxal 5'-phosphate cofactor are very small. These changes occur at lower concentrations of GdnHCl than the changes in the far-u.v. This was also found for the cytoplasmic isoenzyme and is consistent with a general observation that the tertiary structure of a protein is a more readily disrupted by GdnHCl than is the secondary structure (Creighton, 1978; Tsou, 1986). The decrease in the value at θ_{355} on addition of 2 M GdnHCl is due to dissociation of the cofactor from the enzyme. This is shown in the next section which looks at the binding of cofactor.

5.3.2.4 Binding of cofactor

The absorption spectra of m. AAT in the absence of GdnHCl and in the presence of 1 M and 2 M GdnHCl were recorded at 5 nm intervals from 280 nm to 420 nm, as in section 5.2.2.5. The spectrum of the native m. AAT is shown in Fig. 5.8. This shows a peak at 355 nm, which corresponds to the bound pyridoxal 5'-phosphate cofactor. This peak did not change if the enzyme was dialysed for 24 hr at 4°C against 20 volumes of buffer (0.05 M potassium phosphate, pH 7.4). In the presence of 1 M GdnHCl the spectrum shows a peak at 355 nm which has a lower intensity than the peak obtained for the native enzyme (Fig. 5.8).

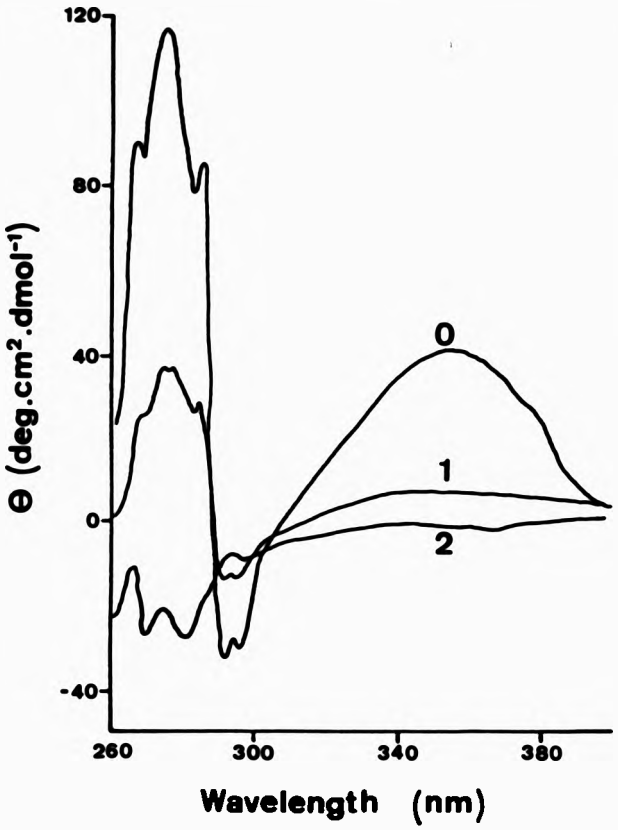


Fig. 5.7 Near-u.v. circular dichroism spectra

Spectra of mitochondrial aspartate aminotransferase (0.8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4 were recorded in the absence of GdnHCl and in the presence of 1 M and 2 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample.

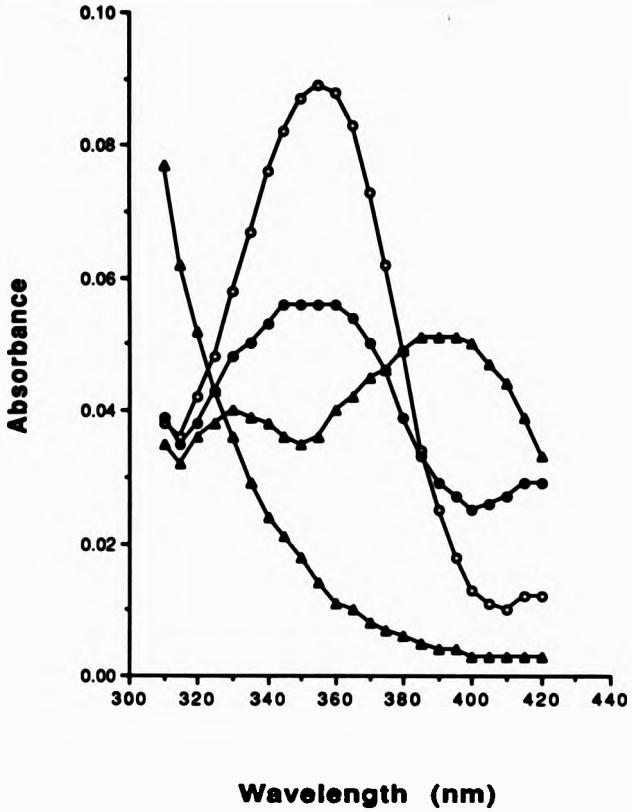


Fig. 5.8 Absorption spectra of mitochondrial aspartate aminotransferase

Absorption spectra of enzyme (0.5 mg/ml) in the absence of GdnHCl (○) and in the presence of 1 M (●) and 2 M (△) GdnHCl were recorded from 280-420 nm, at 5 nm intervals. The spectra from 310-420 nm are shown. (▲) represents the absorption spectrum of enzyme in the presence of 2 M GdnHCl after dialysis.

After dialysis against buffer plus 1 M GdnHCl there was a small decrease in the absorbance value at 355 nm. This suggests that in the presence of 1 M GdnHCl some dissociation of cofactor has occurred, which is consistent with the decrease of the θ_{355} value in the near-u.v. c.d. spectrum (Fig. 5.7).

In the presence of 2 M GdnHCl the spectrum showed two peaks at 330 nm and 390 nm, with the peak at 390 nm having slightly greater intensity (Fig. 5.8). These peaks correspond to the absorption spectrum of pyridoxal 5'-phosphate under these conditions. After dialysis against buffer plus 2 M GdnHCl the peaks at 330 nm and 390 nm were eliminated (Fig. 5.8). This showed that the cofactor had dissociated from the m. AAT and this is confirmed by the results obtained for the near-u.v. c.d. spectrum at 2 M GdnHCl.

5.3.2.5 Determination of reactive thiol groups

The m. AAT was incubated in different concentrations of GdnHCl and the increase in absorbance at 412 nm on reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) was used to calculate the number of reactive thiol groups per subunit. The number of reactive thiol groups per subunit at increasing concentrations of GdnHCl are shown in Fig. 5.9. The native m. AAT possessed virtually no thiol groups (< 0.2 group per subunit) which reacted rapidly with Nbs_2. On addition of 0.1% SDS a total of 7.1 thiol groups per subunit were found to react with Nbs_2. This value corresponds to the known content of cysteine residues in each subunit (Barra et al., 1980).

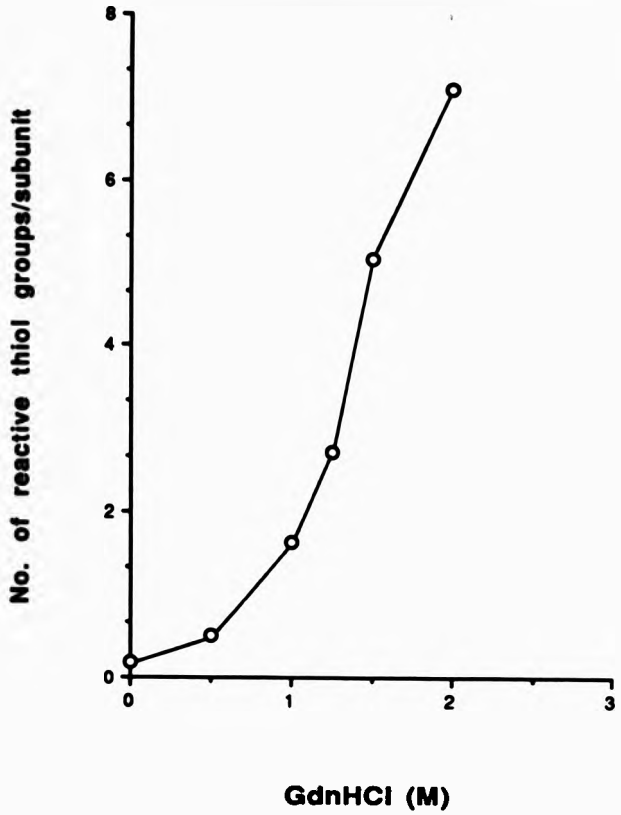


Fig. 5.9 Reactive thiol groups of mitochondrial aspartate aminotransferase

Enzyme (0.1 mg/ml) was incubated in 0.05 M potassium phosphate buffer, pH 7.4 containing increasing concentrations of GdnHCl at 20°C. The increase in A_{412} on reaction with Nbs_2 (250 μM) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

In the presence of increasing concentrations of GdnHCl there is an increase in the number of reactive thiol groups per subunit as shown in Fig. 5.9. The major increase occurs between 1 M and 2 M GdnHCl and at 2 M GdnHCl all the thiol groups have become exposed. The seven thiol groups which are exposed are Cys-80, Cys-166, Cys-191, Cys-251, Cys-253, Cys-274 and Cys-361. These results can be compared with the cytoplasmic isoenzyme which showed two thiol groups exposed in the native enzyme and five thiol groups exposed in the unfolded form of the enzyme. The increase in exposure of the thiol groups in this case occurred at a higher GdnHCl concentration range, between 3 M and 6 M.

5.3.3. Attempted refolding of mitochondrial aspartate aminotransferase after unfolding in GdnHCl

Mitochondrial aspartate aminotransferase was incubated in the presence of GdnHCl to allow unfolding to occur. The ability of the m. AAT to be reactivated was studied by diluting the enzyme 60-fold into 0.05 M potassium phosphate buffer, pH 7.4 after unfolding, as described in section 5.2.2.6. The enzyme activity was assayed 24 hr after dilution and the reactivation was expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 5.10. These results showed that full activity could only be restored if the initial concentration of the GdnHCl was $\leq$ 1 M. As the GdnHCl concentration was increased to 2 M there was a significant loss of the ability of the enzyme to be reactivated. At 2 M GdnHCl only 15% activity could be regained. This decline in reactivation occurred both in the absence and presence of 0.1 mM pyridoxal 5'-phosphate and 1 mM dithiothreitol in the diluting solution.

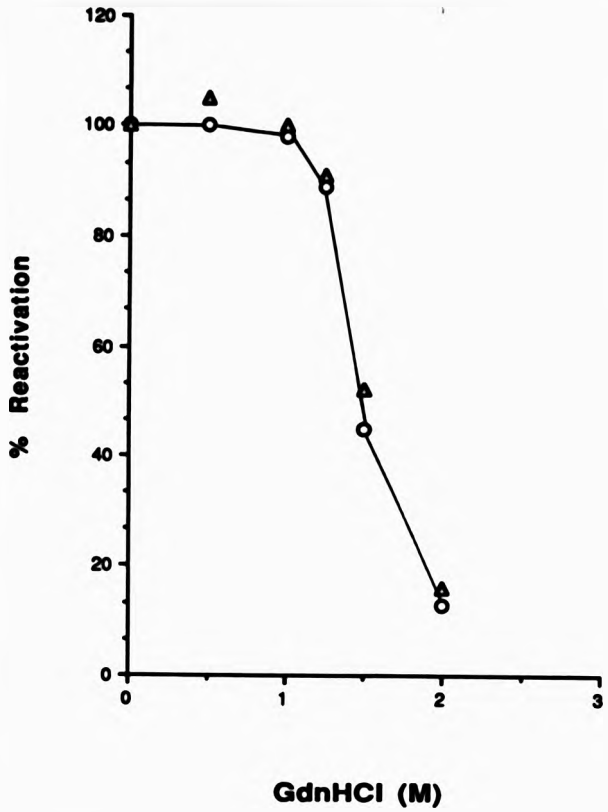


Fig. 5.10 Reactivation of mitochondrial aspartate aminotransferase after unfolding in GdnHCl

The unfolding and refolding of the enzyme were performed as described in Section 4.2.2.6. The enzyme concentration during refolding was 4 µg/ml. The activity was assayed after 24 hr refolding and expressed relative to a control sample from which GdnHCl had been omitted. ○ enzyme refolded in the presence of 0.1 mM PLP and 1 mM DTT; △ enzyme refolded in the absence of these ligands.

The time dependence of reactivation of m. AAT was studied as described in section 5.2.2.7. The reactivation at different time points is shown in Fig. 5.11. This showed there was no difference in the regain of activity over the period 30s to 24 hr after dilution. The results for reactivation of the m. AAT are very different from those observed for the c. AAT (section 4.3.3), which showed reactivation of the enzyme was dependent on the presence of pyridoxal 5'-phosphate and dithiothreitol above 3 M GdnHCl and was time dependent.

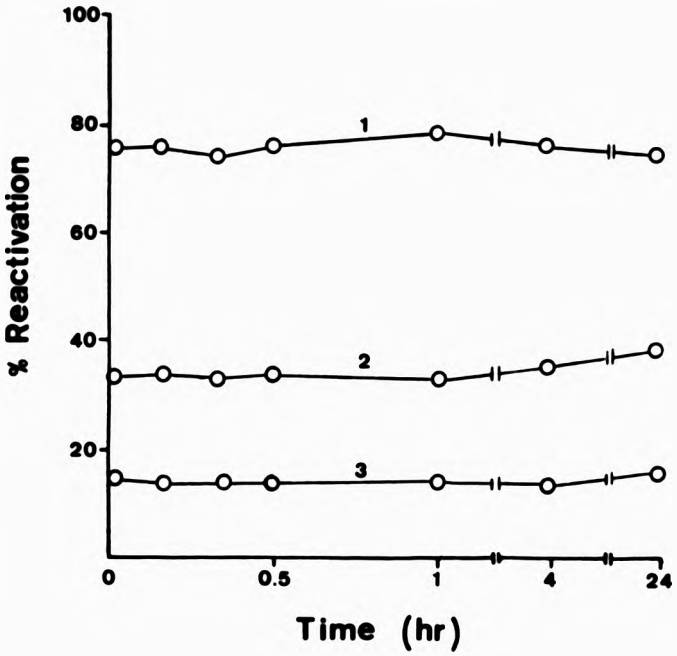


Fig. 5.11 Time-dependence of reactivation

Enzyme was unfolded and refolded as described in Section 5.2.2.7. Activity was assayed at several time points over 24 hr and expressed relative to a control sample from which GdnHCl was omitted. 1, 2 and 3 represent enzyme incubated in the presence of 1.25 M, 1.5 M and 1.75 M GdnHCl respectively before refolding.

5.4. CONCLUSIONS AND COMPARISON OF THE CYTOPLASMIC AND MITOCHONDRIAL ISOENZYMES OF ASPARTATE AMINOTRANSFERASE

The results of the experiments described in this chapter show that mitochondrial aspartate aminotransferase is unfolded by incubation in GdnHCl but cannot be refolded. The results for the changes in structure as detected by fluorescence, circular dichroism and exposure of reactive thiol groups were combined as shown in Fig. 5.12. The changes in enzyme activity (Fig. 5.1) occur at lower concentrations of GdnHCl than those in fluorescence, circular dichroism and exposure of reactive thiol groups. This was observed for the cytoplasmic isoenzyme and has been found for a number of enzymes (Tsou, 1986).

The loss of enzyme activity which occurs at low concentrations of GdnHCl (< 1M) can be caused by slight changes at the active site as discussed by Tsou (1986). Over the same GdnHCl concentration range (0 M-1 M) there are only very small changes in fluorescence, circular dichroism and exposure of reactive thiol groups (Fig. 5.12). The degree of reactivation of the enzyme was found to be high at concentrations of GdnHCl ≤ 1 M. These results show that at GdnHCl between 0 M and 1 M small changes occur at the active site leading to loss of enzyme activity but no changes occur in the structure as detected by fluorescence and far-u.v. circular dichroism.

At concentrations of GdnHCl between 1 M and 2 M there are changes in the secondary structure of the enzyme, which are detected by the fluorescence and circular dichroism measurements. This shows that dissociation and unfolding of the enzyme occurs between 1 M and 2 M GdnHCl. The unfolding of the enzyme between 1 M and 2 M GdnHCl is confirmed by the exposure of reactive thiol groups (Fig. 5.9).

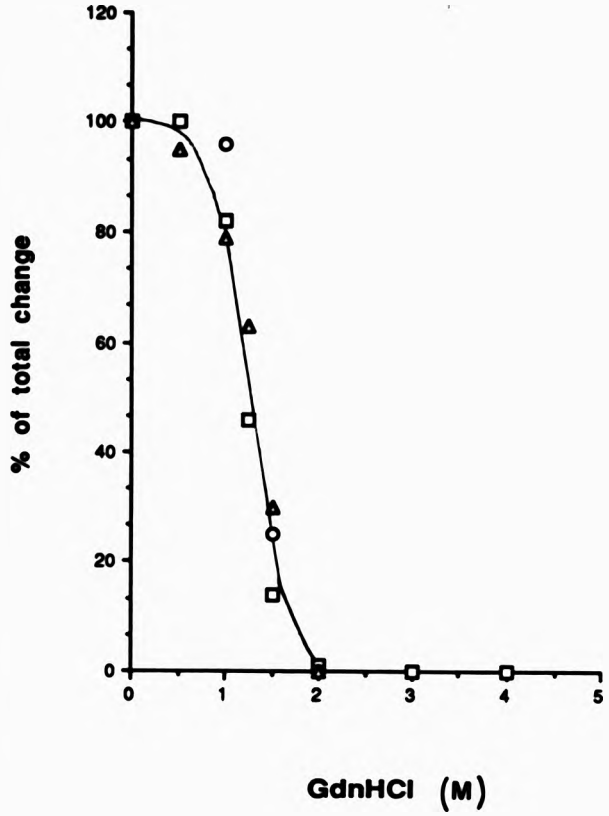


Fig. 5.12 Combined data for mitochondrial aspartate aminotransferase

The changes in fluorescence intensity at 340 nm (□) are expressed relative to the total change between 0 and 4 M GdnHCl. The changes in θ_{225} (○) and the number of reactive thiol groups/subunit (△) are expressed relative to the total change between 0 and 2 M GdnHCl.

The results obtained for the m. AAT isoenzyme show that it is almost totally unfolded in 2 M GdnHCl. The mitochondrial isoenzyme is therefore much less stable than the cytoplasmic isoenzyme, which has lost enzyme activity at 2 M GdnHCl but still retains substantial secondary and tertiary structure, as measured by fluorescence and circular dichroism.

The overall folding of the peptide chain is reported to be similar for the two isoenzymes (Borisov et al., 1985) therefore the reason for the difference in behaviour of the two isoenzyme is unclear. It may be significant that in one of the two regions of intersubunit contact identified in the X-ray crystallographic structure (Ford et al., 1980) there is a lower degree of sequence identity than for the two isoenzymes as a whole (48%). In the contact region formed between the N-terminal arm and one subunit and the cofactor binding domain of the other, there is only 29% sequence identity, compared with 49% in the other contact region. Therefore the strength of the subunit interactions may be significantly different in the two isoenzyme forms which will in turn affect the stability of the secondary and tertiary structure of each polypeptide chain.

The two isoenzymes are also different in their ability to refold after denaturation in GdnHCl. The cytoplasmic isoenzyme was found to refold after denaturation provided pyridoxal 5'-phosphate and dithiothreitol were present during refolding. The mitochondrial isoenzyme did not refold after denaturation. The decline in reactivation of the m. AAT, shown in Fig. 5.10, corresponds to the unfolding of the enzyme as measured by fluorescence, circular dichroism and exposure of reactive thiol groups (Fig. 5.12). The mitochondrial isoenzyme showed no time dependence of regain of activity (Fig. 5.11).

This suggests that it is only the enzyme molecules which retain native or native-like structure which can be reactivated within 30 s of dilution of the denaturing agent. Once the enzyme has unfolded it apparently cannot be re-activated under these conditions. The cytoplasmic isoenzyme differs from the mitochondrial isoenzyme as it showed a time dependent regain of activity, reaching maximum activity at 24 hr.

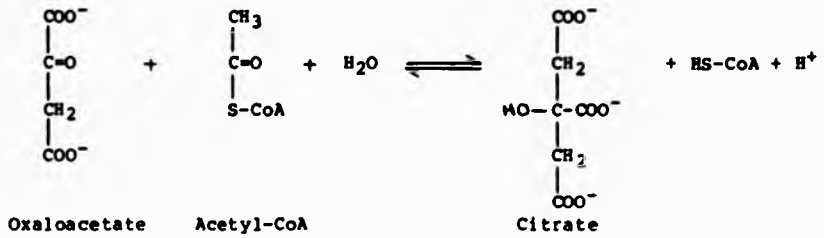
The behaviour of the mitochondrial isoenzyme aspartate aminotransferase is similar to other imported mitochondrial enzymes such as bovine liver glutamate dehydrogenase (Chapter 3) and pig heart citrate synthase (Chapter 6) in that they are unable to refold from isolated, unfolded subunits.

CHAPTER SIX

UNFOLDING AND REFOLDING OF CITRATE SYNTHASE

6.1 INTRODUCTION

Citrate synthase catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate



Oxaloacetate Acetyl-CoA Citrate

Oxaloacetate first condenses with acetyl-CoA to form citryl CoA, which is then hydrolysed to citrate and CoA. Hydrolysis of citryl CoA pulls the overall reaction in the direction of the synthesis of citrate. Citrate synthase occupies a key position within the central metabolic pathway, the citric acid cycle, and is considered the first enzyme in the pathway (Spector, 1972). It is the only enzyme in the cycle that can catalyse the formation of a carbon-carbon bond.

The overall reaction catalysed by citrate synthase can be divided into three chemical reactions:- (1) enolisation of the thioester group of acetyl-CoA by proton abstraction from the acetyl moiety forming an enolate anion, (2) condensation of the enolate anion with the carboxyl group of oxaloacetate forming a citryl-thioester, (3) hydrolysis of the citryl-thioester forming citrate and CoA (Spector, 1972; Wiegand and Remington, 1986). The three reactions catalysed by citrate synthase are shown in Fig. 6.1. Citrate synthase is highly specific for its substrates, oxaloacetate and acetyl-CoA. Oxaloacetate binds first to the enzyme and increases the binding constant for acetyl-CoA.

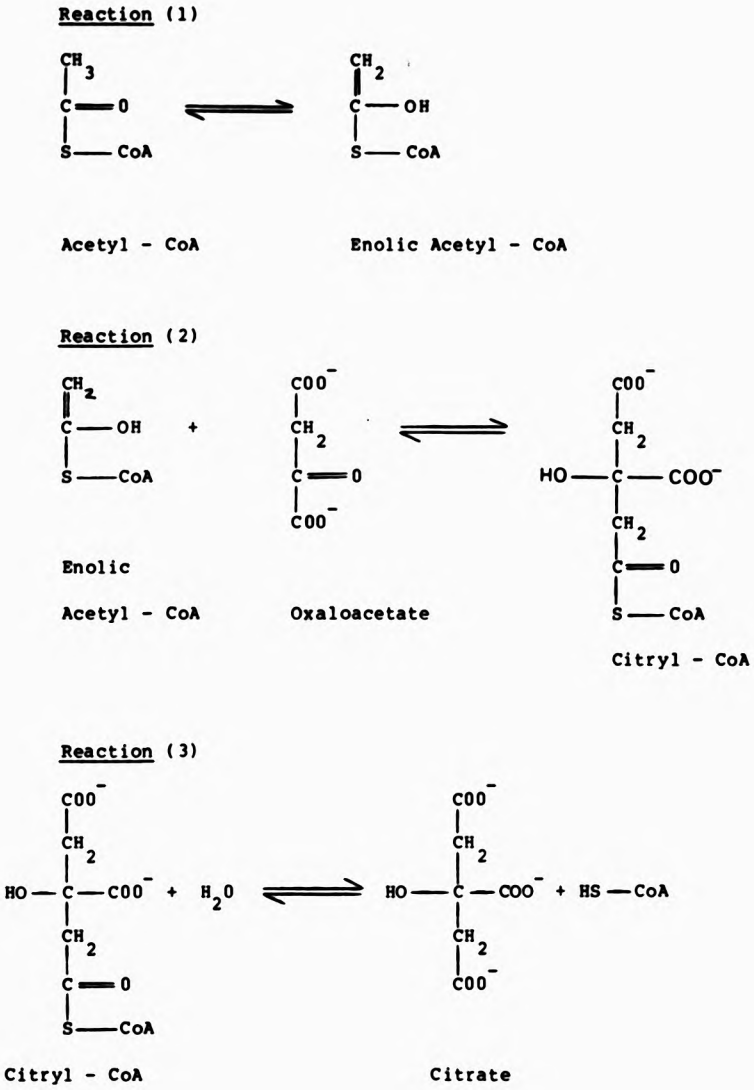


Fig. 6.1 The three reactions catalysed by citrate synthase

As the first enzyme of the citric acid cycle, citrate synthase, under certain conditions is the rate-limiting step of the cycle. Many of the regulatory properties of citrate synthase from various sources have been studied (Danson, 1988). These showed that citrate synthases from gram-negative bacteria are sensitive to allosteric control by NADH, whereas those from gram-positive bacteria and eukaryotes are sensitive to ATP.

Citrate synthases can be divided into two groups, large and small, relating to their size. The large enzyme is found in gram-negative bacteria and it has a tetrameric or hexameric structure. The small enzyme is found in gram-positive bacteria and eukaryotes and it has a dimeric structure. Though there is a difference in the size of the native citrate synthases the subunit M_r is approximately 50 000. The oligomeric state of citrate synthase is thought to determine its regulatory properties (Danson, 1988).

The pig heart citrate synthase is dimeric and consists of two identical subunits, each having a M_r of 48 969. The amino acid sequence has been determined and the enzyme contains 437 amino acids (Bloxham et al., 1982). The X-ray structure of citrate synthase from pig heart has been determined (Remington et al., 1982; Wiegand and Remington, 1986). The enzyme structure is essentially all α-helix as each subunit consists of 20 α-helical segments. Each subunit has two domains, a large domain containing 15 helices and a smaller domain containing 5 helices. In the large domain there are four antiparallel pairs of helices, these pack tightly together to give a globular molecule. The rigid globular domains can move relative to one another effecting a large conformational change, but no change occurs in the helical secondary structure. Citrate synthase exists in either an open or a closed form, which differ by the relative arrangement of the two

domains. In the open form, which occurs in the absence of ligands and in the presence of citrate, there is a large cleft between the small domain of one subunit and the large domain of the other subunit. On binding oxaloacetate and acetyl-CoA the enzyme undergoes a conformational change to the closed form. The change is represented by an 18.5^{o} rotation of the small domain relative to the large domain, so the small domain of one subunit makes contact with the large domain of the other subunit.

There are two active sites in each dimeric citrate synthase molecule which lie in the cleft between the two domains of each subunit. Structural studies show that amino acid residues from both subunits are involved in the binding of citrate and CoA at each active site (Wiegand and Remington, 1986).

Citrate synthase is a mitochondrial enzyme encoded by nuclear DNA and synthesised in the cytosol as a precursor, which is translocated into the mitochondria. In the mitochondrial matrix the enzyme may be bound to the inner mitochondrial membrane. Citrate synthase from pig heart has been shown to dissociate into subunits in the presence of 6M GdnHCl by hydrodynamic and circular dichroism measurements (Wu and Yang, 1970). However, no attempt to reactivate citrate synthase has been reported. The enzyme can also be dissociated into monomers at pH 9.6 as reported by Weitzman and Danson (1976) and on readjustment of the pH to 8.0 partial reactivation occurs. The unfolding and refolding of citrate synthase from pig heart was studied as it is an example of a translocated protein. These studies can be used to compare citrate synthase with other translocated proteins studied, glutamate dehydrogenase (Chapter 3) and aspartate aminotransferase (Chapter 5). The combined studies should give some information about the assembly of translocated proteins.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Citrate synthase from pig heart was purchased as an ammonium sulphate suspension from Sigma Chemical Co.

All substrates were purchased from Sigma Chemical Co.

All other chemicals were analar grade and were purchased from BDH Chemicals or Sigma Chemical Co.

6.2.2 Methods

6.2.2.1 Determination of protein concentration

The protein concentration of citrate synthase was determined from the absorbance at 280 nm using a value of $A^{0.1\%}_{1cm}$ of 1.78 (Bloxham et al., 1980).

6.2.2. Assay for citrate synthase activity

Citrate synthase activity was assayed by the method of Srere et al. (1963). The formation of CoA was determined by reaction with Nbs_2 which was measured spectrophotometrically at 412 nm. The assay mixture contained 20 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, 0.1 mM oxaloacetate, 0.15 mM acetyl-CoA and 0.1 mM Nbs_2. The reaction was initiated by addition of citrate synthase to an assay mix volume of 1 ml at 25°C. The rate of change in absorbance at 412 nm was measured. The A_{412} of 1 mM thionitrophenolate is 13.6 (Ellman, 1959).

6.2.2.3 Activity of citrate synthase in the presence of GdnHCl

Citrate synthase (3.5 µg/ml) was incubated in 20 mM Tris-HCl buffer, pH 8.0 in the absence and presence of GdnHCl at 20°C for 15 min. Samples were taken and assayed in the presence of the same concentration of GdnHCl.

6.2.2.4 Reactivation of citrate synthase after unfolding in GdnHCl

Citrate synthase was incubated in 20 mM Tris-HCl buffer, pH 8.0 in the absence or presence of GdnHCl for 15 min at 0°C. Samples were diluted 60-fold into 20 mM Tris-HCl buffer, pH 8.0 at 20°C to allow refolding to occur.

6.3. RESULTS

6.3.1 Pig heart citrate synthase

The enzyme preparation obtained from Sigma Chemical Co. was at least 95% homogeneous on SDS/polyacrylamide gel electrophoresis as judged by staining with Coomassie blue. The subunit M_r of citrate synthase as determined from SDS/polyacrylamide gel electrophoresis on 12% acrylamide gels was estimated to be 47 000 $\pm$ 2 500. This value can be compared with a value of 48 969 calculated from amino acid sequence (Bloxham et al., 1982). The specific activity of the pig heart citrate synthase at 25°C was 120 µmol/min per mg.

6.3.2. Unfolding of citrate synthase in GdnHCl

6.3.2.1 Enzyme activity in the presence of GdnHCl

Citrate synthase was incubated in the presence of increasing concentrations of GdnHCl and then assayed in the presence of the same concentration of GdnHCl as described in section 6.2.2.3. The activities at different GdnHCl concentration are expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 6.2. There is a progressive loss of activity with increasing concentrations of GdnHCl, with only 10% activity remaining at 0.6 M GdnHCl. The GdnHCl concentration at which 50% loss of activity occurs is 0.33 M.

6.3.2.2. Fluorescence studies

The fluorescence emission spectra of citrate synthase in the absence of GdnHCl and in the presence of 2 M, 4 M and 6 M GdnHCl are shown in Fig. 6.3. The fluorescence emission maximum of the native enzyme, when excited at 290 nm, occurs at 335 nm, characteristic of

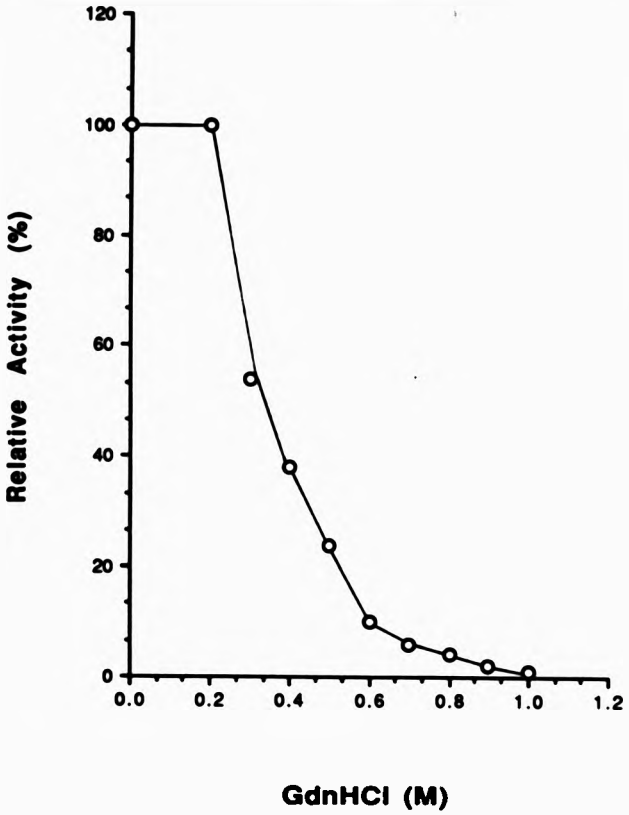


Fig. 6.2 Activity of citrate synthase in the presence of GdnHCl

Enzyme (3.5 µg/ml) was incubated in 20 mM Tris-HCl, pH 8.0 containing increasing concentrations of GdnHCl at 20°C for 15 min. Samples were assayed in the same concentration of GdnHCl. Activities are expressed relative to a control sample from which GdnHCl was omitted.

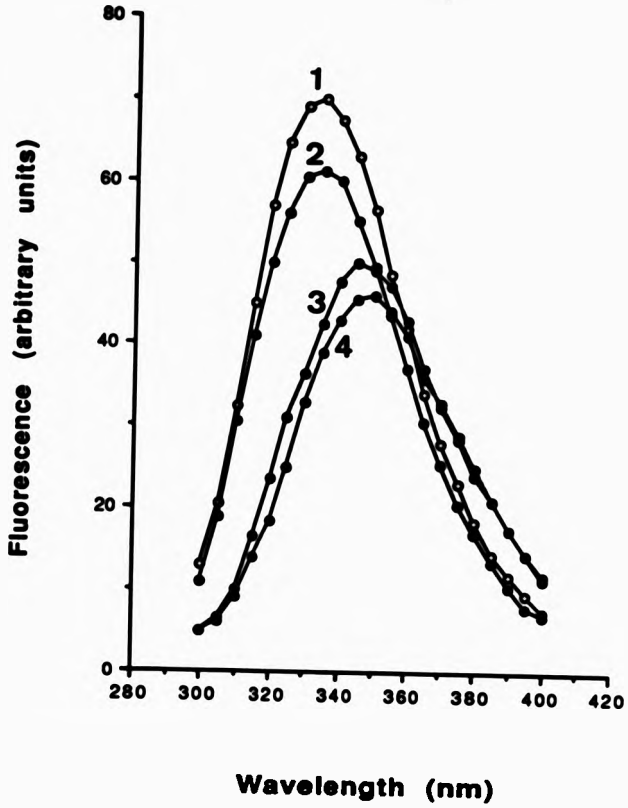


Fig. 6.3 Fluorescence emission spectra of citrate synthase in the absence and presence of GdnHCl

Enzyme (20 µg/ml) was incubated in 20 mM Tris-HCl, pH 8.0, in the absence and presence of GdnHCl at 20°C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm. Curves 1, 2, 3 and 4 represent enzyme in the presence of GdnHCl and enzyme in the presence of 2 M, 4 M and 6 M GdnHCl respectively.

tryptophan side chains partially shielded from aqueous solvent (Teipel and Koshland, 1971). Addition of 1 M GdnHCl caused little change in the spectrum but higher concentrations of GdnHCl caused a decrease in the fluorescence intensity and a shift in the emission maximum. The decrease in the fluorescence intensity occurs between 1 M and 4 M GdnHCl and at 4 M GdnHCl the emission maximum has shifted to 345 nm. If the GdnHCl concentration is increased to 6 M there is only a very small decrease in fluorescence intensity and the emission maximum shifts to 350 nm, characteristic of tryptophan side chains exposed to solvent (Teipel and Koshland, 1971). The exposure of tryptophan side chains is due to the dissociation and unfolding of the enzyme. The changes in fluorescence intensity at 335 nm are shown in Fig. 6.4. This shows the changes occurring between 1 M and 4 M GdnHCl.

6.3.2.3 Circular dichroism studies

The far-u.v. circular dichroism (c.d.) spectrum of the native citrate synthase enzyme is shown in Fig. 6.5. The spectrum shows double minima at 222 nm and 210 nm, characteristic of a protein with significant α-helical content (Chen et al., 1974). The α-helical content was calculated using reference values for the mean residue ellipticity at these wavelengths (Chen et al., 1974) which gave a value of 78 $\pm$ 7%. This value is consistent with the value of 72% determined by X-ray crystallography (Remington et al., 1982). The far-u.v. c.d. spectrum of the native enzyme is similar to that reported by Wu and Yang (1970). Though, their estimate of α-helical content (50-55%) was lower than the value obtained above.

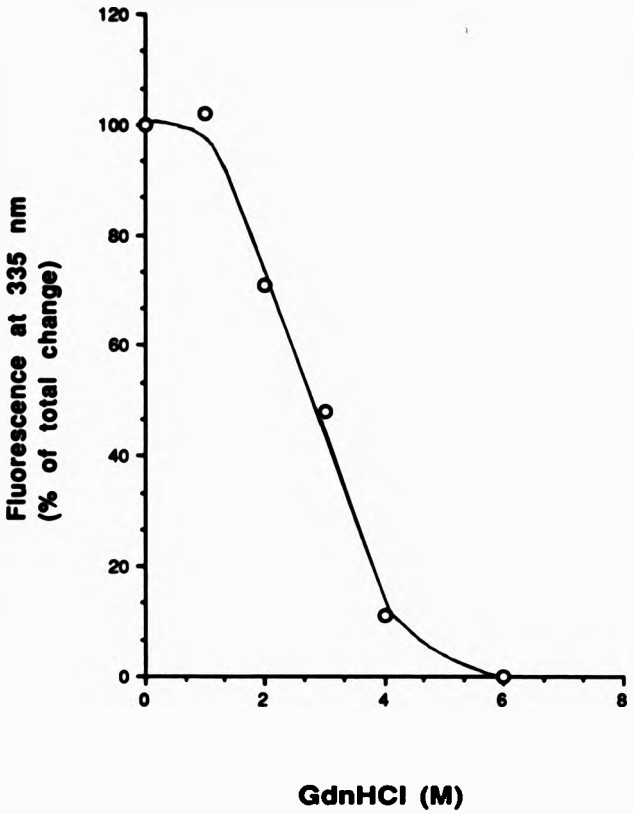


Fig. 6.4 Changes in fluorescence at 335 nm

Citrate synthase (20 μg/ml) was incubated in 20 mM Tris-HCl, pH 8.0 containing increasing concentrations of GdnHCl at 20°C. Spectra were recorded 15 min after addition of GdnHCl. The excitation wavelength was 290 nm. The changes in fluorescence intensity at 335 nm are expressed relative to the total change between 0 and 6 M GdnHCl.

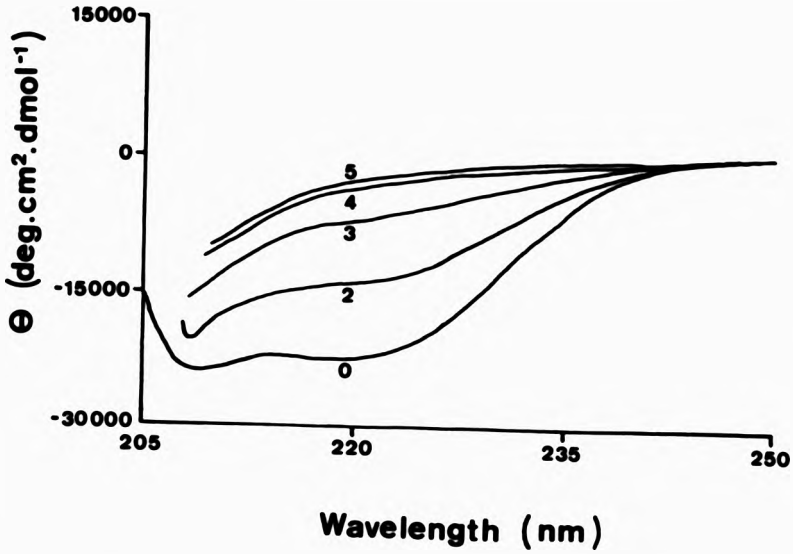


Fig. 6.5 Far-u.v. circular dichroism spectra

Spectra of citrate synthase (0.07 mg/ml) in 20 mM Tris-HCl, pH 8.0 were recorded in the absence of GdnHCl and in the presence of 2 M, 3 M, 4 M and 5 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample.

The far-u.v. c.d. spectra of citrate synthase in the presence of 2 M, 3 M, 4 M and 5 M GdnHCl are shown in Fig. 6.5. In the presence of concentrations of GdnHCl up to 1.5 M there is no significant change in the c.d. spectrum. On increasing the GdnHCl concentration above 1.5 M there is progressive loss of secondary structure and by 4 M GdnHCl there is virtually no secondary structure remaining. When the GdnHCl concentration is increased to 5 M no significant change occurs in the spectrum, which confirms the loss of secondary structure. These results show that changes in the secondary structure of the enzyme occur between 1.5 M and 4 M GdnHCl.

The near-u.v. c.d. spectra of the native enzyme and enzyme in the presence of 2 M and 3 M GdnHCl are shown in Fig. 6.6. In the presence of 1 M GdnHCl there was no significant change in the near-u.v. c.d. spectrum. The changes occur over the GdnHCl concentration range between 1 M and 3 M. These changes represent the disruption of the tertiary structure of the enzyme. The changes in the far-u.v., as measured by the change in θ_{225} and in the near-u.v., as measured by the change in θ_{295} are shown in Fig. No.6.7. In each case the change is expressed relative to the total change observed between the native enzyme and a sample in the presence of 6 M GdnHCl. These results show that the changes in secondary and tertiary structure run broadly in parallel.

6.3.2.4 Determination of reactive thiol groups

Citrate synthase was incubated in different concentrations of GdnHCl and the increase in absorbance at 412 nm on reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) was used to calculate the number of reactive thiol groups per subunit. The number of reactive thiol groups per subunit at increasing concentrations of GdnHCl are shown

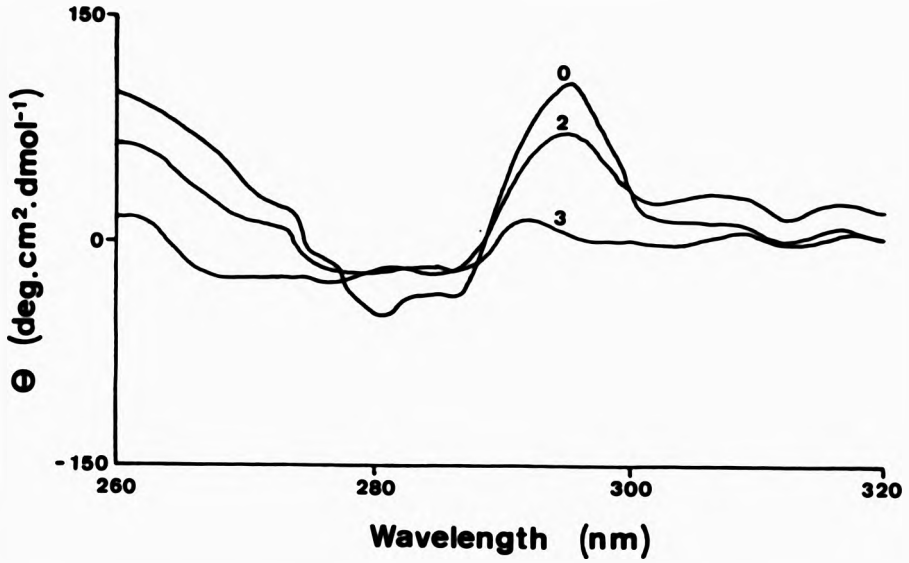


Fig. 6.6 Near-u.v. circular dichroism spectra

Spectra of citrate synthase (0.3 mg/ml) in 20 mM Tris-HCl, pH 8.0 were recorded in the absence of GdnHCl and in the presence of 2 M and 3 M GdnHCl at 20°C. The numbers correspond to the concentration of GdnHCl present in the sample.

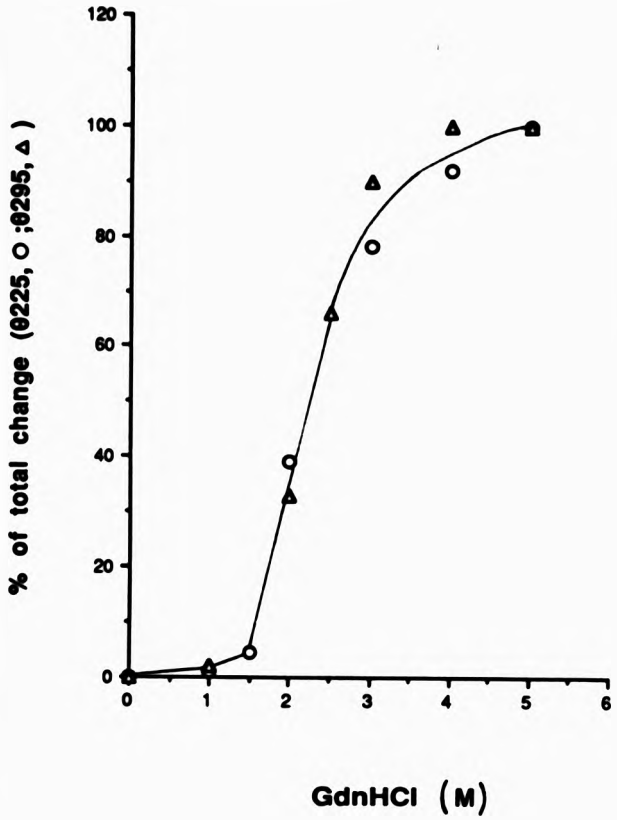


Fig. 6.7 Changes in far-u.v. (θ_{225}) and near-u.v. (θ_{295}) in the presence of GdnHCl

In each case the changes in θ_{225} (O) and θ_{295} (Δ) are expressed relative to the total change between 0 and 6 M GdnHCl.

in Fig. 6.8. The native citrate synthase enzyme showed < 0.1 thiol groups per subunit reactive towards Nbs_2. This agrees with data reported by Srere (1965).

In the presence of 6 M GdnHCl a total of 4.0 thiol groups per subunit were found to react with Nbs_2. This value corresponds to the known number of cysteine residues in each subunit (Bloxham et al., 1982). In the presence of increasing concentrations of GdnHCl there is an increase in the number of reactive thiol groups per subunit as shown in Fig. 6.8. The increase is shown to occur in the GdnHCl concentration range between 1.5 M and 2.5 M. At 3 M GdnHCl all the thiol groups are exposed. The four thiol groups which are exposed are Cys-74, Cys-175, Cys-184 and Cys-332.

6.3.3 Attempted refolding of citrate synthase after unfolding in GdnHCl

Citrate synthase was incubated in the presence of GdnHCl to allow unfolding to occur. The ability of citrate synthase to regain activity was studied by diluting the enzyme 60-fold into 20 mM Tris-HCl, pH 8.0 after unfolding, as described in section 6.2.2.4. The enzyme activity was assayed 2.5 hr after dilution and the reactivation was expressed relative to a control sample from which GdnHCl was omitted, as shown in Fig. 6.9. The results show that nearly all ($\geq$ 90%) activity could be regained if the initial concentration was $\leq$ 1 M. As the GdnHCl concentration was increased to 2 M there was a significant loss of the ability of the enzyme to regain activity. At 2 M GdnHCl only 15% activity could be regained and on further increasing the GdnHCl concentration to 3 M only 3% activity could be regained. When the samples were assayed at 5 min and 24 hr after dilution, no difference in the percent activity regained was found.

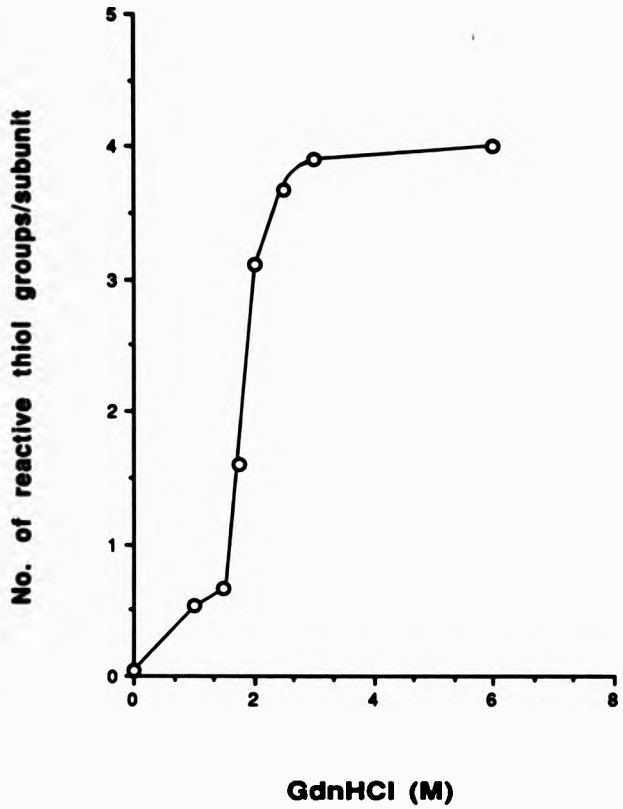


Fig. 6.8 Reactive thiol groups of citrate synthase

Enzyme (0.1 mg/ml) was incubated in 20 mM Tris-HCl, pH 8.0 containing increasing concentrations of GdnHCl at 20°C. The increase in A_{412} on reaction with Nbs_2 (250 μM) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

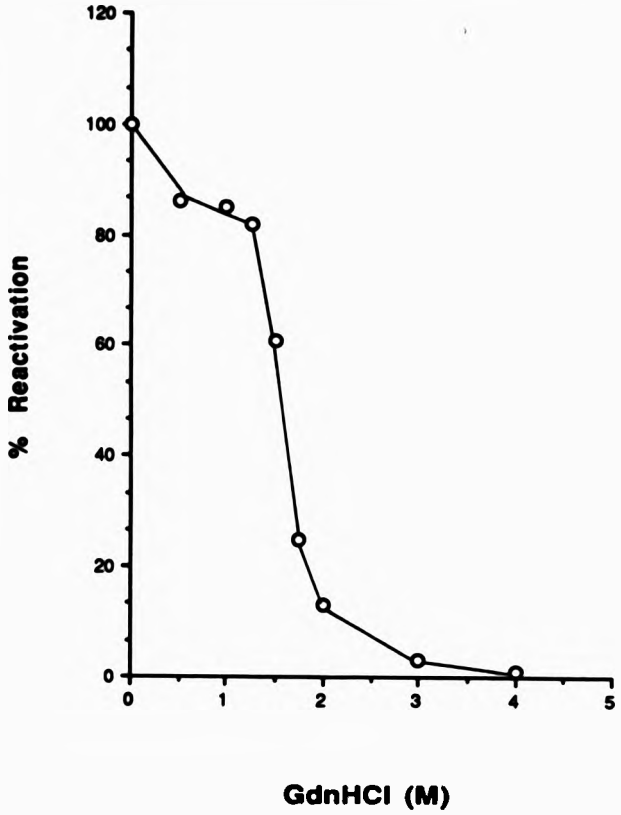


Fig. 6.9 Reactivation of citrate synthase after unfolding in GdnHCl

The unfolding and refolding of the enzyme were performed as described in (Section 6.2.2.4). The enzyme concentration during refolding was 4 µg/ml. The activity was assayed after 2.5 hr refolding and expressed relative to a control sample from which GdnHCl had been omitted.

In a control experiment it was shown that the inclusion of dithiothreitol (1 mM) in the denaturation solution and in the renaturation solution had no effect on the degree of reactivation, when the enzyme had been denatured in 3 M GdnHCl. The inability of the enzyme to regain activity is not a consequence of oxidative damage to the thiol groups in the enzyme.

6.3.4 Reactivation of citrate synthase after exposure to high pH

It has been reported that citrate synthase can be dissociated into monomers at pH 9.6 and partial reactivation of the enzyme occurred on readjustment of the pH to 8.0 (Weitzman and Danson, 1976). Citrate synthase was incubated in 20 mM Tris-HCl, pH 9.6 for 15 min at 0°C then diluted 60-fold into 20 mM Tris-HCl, pH 8.0 at 20°C. The samples were then assayed for activity and activity was expressed relative to a control sample which was incubated at pH 8.0. The results showed that 20% activity could be regained, this confirms that partial reactivation of the enzyme occurs after incubation at pH 9.6.

Circular dichroism (c.d.) spectra of citrate synthase at different pH's were recorded. The far-u.v. c.d. spectra of citrate synthase at pH 8.0 and pH 10.0 are shown in Fig. 6.10. These results show that exposure to pH values as high as 10.0 causes little unfolding of the protein. There is $< 15\%$ change in the θ_{225} value at pH 10.0 as compared to pH 8.0. The pH-induced dissociation into subunits is thus not accompanied by large structural changes in the enzyme.

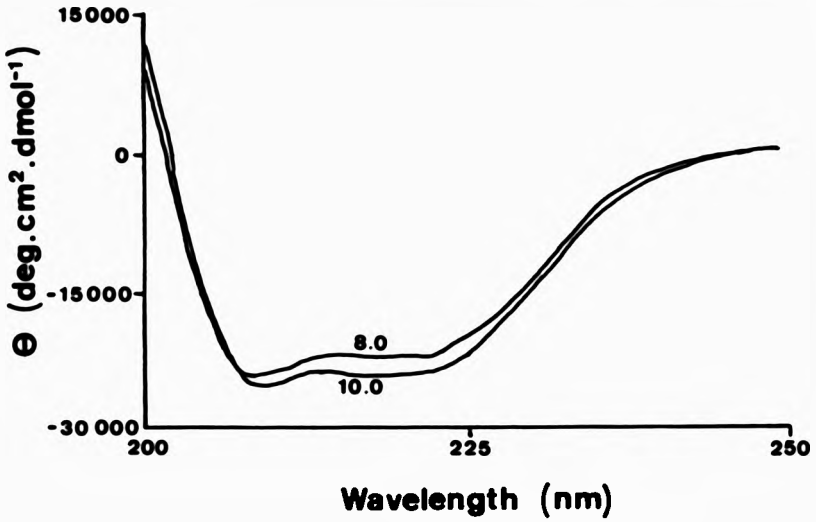


Fig. 6.10 Far-u.v. circular dichroism spectra of citrate synthase at pH 8.0 and pH 10.0

Spectra of citrate synthase (0.07 mg/ml) in 20 mM Tris-HCl, pH 8.0 and pH 10.0 were recorded at 20°C. The numbers correspond to the pH at which the spectra were recorded.

6.4 CONCLUSIONS

The results of the experiments described in this chapter show that citrate synthase is unfolded by incubation in GdnHCl but cannot be refolded. The results for the changes in structure as detected by fluorescence, circular dichroism and exposure of thiol groups were combined, as shown in Fig. 6.11. The changes in enzyme activity (Fig. 6.1) occur at lower concentrations of GdnHCl than those in fluorescence, circular dichroism and exposure of thiol groups. The loss of enzyme activity occurs at concentrations of GdnHCl $<$ 1 M. There are virtually no changes in structure at this low GdnHCl concentration as measured by fluorescence, circular dichroism and exposure of thiol groups. The degree of reactivation of the enzyme was found to be high at concentrations of GdnHCl $\leq$1.25M. These results show that at concentrations of GdnHCl between 0 M and 1 M changes occur at the active site, leading to loss of activity but no changes occur in the structure. This confirms that the active site of the enzyme is more sensitive to denaturing agents than the enzyme molecule as a whole. A similar situation for a number of other enzymes has been reported by Tsou, 1986).

At concentrations of GdnHCl between 1.5 M and 3 M there are changes in the secondary structure of the enzyme, which are detected by fluorescence and circular dichroism measurements. This shows that dissociation and unfolding of the enzyme occurs in this GdnHCl concentration range and is confirmed by the exposure of reactive thiol groups. At these higher GdnHCl concentrations there is a sharp decrease in the ability of the enzyme to refold (Fig. 6.8). This decrease corresponds to the unfolding of the enzyme molecule. This suggests that it is only the enzyme molecules which retain native or native-like

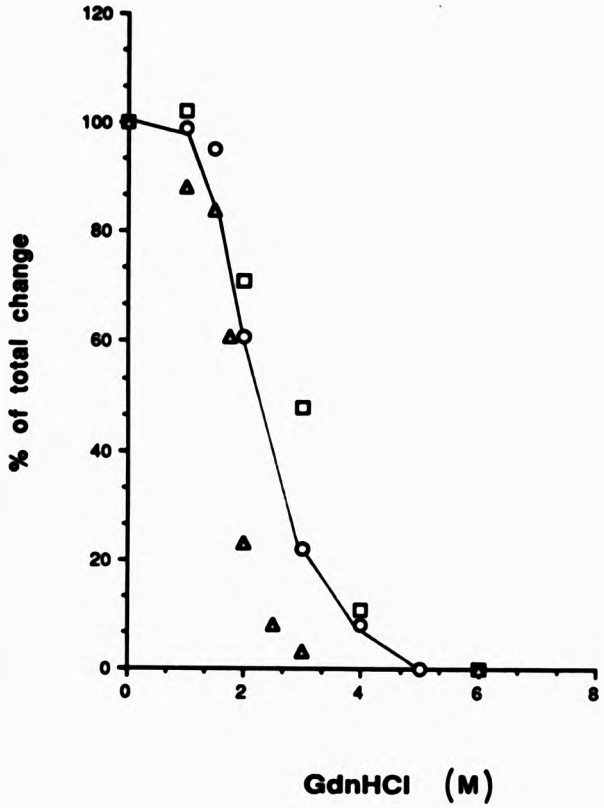


Fig. 6.11 Combined data for citrate synthase

The changes in fluorescence intensity at 335 nm (□), θ_{225} (○) and the number of reactive thiol groups/subunit (△) are expressed relative to the total change between 0 and 2 M GdnHCl.

structure which can be reactivated on dilution of the denaturing agent. Once the enzyme has unfolded it apparently cannot be reactivated under these conditions. The results obtained for the enzyme when it is exposed to high pH are consistent with this conclusion. As there was only a relatively small loss of secondary structure and partial reactivation was observed on readjustment of the pH to 8.0.

The behaviour of citrate synthase is similar to glutamate dehydrogenase (Chapter 3) and mitochondrial aspartate aminotransferase (Chapter 5) enzymes studied in that it is another example of a translocated protein which is unable to refold from its isolated, unfolded subunits.

CHAPTER SEVEN

GENERAL CONCLUSIONS AND DISCUSSION

7.1 GENERAL CONCLUSIONS AND DISCUSSION

In this thesis the unfolding and refolding of translocated and corresponding non-translocated enzymes has been studied. The results obtained for the glutamate dehydrogenase enzymes, cytoplasmic aspartate aminotransferase, mitochondrial aspartate aminotransferase and citrate synthase were presented in chapters 3-6. The degree of unfolding of each of these enzymes in GdnHCl was measured by loss of enzyme activity and changes in structure were monitored by circular dichroism, fluorescence and exposure of thiol groups. In each case the loss of enzyme activity occurred at a lower concentration of GdnHCl than any major changes in the structure. This situation has been reported for a number of enzymes (Tsou, 1986). Thus, it can be concluded that the active sites of the enzymes are more sensitive to GdnHCl than the enzyme molecules as a whole reflecting the requirement for a precise arrangement of groupsat the active site for catalytic activity.

As the GdnHCl concentration was increased, changes in the enzyme structure were detected by circular dichroism, fluorescence and exposure of reactive thiol groups. The unfolding of the enzyme molecules as the GdnHCl concentration increases probably occurs in a number of stages. The first stage is dissociation of the subunits which can be measured by light-scattering. The second stage is loss of tertiary structure which can be measured by fluorescence and exposure of reactive thiol groups. The third stage is loss of secondary structure which can be measured by far-u.v. circular dichroism. The stages leading to unfolding of the enzyme molecules reflect a reversal of the pathway of folding and assembly. In the

case of the hexameric glutamate dehydrogenase enzymes dissociation to trimers occurred without unfolding whereas on dissociation to monomers subsequent unfolding of the polypeptide chains occurred.

The refolding of each of these enzymes was studied by measuring the regain of enzyme activity after removal of the denaturing agent. It was found that only the c. AAT enzyme was able to regain activity from the unfolded state. The reactivation of c. AAT was dependent on the presence of PLP and DTT at high concentrations of GdnHCl and was also time dependent, reaching maximum activity (65%) at 24 hr. These results showed that c. AAT, a non-translocated enzyme could undergo refolding and reactiv ation after denaturation. By contrast, the translocated proteins studied; bovine liver GDH, m. AAT and CS could not be refolded after denaturation. These proteins could only refold from low concentrations of GdnHCl in which the overall protein structures had not been disrupted. The GdnHCl concentration range where the decline in reactivation occurs, corresponds to the unfolding of the enzyme molecules. In this GdnHCl concentration range it is only the enzyme molecules which retain native or native-like structures which can be reactivated on dilution of the denaturing agent. Thus, the translocated proteins studied are unable to refold under these conditions from isolated, unfolded subunits.

This raises the question of how are such translocated proteins folded and assembled *in vivo*. The mechanism of folding and assembly is of interest as a number of studies reviewed by Meyer (1988) and Bychkova *et al*. (1988) have shown that translocated proteins cross the mitochondrial and other membranes in a non-native, or unfolded state. It is probable that the folding and assembly of these imported proteins involves the participation of

specific binding proteins ('molecular chaperones') which have been implicated in the assembly of imported proteins such as Rubisco (Hemmingsen et al., 1988; Ellis and Hemmingsen, 1989).

Molecular chaperones are proteins whose proposed role is to mediate the folding and assembly of other target proteins into the correct oligomeric structures. The essential function of molecular chaperones is to prevent the formation of incorrect structures and to disrupt any that do form. These may occur during the transient exposure of charged or hydrophobic surfaces normally involved in interactions between or within polypeptide chains (Ellis et al., 1989). This transient exposure may occur during the synthesis of polypeptides, the unfolding and refolding that occurs during their translocation across membranes and recovery from heat shock. To date a number of proteins have been identified as molecular chaperones these include; hsp 70, immunoglobulin heavy-chain binding protein (BiP), rubisco subunit binding protein, gro EL and gro ES proteins and hsp 60.

The major heat-shock protein of animal cells, hsp 70, was suggested to be involved in the disassembly of aggregated structures which form during heat shock (Pelham, 1986). Many nuclear proteins become insoluble on heat shock, especially pre-ribosomes. The binding of hsp 70 to exposed, hydrophobic regions of partially denatured proteins can be reversed in vitro by ATP. The binding and release of hsp 70 by ATP hydrolysis prevents the formation of insoluble aggregates and promotes disaggregation. Animal and yeast cells contain proteins related to hsp 70 which are synthesised in the absence of stress and remain in the cytoplasm. These constitutive forms of hsp 70 are involved in maintaining precursor

proteins destined for the endoplasmic reticulum (ER) or mitochondria in an import-competent conformation (Deshaies *et al.*, 1988; Chirico *et al.*, 1988).

The hsp 70 of animal cells is related in primary structure to BiP (Munro and Pelham, 1986). BiP is present in the lumen of the ER of lymphoid cells and binds to newly synthesised immunoglobulin heavy chains which do not have light chains attached (Hass and Wabl, 1983). BiP seems to recognise the hydrophobic region of the heavy chains that associates with a hydrophobic region of light chains. Addition of ATP *in vitro* causes release of BiP from heavy chains allowing light chains to associate with heavy chains in a controlled fashion. The role of BiP in antibody-secreting cells is to prevent the formation of heavy chain aggregates and to aid in the assembly of immunoglobulin molecules (Munro and Pelham, 1986).

BiP is identical to grp 78 a protein produced in the ER when fibroblasts are starved of glucose. Thus, grp 78 may bind to abnormal underglycosylated proteins that accumulate in such fibroblasts and prevent them from forming insoluble aggregates (Pelham, 1986). BiP has also been shown to be a binding protein for misfolded, unassembled or otherwise aberrant proteins in the ER (Kassenbrock *et al.*, 1988; Hurtley *et al.*, 1989). The results suggested that one of the major functions of BiP is the recognition of incorrectly folded or aggregated proteins and to remove them from the ER. BiP and hsp 70 belong to one class of proteins within the molecular chaperone family.

A second class of molecular chaperones are termed chaperonins and are found in chloroplasts, prokaryotes and mitochondria. They are highly abundant proteins, have a M_r of about 60 000, are strongly homologous in sequence and are required for assembly in a wide range of cells. Rubisco subunit binding protein is present in chloroplasts of higher plants and its role in assembly of rubisco was mentioned in Chapter 1. Rubisco subunit binding protein consists of two subunits, alpha and beta, the alpha subunit has 46% homology to the E. coli gro EL protein. Thus, it was concluded that the two proteins are homologous in evolutionary terms (Hemmingsen et al., 1988). The gro E locus of E. coli contains two genes, gro EL and gro ES which are essential for cell viability. The heat shock gro EL and gro ES proteins are required for the assembly of the heads of bacteriophages lambda and T_4 in infected cells. The role of the gro E proteins in uninfected cells is not clear, though an in vitro study showed that the gro EL protein forms a complex with newly synthesised, unfolded cytoplasmic and secretory proteins in E. coli (Bochkareva et al., 1988). The gro EL protein may play a role in maintaining the secretory protein in a form that is competent for membrane translocation. Dissociation of the bound polypeptide from gro EL involves ATP hydrolysis.

The gro E heat-shock proteins have also been shown to promote the assembly of foreign prokaryotic rubisco oligomers in E. coli (Goloubinoff et al., 1989a). This dependence of a foreign oligomeric protein on host heat-shock protein for correct assembly provides experimental evidence for the existence of a conserved mechanism capable of controlling the assembly of a variety of

oligomeric proteins. More recently, it has been shown that the *in vitro* reconstitution of the catalytically functional dimeric form of rubisco from unfolded polypeptides depends on the presence of both *gro EL* and *gro ES* chaperonin proteins and Mg-ATP (Goloubinoff *et al.*, 1989b). The chaperonins enhance the rate of reconstitution of rubisco and the results are consistent with the notion that chaperonins facilitate some part of the folding step, with assembly of the dimer occurring subsequently and independently of chaperonins.

A mitochondrial heat-shock protein, hsp 60, is structurally related to the alpha subunit of Rubisco subunit binding protein and *gro EL*. The presence of this chaperonin in the mitochondrial matrix indicates that it may be involved in the folding and assembly of imported proteins. In support of this idea, it has been shown that hsp 60 is required for the correct assembly of various oligomeric proteins imported into yeast mitochondria (Cheng *et al.*, 1989). More recently, it was shown that hsp 60 has a role in the folding of proteins imported into the mitochondria (Ostermann *et al.*, 1989). The folding of proteins occurs at the surface of hsp 60 in an ATP-mediated reaction, followed by release of the bound polypeptide. This type of reaction has been described as protein-catalysed protein folding. Thus, the hsp 60 protein may possess two activities involved in the mechanism of protein folding the 'chaperoning' function, preventing illegitimate interactions that could prevent a polypeptide from reaching an active conformation, and a catalytic function, in which hsp 60 increases the reaction rates of the pathway leading to an active conformation (Horwich *et al.*, 1990).

The first three-dimensional X-ray structure of a chaperone protein was reported by Holmgren and Bründen (1989). They studied the chaperone protein Pap D which mediates the assembly of pilli in E. coli. The structure contains two globular domains each consisting of an antiparallel structure formed from packed β-sheets. This structure is similar to the constant domain of immunoglobulins. It is suggested that the region which interacts with the target protein is the wide crevice between the two domains that contains an exposed hydrophobic patch flanked by basic and acidic residues. This region may serve as a flexible hinge that can open up to accommodate target sequences of various sizes (Sambrook and Gething, 1989). As Pap D is smaller and more specific than other chaperone proteins, that fulfil a more generalised function in protein folding and assembly, the potential polypeptide binding site between the two domains may differ in detail.

Future studies on the refolding and assembly of translocated proteins would aim to achieve successful refolding and assembly in vitro. The results of these studies would provide more information about the pathway of folding and assembly of translocated proteins in vivo. Factors which influence the folding and assembly of translocated proteins in vivo include co-translational folding, post-translational modifications and chaperone proteins. Studies are required to establish how and to what extent each of these factors are involved in the formation of the native protein. The involvement of chaperone proteins is currently of great interest. In vitro studies of protein refolding using isolated chaperone proteins would show if chaperone proteins could assist in mediating the correct structural changes to allow proper folding and assembly.

REFERENCES

Alder, A.J., Greenfield, N.J. and Fasman, G.D. (1973)
Methods Enzymol. **27**, 675-735

Altieri, F., Mattingly, J.R.jr., Rodriguez-Berrocal, F.J., Youssef, J., Iriarte, A., Wu, T. and Martinez-Carrion, M. (1989)
J. Biol. Chem. **264**, 4782-4786

Anfinsen, C.B. (1966)
Harvey Lectures **61**, 95-116

Anson, M.L. and Mirsky, A.E. (1934a)
J. Gen. Physiol. **17**, 393-398

Anson, M.L. and Mirsky, A.E. (1934b)
J. Gen. Physiol. **17**, 399-408

Arnone, A., Rogers, P.H., Schimidt, J., Han, C.-N., Harris, C.M. and Metzler, D.E. (1977)
J. Mol. Biol. **112**, 509-513

Arnone, A., Rogers, P.H., Hyde, C.C., Briley, P.D., Metzler, C.M. and Metzler, D.E. (1985)
in Transaminases (Christen, P. and Metzler, D.E., eds.), pp.138-155
John Wiley, New York.

Banks, B.E.C., Doonan, S., Gauldie, J., Lawrence, A.J. and Vernon, C.A. (1968a)
Eur. J. Biochem. **6**, 507-513

Banks, B.E.C., Doonan, S., Lawrence, A.J. and Vernon, C.A. (1968b)
Eur. J. Biochem . **5**, 528-539

Barra, D., Bossa, F., Doonan, S., Fahmy, H.M.A., Martini, F. and Hughes, G.J. (1976)
Eur. J. Biochem. **64**, 519-526

Barra, D., Bossa, F., Doonan, S., Fahmy, H.M.A., Hughes, G.J., Martini, F., Petruzzelli, R. and Wittman-Liebold, B. (1980)
Eur. J. Biochem. **108**, 405-414

Bell, E.T. and Bell, J.E. (1984)
Biochem. J. **217**, 327-330

Bergman, L.W. and Kuehl, W.M. (1979)
J. Biol. Chem. **254**, 8869-8876

Bernal, J.D. (1958)
Discuss. Faraday Soc. **25**, 7-18

Bertland, L.H. and Kaplan, N.O. (1970)
Biochemistry **9**, 2653-2665

Birchmeier, W., Wilson, K.J. and Christen, P. (1973)
J. Biol. Chem. **248**, 1751-1759

Bloxham, D.P., Ericsson, L.H., Titani, K., Walsh, K.A. and Neurath, H. (1980)
Biochemistry **19**, 3979-3985

Bloxham, D.P., Parmlee, D.C., Kumar, S., Walsh, K.A. and Titani, K. (1982)
Biochemistry **21**, 2028-2036

Bochkareva, E.S., Lissin, N.M. and Girshovich, A.S. (1988)
Nature **336**, 254-257

Borisov, V.V., Borisova, S.N., Sosfenov, N.I. and Vainshtein, B.K. (1980)
Nature **284**, 189-190

Borisov, V.V., Borisova, S.N., Kachalova, G.S., Sosfenov, N.I. and Vainshtein, B.K. (1985)
in Transaminases (Christen, P. and Metzler, D.E., eds.) pp.155-164, John Wiley, New York.

Bothwell, M.A. and Schachman, H.K. (1980a)
J. Biol. Chem. **255**, 1962-1970

Bothwell, M.A. and Schachman, H.K. (1980b)
J. Biol. Chem. **255**, 1971-1977

Brandts, J.F., Halvorson, H.R. and Brennan, M. (1975)
Biochemistry **14**, 4953-4963

Braunstein, A.E. (1973)
The Enzymes 3rd Ed. **9**, 379-481

Bulleid, N.J. and Freedman, R.B. (1988)
Nature **335**, 649-651

Bychkova, V.E., Pain, R.H. and Ptitsyn, O.B. (1988)
FEBS Lett. **238**, 231-234

Chan, W.W.-C., Mort, J.S., Chang, D.K.K. and Macdonald, P.D.M. (1973)
J. Biol. Chem. **248**, 2778-2784

Chang, C.T., Wu, C.-S.C. and Yang, J.T. (1978)
Anal. Biochem. **91**, 13-31

Chen, W.-J. and Douglas, M.G. (1987a)
Cell **49**, 651-658

Chen, W.-J. and Douglas, M.G. (1987b)
J. Biol. Chem. **262**, 15605-15609

Chen, Y.-H., Yang, J.T. and Chau, K.H. (1974)
Biochemistry **13**, 3350-3359

Cheng, M.Y., Hartl, F.-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989)
Nature **237**, 620-625

Chirico, W.J., Waters, M.G. and Blobel, G. (1988)
Nature, **332**, 805-810

Chou, P.Y. and Fasman, G.D. (1978)
Adv. Enzymol. **47**, 45-148

Christen, P. and Metzler, D.E. (eds.) (1985)
Transaminases. John Wiley, New York.

Cook, R.A. and Koshland, D.E.jr. (1969)
Proc. Natl. Acad. Sci. USA **64**, 247-254

Creighton, T.E. (1978)
Prog. Biophys. Mol. Biol. **33**, 231-279

Creighton, T.E. (1990)
Biochem. J. **270**, 1-16

Danson, M.J. (1988)
Adv. Microb. Physiol. **29**, 165-230

de Duve, C., Wattiaux, R. and Baudhuin, P. (1962)
Adv. Enzymol. **24**, 291-358

de la Morena, E., Santos, I. and Grisolia, S. (1968)
Biochim. Biophys. Acta **151**, 526-528

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. and Schekman, R. (1988)
Nature **332**, 800-805

Doherty, D. (1970)
Methods Enzymol. **17A**, 850-856

Dolgikh, D.A., Gilmanshin, R.I., Braznikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venjaminov, S. Yu. and Ptitsyn, O.B. (1981)
FEBS Lett. **136**, 311-315

Doonan, S., Vernon, C.A. and Banks, B.E.C. (1970)
Prog. Biophys. Mol. Biol. **20**, 247-327

Eftink, M.R. and Ghiron, C.A. (1984)
Biochemistry **23**, 3891-3899

Egan, R.R. and Dalziel, K. (1971)
Biochim. Biophys. Acta **250**, 47-50

Eilers, M. and Schatz, G. (1986)
Nature **322**, 228-232

Ellis, R.J. (1987)
Nature **328**, 378-379

Ellis, R.J. and Hemmingsen, S.M. (1989)
Trends Biochem. Sci. **14**, 339-342

Ellis, R.J., van der Vies, S.M. and Hemmingsen, S.M. (1989)
Biochem. Soc. Symp. **55**, 145-153

Ellman, G.L. (1959)
Arch. Biochem. Biophys. **82**, 70-77

Fischer, G. and Bang, H. (1985)
Biochim. Biophys. Acta **828**, 39-42

Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989)
Nature **337**, 476-478

Fischer, G. and Schmid, F.X. (1990)
Biochemistry **29**, 2205-2212

Fiskin,A.M., van Bruggen, E.F. and Fisher, H.F. (1971)
Biochemistry **10**, 2396-2408

Ford, G.C., Eichele, G. and Jansonius, J.N. (1980)
Proc. Natl. Acad. Sci. USA **77**, 2559-2563

Freedman, R.B. (1984)
Trends Biochem. Sci. **9**, 438-441

Freedman, R.B., Bulleid, N.J., Hawkins, H.C. and Paver, J.L. (1989)
Biochem. Soc. Symp. **55**, 167-192

Fridovich, I. (1965)
Biochemistry **4**, 1098-1101

Garel, J.-R. and Baldwin, R.L. (1973)
Proc. Natl. Acad. Sci. USA **70**, 3347-3351

Garnier, J., Osguthorpe, D.J. and Robson, B. (1978)
J. Mol. Biol. **120**, 97-120

Gerl, M., Rudolph, R. and Jaenicke, R. (1985)
Biological Chemistry Hoppe-Seyler **366**, 447-454

Ghélis, C. (1980)
Biophys. J. **32**, 503-514

Ghélis, C. and Yon, J. (1982)
Protein Folding. New York, Academic Press.

Gieg, R., Rudolph, R. and Jaenicke, R. (1981)
Eur. J. Biochem. **119**, 301-305

Girg, R., Jaenicke, R. and Rudolph, R. (1983)
Biochemistry International **7**, 433-441

Godinot, C. and Lardy, H.A. (1973)
Biochemistry **12**, 2051-2060

Goldberg, M.E. (1985)
Trends Biochem. Sci. **10**, 388-391

Goldenberg, D.P. (1988)
Biochemistry **27**, 2481-2489

Goloubinoff, P., Gatenby, A.A. and Lorimer, G.H. (1989a)
Nature **337**, 44-47

Goloubinoff, P., Chriteller, J.T., Gatenby, A.A. and Lorimer, G.H. (1989b)
Nature **342**, 884-889

Grisolia, S., Quijada, C.L. and Fernandez, M. (1964)
Biochim. Biophys. Acta **81**, 61-70

Hand, S.C. and Somero, G.N. (1982)
J. Biol. Chem. **257**, 734-741

Hartl, F.-U., Ostermann, J., Guiard, B. and Neupert, W. (1987)
Cell **51**, 1027-1037

Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989)
Biocim. Biophys. Acta **988**, 1-45

Hartl, F.-U. and Neupert, W. (1990)
Science **247**, 930-938

Hass, I.G. and Wabl, M. (1983)
Nature **306**, 387-389

Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. and Neupert, W. (1988)
Cell **53**, 795-806

Hemmingsen, S.M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W. and Ellis, R.J. (1988)
Nature **333**, 330-334

Hermann, R., Jaenicke, R. and Rudolph, R. (1981)
Biochemistry **20**, 5195-5201

Hibbard, L.S. and Tulinsky, A. (1978)
Biochemistry **17**, 5460-5468

Holbrook, J.J., Liljas, A., Steindel, S.J. and Rossmann, M.G. (1975)
The Enzymes 3rd Ed. **11**, 191-192

Hollenberg, C.P., Riks, W.F. and Borst, P. (1970)
Biocim. Biophys. Acta **201**, 13-19

Holley, L.H. and Karplus, M. (1989)
Proc. Natl. Acad. Sci. USA **86**, 152-156

Holmgren, A. and Branden, C.-I. (1989)
Nature **342**, 248-251

Horwich, A.L., Neupert, W. and Hartl, F.-U. (1990)
Tibtech **8**, 126-131

Hurt, E.C. and van Loon. A.P.G.M. (1986)
Trends Biochem. Sci. **11**, 204-207

Hurtley, S.M., Bole, D.G., Hoover-Litty, H., Helenius, A. and Copeland, C.S. (1989)
J. Cell. Biol. **108**, 2117-2126

Inoue, T., Fukushima, K., Tastumoto, T. and Shimozawa, R. (1984)
Biochim. Biophys. Acta **786**, 144-150

Jaenicke, R. (1984)
Angew. Chem. Int. Ed. Engl. **23**, 395-413

Jaenicke, R. (1987)
Prog. Biophys. Molec. Biol. **49**, 117-237

Jaenicke, R. and Rudolph, R. (1986)
Methods Enzymol. **131**, 218-250

Jansonius, J.N., Eichele, G., Ford, G.C., Kirsch, J.F., Picot, D., Thaller, C., Vincent, M.G., Gehring, H. and Christen, P. (1984)
Biochem. Soc. Trans. **12**, 424-427

Jaussi, R., Cotton, B., Juretic, N., Christen, P. and Schumperli, D. (1985)
J. Biol. Chem. **260**, 16060-16063

Jaussi, R., Behra, R., Giannattasio, S., Flura, T. and Christen, P. (1987)
J. Biol. Chem. **262**, 12434-12437

Kabsch, W. and Sander, C. (1983)
FEBS Lett. **155**, 179-182

Karplus, M. and Weaver, D.L. (1976)
Nature **260**, 404-406

Kassenbrock, C.K., Garcia, D.D., Walter, P. and Kelly, R.B. (1988)
Nature **333**, 90-93

Kim, P.S. (1986)
Methods Enzymol. **131**, 136-156

Kim, P.S. and Baldwin, R.L. (1982)
Ann. Rev. Biochem. **51**, 459-489

Koivu, J. and Myllylä, R. (1987)
J. Biol. Chem. **262**, 6159-6164

Koren, R. and Hammes, G.G. (1976)
Biochemistry **15**, 1165-1171

Labhardt, A.M. (1986)
Methods Enzymol. **131**, 126-135

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

Lang, K., Schmid, F.X. and Fischer, G. (1987)
Nature **329**, 268-270

Lang, K. and Schmid, F.X. (1988)
Nature **331**, 453-455

Lehrer, S.S. (1971)
Biochemistry **10**, 3254-3263

Lim, V.I. (1974)
J. Mol. Biol. **88**, 873-894

Lin, L.-N., Hasumi, H. and Brandts, J.F. (1988)
Biocim. Biophys. Acta **956**, 256-266

Linderstrøm-Lang, K.U. (1952)
'Proteins and Enzymes'. Lane Medical Lectures, No.VI, Stanford Univ. Press, Stanford, California.

McNally, T., Purvis, I.J., Fothergill-Gilmore, L.A. and Brown, A.J.P. (1989)
FEBS Lett. **247**, 312-316

Martinez-Carrion, M., Tiemeier, D.C. and Peterson, D.L. (1970)
Biochemistry **9**, 2574-2582

Meyer, D.I. (1988)
Trends Biochem. Sci. **13**, 471-474

Michida, C.M. and Martinez-Carrion, M. (1969)
Biochemistry **8**, 1095-1105

Mitchinson, C. and Pain, R.H. (1985)
J. Mol. Biol. **184**, 331-342

Moon, K. and Smith, E.L. (1973)
J. Biol. Chem. **248**, 3082-3088

Muller, K. and Jaenicke, R. (1980)
Z. Naturforsch. **35c**, 222-228

Munro, S. and Pelham, H.R.B. (1986)
Cell **46**, 291-300

Newsholme, E.A. and Start, C. (1973)
Regulation in Metabolism, pp.94-95, John Wiley, London.

Nishi, T., Nagashima, F., Tanase, S., Fukumoto, Y., Joh, T., Shimada, K., Matsukado, Y., Ushio, Y. and Morino, Y. (1989)
J. Biol. Chem. **264**, 6044-6051

Noelken, M.E. and Timasheff, S.N. (1967)
J. Biol. Chem. **242**, 5080-5085

Northrop, J.H. (1932)
J. Gen. Physiol. **16**, 323-337

Nozaki, Y. (1972)
Methods Enzymol. **26**, 43-50

O'Donovan, K.M.C., Doonan, S., Marra, E. and Passarella, S. (1984)
Biochem. Soc. Trans. **12**, 444-445

Ohgushi, M. and Wada, A. (1983)
FEBS Lett. **164**, 21-24

Opitz, U., Rudolph, R., Jaenicke, R., Ericsson, L. and Neurath, H. (1987)
Biochemistry **26**, 1399-1406

Ostermann, J., Horwich, A.L., Neupert, W. and Hartl, F.-U. (1989)
Nature **341**, 125-130

Parr, G.R. and Hammes, G.G. (1975)
Biochemistry **14**, 1600-1605

Pelham, H.R.B. (1986)
Cell **46**, 959-961

Peters, T.jr. and Davidson, K. (1982)
J. Biol. Chem. **257**, 8847-8853

Pfaller, R., Steger, H.F., Rassow, J., Pfanner, N. and Neupert, W. (1989)
J. Cell. Biol. **107**, 2483-2490

Pfanner, N. and Neupert, W. (1986)
FEBS Lett. **209**, 152-156

Pfanner, N., Tropschug, M. and Neupert, W. (1987)
Cell **49**, 815-823

Pfanner, N., Pfaller, R., Kleene, R., Ito, M., Tropschug, M. and Neupert, W. (1988)
J. Biol. Chem. **263**, 4049-4051

Price, N.C. (1990)
'Folding and assembly of multi-subunit proteins' in 'Receptor subunits and complexes' (Barnard, E.A., Burgen, A.S.V. and Roberts, G.C.K., eds.)
Cambridge University Press, Cambridge. (in press.)

Price, N.C. and Stevens, L. (1982)
Fundamentals of Enzymology, pp.136-142, Oxford University Press, Oxford.

Ptitsyn, O.R. (1987)
J. Prot. Chem. **6**, 273-293

Ptitsyn, O.B., Pain, R.H., Semisotnov, G.V., Zerovnik, E. and Razgulyaev, O.I. (1990)
FEBS Lett. **262**, 20-24

Purvis, I.J., Bettany, A.J.E., Santiago, T.C., Coggins, J.R., Duncan, K., Eason, R. and Brown, A.J.P. (1987)
J. Mol. Biol. **193**, 413-417

Remington, S., Wiegand, G. and Huber, R. (1982)
J. Mol. Biol. **158**, 111-152

Rice, D.W., Hornby, D.P. and Engel, P.C. (1985)
J. Mol. Biol. **181**, 147-149

Rice, D.W., Baker, P.J., Farrants, G.W. and Hornby, J. (1987)
Biochem. J. **242**, 789-795

Roder, H.R., Elove, G.A. and Englander, S.W. (1988)
Nature **335**, 700-704

Roise, D. and Schatz, G. (1988)
J. Biol. Chem. **263**, 4509-4511

Roth, R. and Pierce, S. (1987)
Biochemistry **26**, 4179-4182

Rothman, J.E. and Kornberg, R.D. (1986)
Nature **332**, 209-210

Rudolph, R., Bohrer, M. and Fischer, S. (1983)
Eur. J. Biochem. **131**, 383-386

Sambrook, J. and Gething, M.-J. (1989)
Nature **342**, 224-225

Schleyer, M. and Neupert, W. (1985)
Cell **43**, 339-350

Schmid, F.X., Grafl, R., Wrba, A. and Beintema, J.J. (1986)
Proc. Natl. Acad. Sci. USA **83**, 872-876

Sedmak, J.J. and Grossberg, S.E. (1977)
Anal. Biochem. **79**, 544-552

Smith, E.L., Austen, B.M., Blumenthal, K.M. and Nyc, J.F. (1975)
The Enzymes 3rd Ed. **11**, 293-367

Spector, L.B. (1972)
The Enzymes 3rd Ed. **7**, 357-389

Srere, P.A. (1965)
Biochim. Biophys. Res. Commun. **18**, 87-91

Srere, P.A., Brazil, H. and Gonen, L. (1963)
Acta Chem. Scand. **17**, S129-S134

Stark, G.R., Stein, W.H. and Moore, S. (1960)
J. Biol. Chem. **235**, 3177-3181

States, D.J., Creighton, T.E., Dobson, C.M. and Karplus, M. (1987)
J. Mol. Biol. **195**, 731-739

Strambini, G.B., Cioni, P. and Puntoni, A. (1989)
Biochemistry **28**, 3808-3814

Sund, H., Pilz, I. and Herbst,M. (1969)
Eur. J. Biochem. **7**, 517-525

Takahashi, N., Hayano, T. and Suzuki, M. (1989)
Nature **337**, 473-475

Tashiro, R., Inoue, T. and Shimozawa, R. (1982)
Biochim. Biophys. Acta **706**, 129-135

Teipel, T.W. and Koshland, D.E.jr. (1971)
Biochemistry **10**, 789-805

Teschner, W., Rudolph, R. and Garel, J.-R. (1987)
Biochemistry **26**, 2791-2796

Tsou, C.L. (1986)
Trends Biochem. Sci. **11**, 427-429

Tsou, C.L. (1988)
Biochemistry **27**, 1809-1812

Udgaonkar, J.B. and Baldwin, R.L. (1988)
Nature **335**, 694-699

Venard, R. and Fourcade, A. (1972)
Biochimie **54**, 1381-1389

Verner, K. and Schatz, G. (1987)
EMBO J. **6**, 2449-2456

Wada, H. and Morino, Y. (1964)
Vitamins and Hormones **22**, 411-444

Ward, L.D. (1985)
Methods Enzymol. **117**, 400-414

Weitzman, P.D.J. and Danson, M.J. (1976)
Curr. Top. Cell. Regulation **10**, 161-204

Wetlaufer, D.B. (1981)
Adv. Prot. Chem. **34**, 61-92

Wiegand, G. and Remington. S.J. (1986)
Ann. Rev. Biophys. Biophys. Chem. **15**, 97-117

Wu, J.-Y. and Yang, J.T. (1970)
J. Biol. Chem. **245**, 212-218

Zettlmeissl, G., Rudolph, R. and Jaenicke, R. (1979)
Biochemistry **18**, 5567-5571

Zimmermann, R. and Meyer, D.I. (1986)
Trends Biochem. Sci. **11**, 512-515

Zhou, H.M. and Tsou, C.L. (1986)
Biochim. Biophys. Acta **869**, 69-74

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