ANALYSIS OF THE STABILITY OF NATIVE AND CHEMICALLY-MODIFIED ENZYMES

Thesis

Presented for the degree of

DOCTOR OF PHILOSOPHY

by

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TO LUKE

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I declare that all the work reported in this thesis was performed by Helen Sheehan, unless otherwise stated.

Helen Sheehan

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ABSTRACT

Bovine heart creatine kinase was purified to homogeneity and then chemically modified with a number of different reagents in an attempt to improve the thermal stability of the enzyme. The bifunctional crosslinking reagent, dimethyl suberimidate, successfully enhanced the stability of the enzyme towards heat denaturation at several concentrations. Following optimization of the procedure, accelerated storage studies were performed and the results used to predict the storage time of the native and modified enzyme at lower temperatures. The crosslinked derivative was predicted to have a longer shelf-life at 4 °C than the native enzyme. The pH profile was altered following crosslinking, but the Michaelis constants were not changed. The modified enzyme also exhibited a marked resistance to the action of some denaturing agents.

Salt bridges were implicated as contributing significantly to the intrinsic stability of the native protein. Modifying reagents which interferred with these electrostatic interactions caused destabilization of the enzyme to occur.

Analysis of the processes causing thermal inactivation of creatine kinase, ruled out aggregation as a cause of inactivation. It was revealed that conformational, as opposed to covalent processes were responsible for the inactivation of the enzyme. The role of conformational processes in deactivation was confirmed using a number of different approaches. It was shown that heat-induced oxidation of thiol groups gave rise to incorrect structures through disulfide formation, which resulted in the subsequent loss of enzymic activity.

A microassay was developed for the determination of creatine kinase activity. The assay utilized a microplate reader and micro-titre plates, and compared extremely well with the results obtained using other assay procedures.

Following reduction and carboxymethylation, crude creatine kinase was purified by electrophoresis and electroblotting. The purified protein was then sequenced and the first sixteen residues of the N-terminus were determined.

The techniques used in the stabilization of creatine kinase were applied to two other enzymes, lactate dehydrogenase and aspartate aminotransferase. While only limited studies were performed, some stabilization was achieved, enabling the results to be used as a basis for further studies on these enzymes.

LIST OF ABBREVIATIONS

AAT	aspartate aminotransferase
ADP	adenine dinucleotide phosphate
Asn	Asparagine
Asp	Aspartate
BSA	bovine serum albumin
CK	creatine kinase
CM	carboxymethyl
CP	creatine phosphate
DEAE	diethylaminoethyl
DMA	dimethyl adipimidate
DMP	dimethyl pimilimidate
DMS	dimethyl suberimidate
DMSO	dimethyl sulphoxide
DTE	dithioerythritol
DTNB	5,5-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
Gd-HCl	guanidine hydrochloride
GdmCl	guanidinium chloride
Gln	Glutamine
G6PDH	glucose-6-phosphate dehydrogenase
His	Histidine
HK	hexokinase
IgG	immunoglobulin G
Ile	Isoleucine
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
MW	molecular weight
NBS	N-bromosuccinimide
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
RCA	relative catalytic activity

Ser	Serine
SDS	sodium dodecyl sulfate
TEMED	NNN N -tetramethyl ethylene diamine
Thr	Threonine
T_m	melting temperature
TNP	2,4,6-trinitrobenzene sulphonic acid

CONTENTS:

CHAPTER 3. ANALYSIS OF THE STABILITY OF NATIVE CREATINE KINASE

PUBLICATIONS ASSOCIATED WITH THIS THESIS:

O'Fagain, C., Sheehan, H., O'Kennedy, R. and Kilty, C. (1988). Maintenance of enzyme structure. Process Biochemistry. 23 (6), 166.

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GENERAL INTRODUCTION

1.1 INTRODUCTION

The poor conformational stability of many enzymes is an actual bottleneck for most industrial applications. This is the reason for the increasing interest shown towards protein deactivation phenomena and enzyme stabilization techniques (Greco et al.,1991; O'Fagain et al.,1991).

The catalytic action of an enzyme is directly related to its three dimensional structure. In turn, the latter is determined by a complex set of low-energy interactions among various active groups present in the primary structure. Different inactivating interactions occur spontaneously within the protein molecule among amino acid residues, as a result of thermal agitation (Klibanov, 1968). Interactions among enzyme molecules in solution can yield aggregation and subsequent precipitation of the enzymatic protein (Moskvichyov et al.,1986). External environmental modifications can also produce an increase in the rate of enzyme inactivation. For example, the structural and functional integrity of the active domain of a protein can be irreversibly disrupted by physical, chemical and biological forces, such as heat, freezing, radiation, oxidation, reduction, solvents, metal ions, ionic strength, and enzymatic modification and degradation.

The most common form of in vivo inactivation of many proteins is enzymatic modification, particularly enzymic proteolysis (Tombs, 1985). The half-life of injected proteins is often

relatively short (10-20 minutes) such that clinical application requires multiple injections or methods of continuous infusion. Proteins made resistant to proteolysis would obviously be more effective.

Another use of active proteins (hormone and growth factors) that could benefit from their stabilization is as supplements in media for mammalian cell culture (Murakami et al.,1981). Inactivation of proteins in media is by proteolysis due to lysosomal enzymes released from lysed cells, membrane bound proteinases and hormone specific proteases (Baumann et al.,1986).

Temperature stability of enzymes used industrially is an important factor in productivity. For example, proteases, amylases and lipases which are used in laundry detergents at elevated temperatures and in the presence of an oxidising agent, make resistance to heat and oxidation desirable. Depending on the system in which they are used, enzymes in vitro may be exposed to all of the forms of inactivation mentioned above, though heat and oxidation are the most common.

These examples clearly necessitate the use of enzymes which are stable. There are two different strategies employed for obtaining more stable enzymes : (1) find new microbial sources (eg. extreme thermophiles) which have very thermoresistant enzymes, or (2) stabilize enzymes that already exist. However, before undertaking either of these two strategies, an understanding of the molecular reasons for protein stability is necessary.

1.2 The Relationship Between Protein Structure and Stability.

The structure-stability relationship in proteins has been studied experimentally. A common approach to this problem is to choose functionally related proteins of different stabilities and to compare their structures in order to find out the structural features responsible for the higher stability of one of them. The functionally related proteins differing in stability can be found in nature. For example, two functionally related proteins from a mesophilic source may be compared, or one protein from a mesophilic source may be compared with a protein from a thermophilic microorganism. Also, a protein from a wild strain may be compared with its counterpart from a mutant strain. Finally, a native protein may be compared with a protein prepared in the laboratory by site-directed mutagenisis, immobilization, or chemical modification.

Using the above approaches, in particular the comparison of mesophilic and thermophilic proteins, a number of molecular reasons for protein stability have been advanced.

1.3 Molecular Reasons For Protein Stability.

Mesophilic enzymes usually retain their native structure over a rather narrow range of conditions, e.g. temperatures from 0 or 5 $^{\circ}$C to 40 to 50 $^{\circ}$C (Kauzmann,1959; Shulz and Schirmer,1979). For

thermophilic enzymes, the upper limit of thermostability is usually 20-30°C higher. Thermodynamic calculations (Finney,1980) show that this corresponds to an increase in the stability of a protein by 5-7 kcal mol^{-1}, which is a small change. A ΔG of this order may be derived from only one or two additional salt bridges inside the protein globule (Perutz,1978) or several additional hydrogen bonds, (Shulz and Schirmer, 1979) or seven to ten additional CH_3-groups in the hydrophobic nucleus of the protein (Tanford,1980). Therefore, it is unlikely that such a gain in the stability of the protein will require drastic rearrangement in its structure. Indeed, a thermophilic protein usually differs from its mesophilic counterpart by only insignificant alterations of its primary structure. Moreover, the three dimensional structures of such proteins are, as a rule, largely similar. Some of the most common reasons for the enhanced stability of proteins are the following.

1. Binding of metal ions, substrates, cofactors, and other low molecular weight ligands to proteins.
2. Protein-protein and protein-lipid interactions.
3. Salt bridges.
4. Hydrogen bonds.
5. Disulfide bonds.
6. Low content of amino acids sensitive to oxidative modification.
7. Compact packing of amino acid residues.
8. Hydrophobic interactions.

1.3.1 Binding of metal ions, substrates, cofactors, and other low molecular weight ligands to proteins.

Metal ions dramatically increase protein stability due to their binding to the labile fragments of the polypeptide chain, in particular to the bends, (Matthews et al.,1979) making the overall structure of the protein more compact and rigid (Dahlquist et al.,1976, Hachimori et al.,1979). For example, some thermophilic enzymes having proteolytic (Ohta,1967), amylolytic (Hasegawa and Imahori,1976) and synthetase (Wedler and Hoffman,1976) action are stabilized by Ca^{++}, Mg^{++}, Zn^{++} and other ions. An increase in protein stability is also observed when enzymes interact with substrates, cofactors and other low molecular weight ligands (Parfait,1973, Hibino et al.,1974, Wedler et al., 1976 and Bendzko and Hintsche,1978).

The most general explanation of this phenomenon has been presented by Schellman, (1976). Proteins can exist in at least two states - in the native state and in the denatured state - and any ligand can bind to both these protein forms. The preferential binding of the effector molecule to one of the forms results in stabilization, e.g. binding to the native form rather than the denatured one. The binding of substrates, cofactors, and other effectors of enzyme activity occurs, as a rule, in either the active site or its vicinity. Hence, the enzyme should be in the native (catalytically active) state for the complex to form.

Therefore specific low molecular weight ligands usually stabilize proteins.

1.3.2 Protein-protein and protein-lipid interactions.

In vivo proteins very often interact with lipids, proteins and other compounds. (Sandermann,1978). The resulting complexes fall into two groups:

(1) complexes with a limited number of either protein-protein solitary contacts (e.g. intersubunit contacts in oligomeric enzymes (Klotz,1970) or protein-lipid solitary contacts (as in the complex of serum albumin with fatty acids (Boyer,1947)).

(2) systems with a large number of interrelated and concerted contacts of different types (protein-protein, protein-lipid, lipid-lipid) e.g. biological membranes (Singer, 1972). The interaction with other molecules in both cases can significantly increase the stability of the proteins.

The stabilization mechanism involved in protein-protein or protein-lipid contacts is as follows. As well as a great number of polar and charged groups on the surface of proteins, there are some non-polar residues whose contact with water is unfavourable thermodynamically (Kauzmann, 1959; Shulz and Schirmer,1979). When a protein forms a complex, the contact area of the nonpolar fragments with water and the free energy of the system diminishes and hence enzyme stabilization takes place (Chothia and

Janin,1975).

The mechanism of enzyme stabilization which is due to the incorporation of enzymes into biological membranes is more complex. In addition to the shielding of the hydrophobic regions on the surface of the protein from the thermodynamically unfavourable contacts with water as just discussed, many other factors must be considered, e.g. the microenvironment of the protein and the change of the rigidity of the protein molecule resulting from its multipoint interaction with other proteins and lipids.

The rigidity of the protein molecule is directly related to protein stability. Immobilization (both natural and artificial) can influence the rigidity and stability of proteins. Membrane proteins function in vivo in a naturally immobilized state. It has been shown (Welker,1976) that the more rigid the membrane into which an enzyme is incorporated, the higher the temperature optimum of the catalytic activity. This has also been shown to be true with artificially immobilized enzyme (Mozhaev et al.,1983).

1.3.3 Salt bridges.

The number of salt bridges in proteins is rather small (Perutz,1978; Barlow and Thornton,1983). However, they make a significant contribution to protein stability i.e. up to 5 kcal/mol when they are localized inside the globule, and 1 to 2

kcal/mol when they are on its surface (Perutz,1978). Additional electrostatic interactions have been detected in thermophilic enzymes e.g. glyceraldehyde 3-phosphate dehydrogenase from the thermophilic strain of *B. stearothermophilus* has a three-dimensional structure very similar to that of the enzyme from rabbit muscle. However, the thermophilic enzyme has a cooperative system of salt bridges in its intersubunit region which is absent in the mesophilic enzyme (Walker,1978). This difference in structure is reflected in a higher denaturation and optimum temperature (20°C higher) in the thermophilic enzyme.

Additional salt bridges can be artificially introduced into the protein molecule (by site-directed mutagenisis or chemical modification). Genetic engineering has helped to synthesise mutationally altered haemoglobin and ferrodoxin (Perutz and Raidt,1975) with additional salt bridges on the surface of the globule, resulting in more stable proteins. This approach is thought by some (Mozhaev et al.,1988) to be very promising, particularly as new data on protein structure is being obtained.

1.3.4 Hydrogen bonds.

Hydrogen bonds play an important role in proteins since they support secondary structures i.e. α-helices, β-sheets, turns, etc. Evidence has been presented to suggest that there are a greater number of hydrogen bonds in some thermophilic enzymes (Hachimori

et al.,1974; Boccu et al., 1976). However, the data is of limited value until a correlation can be established between stability and the content of various secondary structures in proteins. This has not been available to date.

1.3.5 Disulphide bonds.

Additional intramolecular S-S bonds also enhance the stability of proteins by increasing their rigidity (Wedler et al.,1980; Sundarum et al.,1980). This crosslinking can be artificially introduced into proteins by (1) bifunctional reagents. Examples of proteins stabilized by crosslinking have been reported by Martinek and Torchilin, (1988). (2) The second approach makes use of site-directed mutagenisis eg. the insertion of an S-S bond into the molecule of lysozyme T4 which normally has no S-S bonds (Perry and Wetzel,1984).

1.3.6 Low content of amino acids sensitive to oxidative modification.

One of the most frequently occurring mechanisms of protein inactivation is the oxidation of structurally important amino acids (Mozhaev and Martinek,1982; Klibanov,1983). The -SH group of cysteine and the indole ring of tryptophan are especially sensitive to oxidation. The number of these labile amino acids

(cysteines) has often been found to be significantly lower in highly stable thermophilic proteins compared with proteins from mesophiles (Mozhaev and Martinek,1984).

1.3.7 Compact packing of amino acid residues.

The contact of polar water molecules with the hydrophobic core of a globule will result in protein destabilization. The more compact the packing of amino acid residues in a protein globule, the less water molecules can occupy the cavities of the protein structure. Hence, the protein will be more stable. Nature stabilizes the structure of some thermophilic enzymes by replacing some of the amino acids in the hydrophobic core with amino acids of larger volume (Argos et al.,1979). Site-directed mutagenisis can also be used to replace amino acids with bulkier ones in an effort to increase the thermostability of enzymes (Malcolm et al.,1990).

1.3.8 Hydrophobic interactions.

Hydrophobic interactions make an important, if not decisive, contribution to the maintenance of the native structure of proteins (Kauzmann,1959; Schulz and Schirmer,1979). Enhanced stability of many proteins from thermophilic strains correlates well with their high hydrophobicity (Harris,1978; Walker et

al.,1980). This has also been observed in a number of β-lactoglobulins from different mesophilic sources (Alexander and Pace,1971). However, discrepancies were observed with this correlation theory (Biffen and Williams,1976; Wedler et al.,1976). Chothia (1976) was the first to analyse the reasons for the discrepancy. It emerged that three factors affect hydrophobic interactions in proteins. These are (1) the necessity of a rather compact packing of amino acids in the protein globule, (2) the microenvironment of amino acid residues affecting their geometry and energy, and (3) the requirement that hydrophobic clusters remain on the surface during folding since in vivo they are responsible for hydrophobic interactions of proteins with other molecules. Hence, the relationship between protein stability and total hydrophobicity is often absent.

The use of the content of aliphatic amino acids as a more appropriate measure of protein hydrophobicity was proposed by Singleton (1976) and a correlation between the content of aliphatic amino acids and protein stability has been found in some thermophilic proteins (Ikai,1980). It was observed that the greater the number of nonpolar residues localized inside the protein globule and the less the number of residues exposed to the solvent, then the more stable the protein (Mozhaev et al.,1988).

Having reviewed the molecular reasons for protein stability, an analysis of the processes causing thermal inactivation of enzymes is necessary before a strategy for enhancing thermostability of enzymes can be undertaken.

1.4 ANALYSIS OF PROCESSES CAUSING THERMAL INACTIVATION OF ENZYMES.

It has been well established that when an aqueous solution of an enzyme is heated, a number of molecular events begin to take place (Klibanov,1983). Initially, the enzyme molecules partly unfold as a result of a heat-induced disruption of the non-covalent interactions that maintain the catalytically active conformation at room temperature (Creighton,1983). This process, which almost invariably leads to enzyme inactivation , is reversible in that the native conformation and the enzymatic activity are completely recovered when the enzyme solution is promptly cooled. However, if heating persists, only a decreasing fraction of the enzymatic activity returns upon cooling, signifying that other irreversible processes take place.

Reversible thermal unfolding (denaturation) of enzymes has been a subject of intense investigation for several decades and is conceptually well understood (Kauzmann, 1959; Tanford, 1968, 1970; Privalov,1979; Pfeil, 1981). However, the irreversible thermoinactivation of enzymes had until very recently remained

obscure until Klibanov and his coworkers elucidated the molecular mechanisms of irreversible thermal inactivation of several enzymes (Ahern and Klibanov,1985; Zale and Klibanov,1986; Ahern et al.,1987).

1.5 DISTINGUISHING BETWEEN REVERSIBLE, POTENTIALLY REVERSIBLE AND IRREVERSIBLE MECHANISMS.

The most marked irreversible effect of elevated temperature on an enzyme is the increase in motion of its constituent parts to such a degree that what is known as the ordered "native" conformation can be said to be lost, replaced by largely disordered, "denatured" conformations. This can be represented as follows:

$$N \Longleftrightarrow U \qquad \text{Reaction (1)}$$

in which N and U are the native and unfolded forms of an enzyme, respectively.

Proteins are constantly in motion. Even at subzero temperatures and in the crystalline state, their constituent atoms undergo vibrations, rotations, and even small translations of the order of 0.2-0.5 Å (Frauenfelder et al.,1974). At higher temperatures, within the range at which most enzymes exhibit their optimal activity (0-60°C), reversible displacements of whole segments of protein structure, or "breathing" are observed in aqueous solutions. Also, concerted motions required for substrate binding,

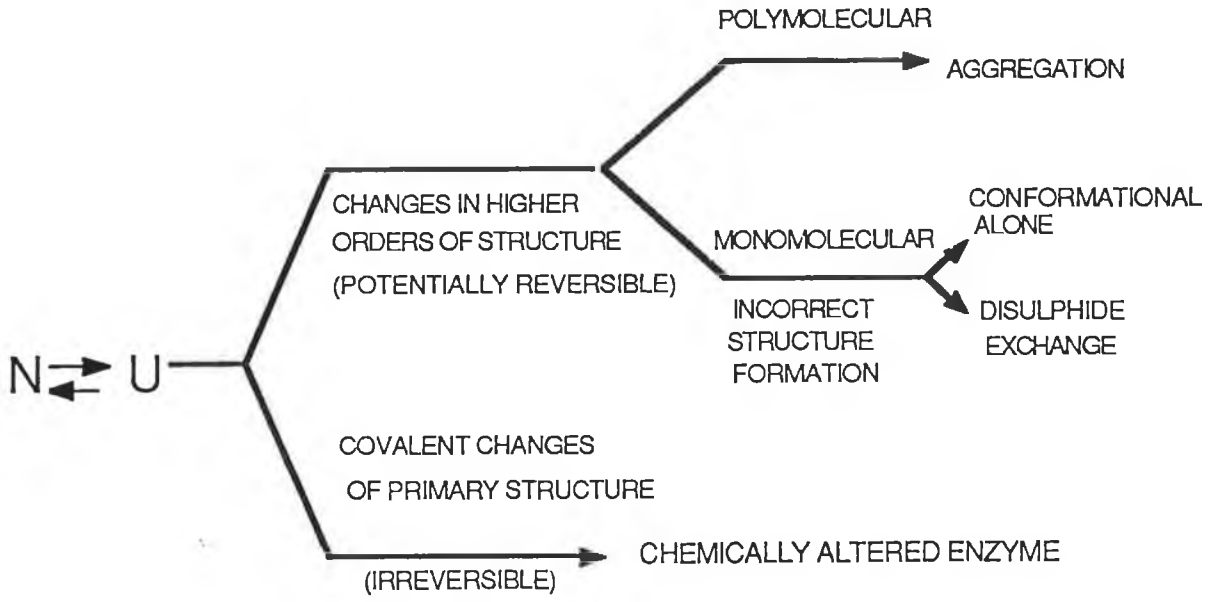
catalysis and product release are normal phenomena for an active enzyme (Karplus and McCammon, 1981). Although the protein has some freedom of movement under these conditions, the predominant conformations are dictated by a complex balance of intramolecular, noncovalent interactions: eg. hydrogen bonds, hydrophobic, ionic and Van der Waals interactions. At elevated temperatures (45-85°C), radical cooperative intramolecular motions take place. At the temperature at which the noncovalent forces maintaining the native structure of a protein can no longer prevail against the increase in entropy, the protein loses most of its ordered secondary and tertiary structure and is said to have denatured (Kauzmann,1959; Tanford,1968,1970; Privalov,1979; and Pfeil,1981). Being less hampered by spatial constraints, the amino acids move more freely and acquire more of the characteristics of free amino acids in solution, although they are still held together in a polypeptide chain. In the case of enzymes, denaturation inevitably results in the dispersal of the residues comprising the active site, and as a consequence, catalytic activity is lost.

Although an enzyme above its midpoint temperature (T_m) for thermal denaturation is no longer active, the transition is usually reversible for globular proteins. If an enzyme is subsequently cooled below its T_m, it refolds to the native structure and regains catalytic activity. If heating is prolonged however, not all of the initial activity returns upon cooling, and eventually the entire enzymatic activity is lost. Thermal

inactivation, therefore consists of two parts: reversible denaturation (partial unfolding) and subsequent irreversible thermoinactivation. Reaction (2) represents the framework distinguishing the various conformational and covalent processes leading to the thermal inactivation of enzymes.

Once unfolded, many enzymes become insoluble and form large particulates. Aggregation, being polymolecular, is a concentration-dependent process, and it can be explained by the amphiphilicity of the surface of a disordered enzyme as follows. Portions of the polypeptide chain that are normally buried tend to be much more hydrophobic than those exposed to solvent in the native structure (Rose et al.,1985). Once an enzyme has been denatured, the exposed hydrophobic surfaces tend to avoid interaction with the aqueous solvent, since water forms ordered clathrate structures around hydrophobic residues. The imposition of order on the solvent results in a decrease in entropy of the system as a whole. Thus, provided enzyme concentrations are high enough, such hydrophobic surfaces may form intermolecular interfaces via aggregation in an attempt to maximize the entropy of the solvent and thereby reduce the free energy of the system.

However, the activity of dilute solutions of enzymes often cannot be recovered after prolonged heating followed by cooling. This irreversible thermal inactivation follows first-order kinetics and can be independent of the initial enzyme concentration (Ahern and Klibanov,1985). It does not result in the



Reaction (2). Processes leading to the thermal inactivation of enzymes. N and U represent the native and unfolded forms of the enzyme, respectively. Once unfolded, an enzyme may be inactivated by conformational processes, covalent processes or a mixture of the two processes. The processes causing irreversible thermoinactivation may be either polymolecular (aggregation) or monomolecular. Processes of the latter type may result in a potentially reversible scrambling of the conformation, or a covalent modification of the primary structure of the enzyme. (Disulfide exchange, although covalent, is potentially reversible and is therefore distinct from the other covalent processes).

formation of aggregates and the loss of activity can be attributed to monomolecular processes.

It is important to determine whether monomolecular, apparently irreversible thermoinactivation, is caused by covalent changes of the primary structure or by changes in higher orders of structure, since the activity of conformationally altered enzymes that have undergone no irreversible deterioration has the potential to be regained.

The existence of monomolecular, incorrect structure formation at high temperatures can be explained by noting that there is more than one way to fold a protein. Upon denaturation, the tendency to bury hydrophobic groups, combined with the freedom of a protein to sample many conformational states, results in new, kinetically or thermodynamically stable structures that are catalytically inactive. Even after cooling, these incorrectly folded "scrambled" structures may remain because a high kinetic barrier prevents spontaneous refolding to the native conformation (Klibanov and Mozhaev,1986). (Disulphide exchange, resulting in the mismatching of cisteinyl residues, can play a role in the formation of these scrambled structures (Zale and Klibanov,1986). Such processes must be distinguished from covalent mechanisms resulting in destruction of the polypeptide chain or chemical deterioration of the side chain residues. These processes are truly irreversible and define the basal rate of irreversible enzyme thermoinactivation.

In order to distinguish between a potentially reversible

inactivation process and covalent mechanisms, Ahern and Klibanov, (1987) proposed four approaches which are as follows:
(1) preventing the conformational inactivation (by incubation in the presence of denaturants - see Section 3.16 for a fuller discussion on the rationale behind this approach) and recovering the activity lost due to scrambling by (2) reducing any disulphide bonds in the presence of strong denaturant, followed by reoxidation and refolding to reform native structure, or by (3) waiting for a period of time sufficient to permit the enzyme to correctly refold, and (4) identifying the contribution of conformational processes based on the rate of irreversible thermoinactivation (the half-lives of covalent processes found to cause irreversible thermoinactivation are relatively large - of the order of 10 min to more than an hour (Ahern and Klibanov,1985)).

1.6 POTENTIALLY REVERSIBLE MECHANISMS.

Having established that potentially reversible mechanisms may be responsible for the thermoinactivation of an enzyme, it is necessary to study this mechanism in more detail. The loss of activity due to the formation of incorrect structures that are potentially reversible may be due to disulfide exchange in enzymes containing cystine. This reaction which is known to occur in proteins at neutral and alkaline pH (Cecil and McPhee,1959; Lumper

and Zahn, 1965), requires the presence of catalytic amounts of thiols, which promote the interchange by nucleophilic attack on the sulfur atoms of a disulfide group (Torchinsky, 1981). The contribution of disulfide exchange in the formation of incorrect structures can be prevented if the enzyme is heated in the presence of thiol scavengers, such as p-mercuribenzoate and N-ethylmaleimide (Ryle and Sanger,1955) or copper ion, which catalyses the spontaneous air oxidation of thiols (Torchinsky,1981).

In addition to enzymes that form incorrect structures due to disulfide exchange, enzymes that contain no disulfide bridges undergo potentially reversible thermoinactivation. For example, α-amylases from *Bacillus amyloliquifaciens* and *B. stearothermophilus* contain 0 and 1 cysteinyl residue each, respectively, yet both are stabilized at least threefold against irreversible thermoinactivation at 90°C, pH 6.5, by the presence of 8M acetamide (Tomazic and Klibanov,1988). Therefore, it appears that not all potentially reversible thermoinactivation is due to disulfide exchange. The more detailed nature of incorrect structure formation is as yet unclear and will have to await further investigation.

1.7 IRREVERSIBLE MECHANISMS.

Covalent processes can affect enzyme structure in the following ways:

1. Cleavage of the polypeptide chain by hydrolysis.
2. Destruction of individual amino acid residues.
3. Destruction of disulfide bonds.
4. Reactions involving metal ions, cofactors, and adducts due to glycosylation.

1.7.1 Peptide chain integrity.

Hydrolysis of the polypeptide chain at Asp residues has been found to account for significant irreversible thermoinactivation in lysozyme (Ahern and Klibanov,1985) and Ribonuclease A (Zale and Klibanov,1986) at 90–100 °C and pH 4. These findings are in agreement with the data on the hydrolysis of proteins in dilute acid solutions which indicate that the Asp-X bond (where X is the amino acid residue bound to the α- carboxyl group of Asp) is the most labile peptide bond under those conditions (Kowit and Maloney,1982; Inglis,1983; Marcus,1985). Prolonged incubation of lysozyme at pH 4 results in release of free aspartic acid in solution. Inglis (1983) has proposed several pathways for the release of aspartic acid from proteins (Fig.1.7.1).

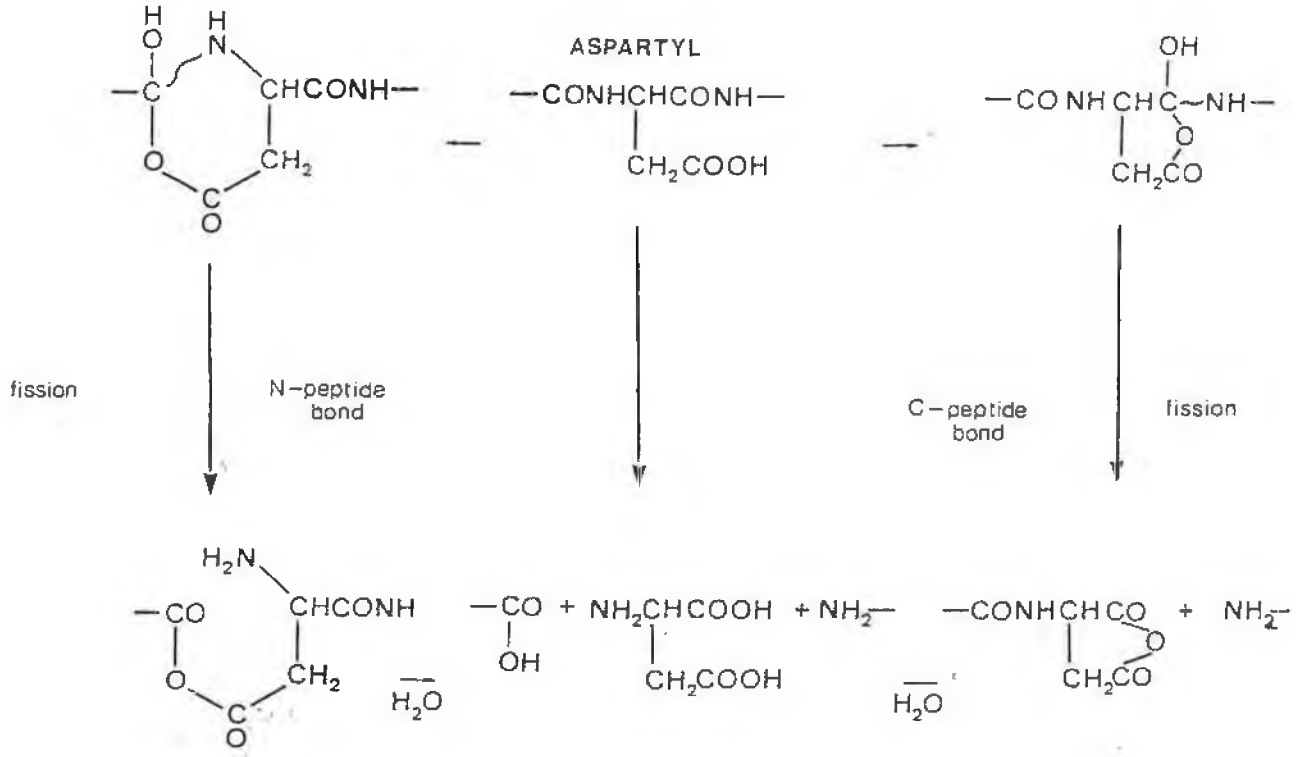


Fig. 1.7.1 Hydrolysis of peptide bonds adjacent to Asp residues causing irreversible enzyme thermoinactivation (Adapted from Inglis, 1983).

1.7.2 Amino acid destruction.

The covalent destruction of amino acid residues in enzymes during thermoinactivation has been studied (Ahern and Klibanov,1985; Zale and Klibanov,1986; Tomazic and Klibanov,1988). These workers found that deamidation of asparagine and\or glutamine residues contributed significantly to the inactivation of lysozyme and ribonuclease A at pH 4, 6 and 8. The proposed mechanism of deamidation of Asn residues is shown in Fig.1.7.2.

1.7.3 Destruction of cystinyl crosslinks.

In addition to the potentially reversible disulfide exchange that occurs in enzymes containing cystine residues when heated above their transition temperatures at alkaline and near-neutral pH (Section 1.6), cystines also undergo irreversible destruction known as β-elimination (Nashef et al.,1977). The base-catalysed abstraction of a proton from the α carbon of a Cys residue forming a disulphide bridge results in the cleavage of the cystine crosslink to form residues of dehydroalanine and thiocysteine (Fig.1.7.3). Dehydroalanine is a very reactive species that can undergo an addition reaction with the ε-amino group of a lysine residue to form the novel intramolecular crosslink, lysinoalanine (Bohak, 1964).

The formation of these degradative products accounts for the

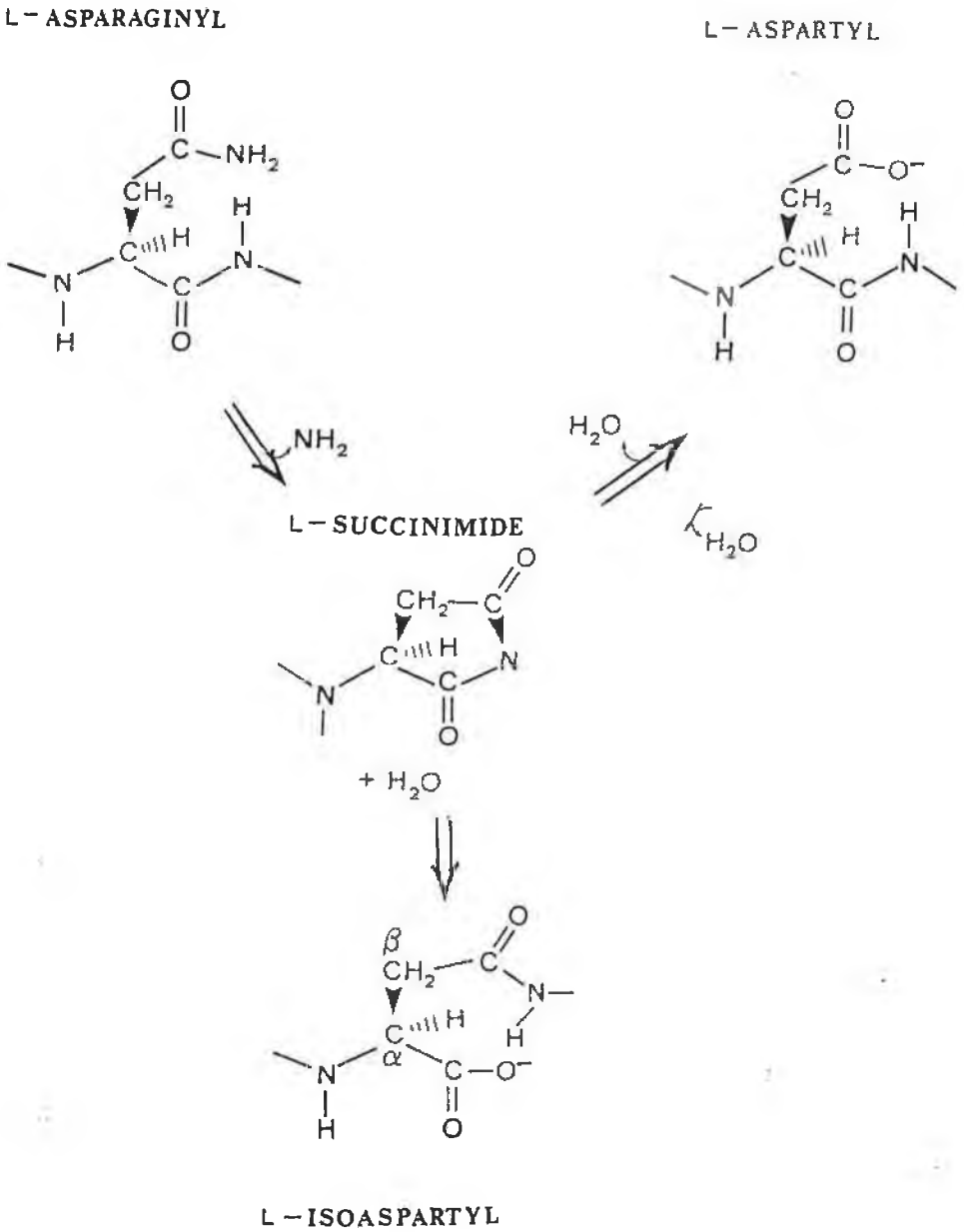


Fig. 1.7.2 Deamidation of Asn residues, resulting in either L-aspartyl or L-isoaspartyl residues, depending on which amide linkage in the proposed succinimide intermediate is hydrolysed (Adapted from Clarke, 1985).

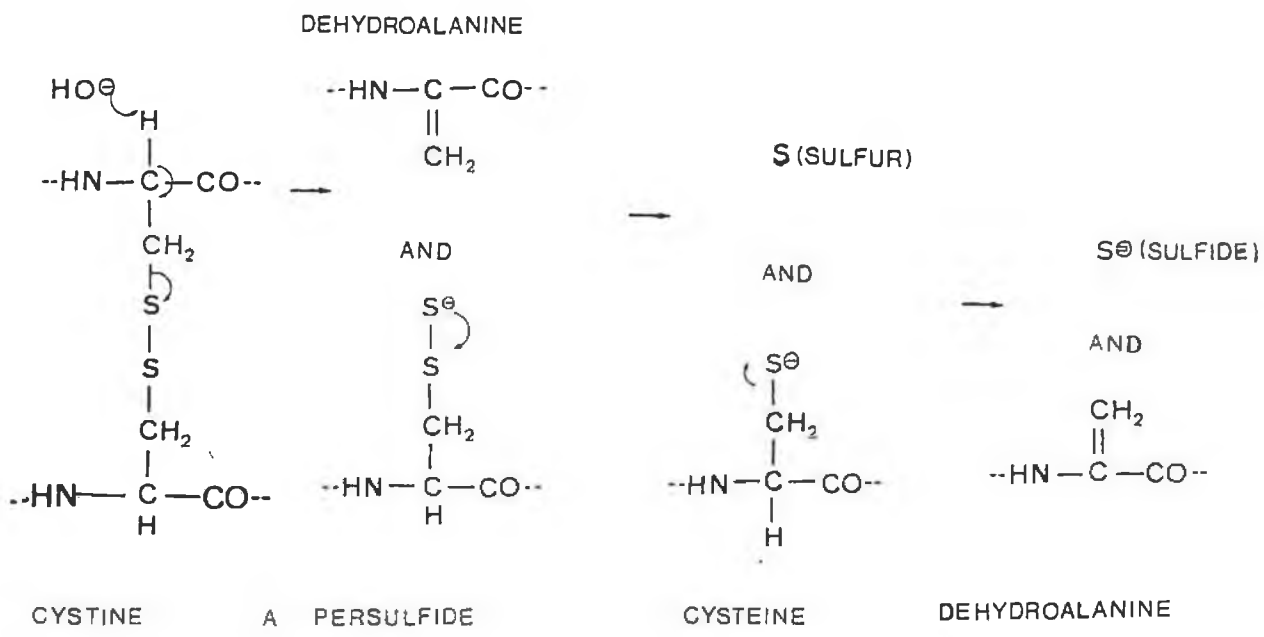


Fig. 1.7.3 Destruction of disulfide linkages via base-catalysed β-elimination (Whitaker, 1980).

fate of approximately 90% of all the cystine lost during the irreversible thermoinactivation of ribonuclease A. Furthermore, the contribution of β-elimination to loss of enzymatic activity was demonstrated by the finding that the degree of stabilization of ribonuclease was proportional to the degree of protection against β-elimination by reversible protection of the Cys residues by chemical modification (Zale and Klibanov,1986).

1.7.4 Other mechanisms.

In addition to the processes causing irreversible thermoinactivation outlined above, many other degradative processes specific to enzymes containing unique constitutive elements may result in thermoinactivation as well. In addition to the 20 common amino acid residues, more than 100 unusual amino acids also exist in proteins (Uy and Wold,1977), and half of them are susceptible to chemical deterioration such as hydrolytic scission of side chain groups bound to indole, phenoxy, thioether, amino, imizadole and sulfhydryl residues, and the derivatives of Ser and Thr (eg. O-glycosyl and O-phosphoryl groups) and Gln and Asn (e.g. methylated and glycosylated); (Feeney, 1980). Of the non-amino acid moieties associated with enzymes, the carbon-nitrogen bonds in purines and pyridines,glycosidic bonds and phosphodiester bonds undergo hydrolytic breakdown at rates comparable to the hydrolysis of peptide bonds (White,1984). If

present, reducing sugars and fatty acid degradative by-products can undergo the Maillard reaction with amino groups in enzymes to produce Schiff bases upon removal of water (Chio and Tappel,1969; Feeney et al.,1975). Metal ions can accelerate hydrolytic cleavage of peptide bonds (Long et al.,1971; Alexander Ross et al.,1979).

In addition to these covalent, deteriorative reactions, simple dissociation of noncovalently bound prosthetic groups during thermally-induced denaturation can be irreversible (Mozhaev and Martinek,1982). For example, once molybdenum has been dissociated from the active center of sulfite oxidase during incubation at high temperature, no reactivation appears possible by cooling the enzyme solution and addition of an excess of the metal ion (Southerland and Rajagopolan, 1978). Nevertheless, it is possible that the activity of some enzymes that lose their cofactors during thermoinactivation may be regenerated. For example, enzymes requiring metal-sulfur compounds can be reactivated after loss of their cofactors by addition of metal salts together with thiols or organic sulfides (Bernhardt and Meisch,1980; Kuhn et al., 1980; Okuno and Fujisawa,1981).

1.8 STABILIZATION OF ENZYME ACTIVITY

Having examined the molecular reasons for protein stability and analysed the processes causing thermal inactivation of proteins, an understanding of the rationale behind the approaches to enzyme stabilization can be more fully comprehended. The most common

approaches to enzyme stabilization are as follows:

1. Screening for stable enzymes.
2. Use of additives.
3. Enzymatic catalysis in anhydrous organic solvents.
4. Site-directed mutagenisis.
5. Immobilization.
6. Chemical modification of enzymes.
7. Crosslinking with bifunctional reagents.

1.8.1 Screening for stable enzymes

The first approach entails the use of enzymes naturally possessing an abnormally high resistance to denaturing factors. Although there is not necessarily a direct correlation between the two, this has led in many cases to the selection of microorganisms from hot springs, very salty media, torrid or glacial regions. For example, glutamine synthetase from *Bacillus caldolyticus* and thermolysin from *Bacillus thermoproteolyticus* have proved much more stable than their mesophilic counterparts (Merkler et al., 1988; Fontana, 1988).

While this approach has resulted in the isolation of several stable enzymes (Daniels et al.,1981), it cannot be considered strictly as a method for stabilization of enzymes. This approach suffers from limitations such as the narrow spectrum of useful proteins that are naturally expressed by microorganisms and the

possibility that the expressed enzymes may have different activity and specificity characteristics (Stewart,1987). It has, however, as discussed in Section 1.2 provided insight into mechanisms of stabilization.

1.8.2 Use of additives

It has been commonly known since the origins of enzyme utilization that the addition of certain compounds allows a very significant increase in enzyme stability. Thus, sugars (sucrose, lactose), polyols (glycerol, sorbitol), salts (ammonium sulfate) and various polymers are widely used for the stabilization of enzyme solutions (Schmid,1979). The use of these additives is described in the following sections.

1.8.2.1 Substrates

Binding of substrates may lead to stabilization or labilization or to no effect at all. In a review of conformational adaptability in enzymes, Citri has tabulated a large number of enzymes, the majority of which were stabilized and a few labilized towards thermal or urea denaturation in the presence of specific ligands (Citri,1973).

For example, asparaginase was protected against denaturation by heat, protease and iodine vapour by binding L-asparagine or

L-aspartate (Citri,1972a,b), and glyceraldehydephosphate dehydrogenase was stabilized towards the action of proteases in the presence of NAD, but labilized by NADH and AMP (Citri,1973). A general model has been proposed to account for the effects of substrates on protein stability (Silverstein and Grisolia,1972). It was postulated that if substrate binding led to a conformation of higher internal energy, denaturation would be facilitated; conversely, if by substrate binding, a conformation of lower internal energy was obtained, the resulting complex would be better protected against denaturation.

1.8.2.2 Solvent additives

There are many potential stabilising co-solutes for proteins (Table 1.8.2.2). In Table 1.8.2.2 compounds are separated into groups that have varying effects on the solvation properties of water such as the dielectric constant, chemical potential, viscosity and the clathric tendency (surface tension).

(a) Osmolytic stabilizers

The first group of compounds in Table 1.8.2.2 are osmolytes which are not strongly charged and have little effect on enzyme activity up to at least 1M concentration (Yancey et al., 1973). Their major effects are on the viscosity and surface tension of water, and hence of solvent ordering.

Table 1.8.2.2 Protein co-solutes and their mode of action in stabilizing proteins (Schein, 1990).

COMPOUNDS	CONCENTRATION USED	MODE OF ACTION
OSMOLYTIC STABILIZERS. These generally have little direct interaction with proteins, but affect the bulk solution properties of water.		
Polyols and sugars E.G. GLYCEROL, ERYTHRITOL, ARABITOL, SORBITOL, MANNITOL, XYLITOL, MANNISDOMMANITOL, GLUCOSEGLYCEROL, GLUCOSE, FRUCTOSE, SUCROSE, TREHALOSE, ISOFLUOROSIDE.	10-40%	THESE STABILIZE THE LATTICE STRUCTURE OF WATER, THUS INCREASING SURFACE TENSION AND VISCOSITY. THEY STABILIZE HYDRATION SHELLS AND PROTECT AGAINST AGGREGATION BY INCREASING THE MOLECULAR DENSITY OF THE SOLUTION WITHOUT CHANGING THE DIELECTRIC CONSTANT.
Polymers E.G. DEXTRANS, LEVANS POLYETHYLENE GLYCOL.	1-15%	POLYMERS INCREASE MOLECULAR DENSITY AND SOLVENT VISCOSITY, THUS LOWERING PROTEIN AGGREGATION IN A SINGLE PHASE SYSTEM. AT HIGH POLYMER CONCENTRATION, A TWO PHASE SYSTEM DEVELOPS AND THE PROTEIN AGGREGATES IN THE PHASE WHERE ITS CONCENTRATION IS THE HIGHEST.
Amino acids and derivatives E.G. GLYCINE, ALANINE, PROLINE, TAURINE, BETAINE, OCTOPINE, GLUTAMATE, SARCOSINE, γ-AMINOBUTYRIC ACID, TRIMETHYLAMINE N-OXIDE (TMAO).	20-500mM	SMALL AMINO ACIDS WITH NO NET CHARGE LIKE GLY AND ALA, HAVE WEAK ELECTROSTATIC INTERACTIONS WITH PROTEINS. OCTOPINE IS A DERIVATIVE OF ARG THAT IS LESS DENATURING TO PROTEINS. TMAO STABILIZES PROTEINS EVEN IN THE PRESENCE OF DENATURANTS LIKE UREA. MOST OF THESE COMPOUNDS INCREASE THE SURFACE TENSION OF WATER.
IONIC COMPOUNDS. These affect enzyme reactions and their stabilizing effects on proteins occur in a much narrower concentration range than the above compounds.		
Stabilizing E.G. CITRATES, ACETATE, PHOSPHATES, QUATERNARY AMINES, SULFATES.	20-400mM	LARGER ANIONS SHIELD CHARGES AND CAN STABILIZE PROTEINS AT LOW CONCENTRATIONS. AT HIGH CONCENTRATIONS THEY LEAD TO PRECIPITATION BY COMPETING FOR WATER MOLECULES.

Table 1.8.2.2 (continued)

COMPOUNDS	CONCENTRATION USED	MODE OF ACTION
Destabilizing		
E.G. CHLORIDES, NITRATES, THIOCYANATES.	20-40mM	THESE ARE GENERALLY LESS STABILIZING THAN LARGE IONS, BUT ARE ALSO USEFUL FOR CHARGE SHIELDING AT LOWER CONCENTRATIONS.
Denaturing (chaotrophs)		
UREA, GUANIDINIUM SALTS, TRICHLOROACETATES, CETLYMETHYLAMMONIUM SALTS, ORGANIC SOLVENTS.	0.2-8M	DENATURANTS EITHER STABILIZE THE UNFOLDED STATES OF PROTEINS (UREA) OR PERTURB PROTEIN STRUCTURE BY INTERFERING WITH HYDROGEN BONDING OR DISTURBING THE HYDRATION SHELL.
OTHER COMMON ADDITIVES.		
2-MERCAPTOETHANOL, DITHIOTHREITOL (DTT), GLUTATHIONE.	1-5mM 0.1-1mM[1] 1-4mM	REDUCTANTS PROTECT FREE SULFHYDRYLS FROM OXIDATION; PREVENT INTERMOLECULAR SULFHYDRYL CROSSLINKING.
PHENYLMETHYLSULFONYL-FLUORIDE (PMSF), BENZAMIDINE.	0.02-0.05mM[2] 1mM	INHIBIT SERINE PROTEASES BY REACTING WITH THE ACTIVE SITE HYDROXYL GROUP.
LEUPEPTIN, PEPTIDES	<1mM	PROTECT FROM PROTEASE ATTACK BY SERVING AS ALTERNATE SUBSTRATES.
ETHYLENEDIAMINE TETRA ACETIC ACID (EDTA), ETHYLENE-BIS(OXYETHLENE-NITRILO) TETRACETIC ACID (EGTA).	0.01-0.1M FOR BUFFERS	CHELATE DIVALENT METAL CATIONS WHICH MAY REACT WITH PROTEINS; INHIBIT METALLOPROTEASES.
CATECHOLS, PHENOLICS, NaN_3	<0.05% <0.1%	BACTERIOCIDES

[1] DTT IS A POTENTIAL DENATURANT OF PROTEINS AT HIGH TEMPERATURES AND HAS LIMITED SOLUBILITY IN HIGH SALT. THE CONCENTRATIONS INDICATED SHOULD NOT BE EXCEEDED.

[2] DISSOLVE PMSF TO 20MM IN ISOPROPANOL. THE INDICATED CONCENTRATIONS REPRESENT THE MAXIMUM SOLUBILITY IN AQUEOUS BUFFERS.

Osmolytes can be polyols, sugars, polysaccharides, neutral polymers, amino acids and their derivatives, and large dipolar molecules like trimethylamine N-oxide (TMAO). Glycerol is the most commonly used osmolyte, as it is easily removed by dialysis and does not interfere with ion-exchange chromatography. It does not alter the dielectric constant of the medium significantly and its stabilization effect on proteins seems to be due to its ability to enter into and strengthen the water lattice structure. High concentrations of glycerol decrease the diffusivity and the partial molar volume of proteins (Gekko and Timasheff,1981), thus lowering the rate of aggregate-producing solute interactions.

(b) Ionic stabilisers

Ionic compounds and salts can stabilise protein structure by shielding surface charges. Much work has been devoted to the effect of salts on protein stability (Hippel and Schleich,1969a; Jenks,1969). All studies in this area can be divided into two groups. The first includes studies of the action of relatively low concentrations of divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, etc., on enzyme stability. In many cases these cations stabilise enzymes as exemplified by Ca^{2+} (Siegel,1973). This effect is usually very specific, e.g. whereas Ca^{2+} greatly increases thermal stability of *Bacillus caldolyticus* α-amylase, Sr^{2+} does not (Heinen and Lauwers, 1976). Thus, because of this specificity, stabilization by divalent cations is a poor candidate as a general method of enzyme

stabilization.

The second group of studies includes the effect of relatively high concentrations of various neutral salts. The latter may bind to protein-charged groups of dipoles increasing the solubility of the protein ("salting in") or reduce the solubility of protein hydrophobic groups by increasing the ionic strength of solution ("salting out"). These interactions can stabilize proteins. For example, salting out of hydrophobic residues from the surface into the interior of an enzyme macromolecule will conformationally "compress" the enzyme by facilitating hydrophobic interactions on the molecules surface layer. Such enzymes will be more resistant to thermal unfolding and thus could demonstrate a higher thermal stability.

With respect to this reversible thermodenaturation of enzymes, the experimental data generally correlate with the Hofmeister lyotropic series (Jencks,1969) concerning the effects of salts on protein solubility. The major common feature of salting in and salting out is that added salts change the activity coefficients of both the noncharged and charged regions of the protein molecule.

In accordance with the Hofmeister lyotropic series, the stabilising effect of cations and anions should decrease in the following order (Hippel and Schleich,1969):

$$(CH_3)_4N^+ > NH_4^+ > K^+, Na^+ > Ca^{2+} > Ba^{2+}$$

$$SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

with anions and cations being additively effective. Therefore, $(NH_4)_2SO_4$ should be a strong stabilizer, whereas NaSCN should destabilize enzymes. There are no systematic data available concerning the effects of Hofmeister salts on irreversible thermoinactivation of enzymes.

(c) Denaturants, chaotrophs, cryoprotectants and other additives.

One can solubilize almost any protein (usually at the expense of its activity) by chemical denaturation with perturbing ions. Urea stabilizes the unfolded states of proteins because essentially all protein parts, from the backbone to the tryptophan side chains, are more soluble in 6M urea than water as evidenced by the free energy of transfer into this solvent (Kamoun,1988). Another class of denaturants, "chaotrophs" like guanidinium, cetyltrimethyl ammonium salts, trichloroacetate, and thiocyanate ions disrupt hydrogen bond formation and disturb the hydration shell around proteins (Gekko and Timasheff,1981). Detergents bind to hydrophobic areas of proteins. They are frequently used for solubilizing proteins from membranes. The only way to isolate most integral membrane proteins is to extract them from their lipid environment with bulky detergents e.g.

Triton X-100. The protein is integrated into a detergent micelle, with detergent replacing phospholipids or proteins that were previously in contact with the hydrophobic surfaces (Hjelmeland and Chrambach, 1984).

Another class of denaturants, organic solvents, lower the dielectric constant of water. The denaturing activity of hydrophobic solvents is due to a limited detergent effect and that they provide a competing interaction for the intramolecular hydrophobic interactions responsible for a stable tertiary structure.

Some organic solvents such as dimethylsulfoxide (DMSO) and ethyleneglycol are powerful cryoprotectants which help to maintain native protein conformations at temperatures below the freezing point of water. They have been extensively used in protein crystallography (Petsko,1975) and enzymology (Douzou, 1973, 1975. 1976).

1.8.3 Enzymatic catalysis in anhydrous organic solvents.

A relatively new and emerging area of protein stabilization concerns the use of enzymes in anhydrous organic solvents (Klibanov,1989; Arnold,1990). It has been demonstrated that the rate of irreversible thermoinactivation is reduced by several orders of magnitude when an enzyme is incubated in organic solvents such as cyclohexane, hexadecane, various esters, and

alcohols (Zaks and Klibanov,1984; Ahern and Klibanov,1986; Ayala et al.,1986).

The rationale behind the use of anhydrous organic solvents is as follows: The native, catalytically active conformation of enzymes is maintained by a delicate balance of various non-covalent interactions, and water participates in all of them (Cantor and Schimmel, 1980; Creighton, 1983). Hence the removal of water should radically alter the enzymes conformation and destroy enzymatic activity. However, a certain amount of water is required for enzymatic activity. The enzyme molecule cannot "see" more than a monolayer or so of water around it. Therefore, if this layer of "essential" water is somehow localized and kept on the surface of the enzyme, then all the bulk water should be replaceable with organic solvents with no adverse effect on the enzyme. (The term anhydrous means that the solvents should have a water content less than 1%).

There are a number of requirements for enzyme activity in organic media. Firstly, the nature of the solvent is critical, with hydrophobic ones being the best reaction media. This requirement has been well illustrated by Zaks and Klibanov, (1988). It was shown that the enzymatic activity of chymotrypsin dropped precipitously upon a decrease in hydrophobicity of the solvent. For example, the reactivity of the enzyme in octane exceeded that in pyridine by more than 10^4. This has been explained by assuming that hydrophilic solvents "strip" the

essential water from enzyme molecules, thereby diminishing their enzymatic activity (Klibanov,1989)

The second requirement is that enzymes to be used in organic solvents should be lyophilized (or precipitated) from aqueous solutions at the pH optimal for enzymatic activity. This requirement is due to the "pH memory" of enzymes in organic solvents (Zaks and Klibanov,1985; Zaks and Klibanov,1988).

The last requirement stems from the insolubility of enzymes in nearly all organic solvents (Singer,1962). Thus enzymes are suspended in non-aqueous solvents. Such heterogeneous dispersions may be plagued by the hindered diffusion of substrates to and through the enzyme particles. However, this problem can be readily eliminated (Klibanov,1986) by (1) vigourous agitation and (2) sonication of the suspensions prior to use to reduce the size of the enzyme particles.

As discussed above, the use of anhydrous organic solvents has been used to reduce the rate of irreversible thermoinactivation of some enzymes and would therefore appear to be a very promising method in the area of protein stabilization in the future.

1.8.4 Site-directed mutagenisis.

The most powerful recent development in enzymology is protein engineering; that is , the redesign of enzymes by site-directed mutagenisis (Fersht et al., 1984; Knowles, 1987; Eijsink, 1991).

Stability in a folded protein is a balance between the stabilizing (mostly hydrophobic) interactions, and the tendency towards destabilization caused by the loss of conformational entropy as the protein adopts the unfolded form. Consequently, the stability of a protein may be altered by changing amino acids which affect either stabilising interactions in the folded protein, or destabilising interactions in the unfolded form, or both (Table 1.8.2.4).

For example, point mutations have been engineered in the dimeric enzyme, yeast triosephosphate isomerase that have improved its thermostability (Ahern et al., 1987). Cumulative replacement of asparagine residues at the subunit interface by residues resistant to heat-induced deterioration and approximating the geometry of asparagine (Asn-14 ——>Thr-14 and Asn-78——>Ile-78) nearly doubled the activity of the enzyme at 100°C, pH 6. Moreover, in an attempt to model the deleterious effects of deamidation, Ahern and coworkers showed that replacement of interfacial Asn-78 by an aspartic acid residue increased the rate constant of irreversible thermal inactivation, drastically decreasing the reversible transition temperature, and reducing the stability against dilution-induced dissociation.

Sandberg and Terwilliger (1989) used site-directed mutagenisis to study the influence of interior packing and hydrophobicity on the stability of V proteins from bacteriophage f1. They found that packing effects appeared to destabilize their three mutant

Table 1.8.2.4 Factors to consider in stabilizing proteins[1] (Nosoh and Sekiguchi, 1990).

Stabilizing interactions or bonds
Disulfide bond
Hydrogen bond
Electrostatic interaction
Hydrophobic interaction
Conformational factors
Stability of secondary structure
Compact packing
Conformational flexibility
Internal hydrophobicity
Entropic stability
Protection
Deamination of carboxyamide
Oxidation of sulfhydryl groups
Intramolecular S-H/S-S exchange
Oxidation of tryptophan or methionine

[1]These factors were deduced by comparing the structures of proteins of different stabilities (proteins isolated from mesophiles and thermophiles, wild-type and amino acid-substituted proteins, wild-type and chemically modified proteins.

proteins by 0.8 to 2.9 kcal/mol, whereas hydrophobic effects stabilized or destabilized them by only 0 to 1.6 kcal/mol. They concluded that simply adding buried hydrophobic groups to proteins did not necessarily increase their stabilities, as the structural distortion caused by repacking of the proteins interiors was a large problem.

Further recent work showing the success of site-directed mutagenisis to increase enzyme thermostability can be seen in the studies of Pakula and Sauer,1990; Koizumi et al., 1990; and Malcolm et al., 1990. It appears, therefore, that protein engineering is a promising tool, not only for stabilizing proteins, but also for studying destabilizing interactions in proteins (Nosoh and Sekiguchi, 1990).

1.8.5 Immobilization

The term immobilized enzymes means the physical localization of the enzyme molecules during a continuous catalytic process. The usual practice is to confine the enzyme to a water-insoluble matrix where it can be recovered for further use (Zaborsky, 1973). More than 100 immobilization techniques have been elaborated (Zaborsky, 1973; Mosbach,1976; Trevan, 1980; Chibata, 1978). They can be divided into the following groups (Fig.1.8.5).

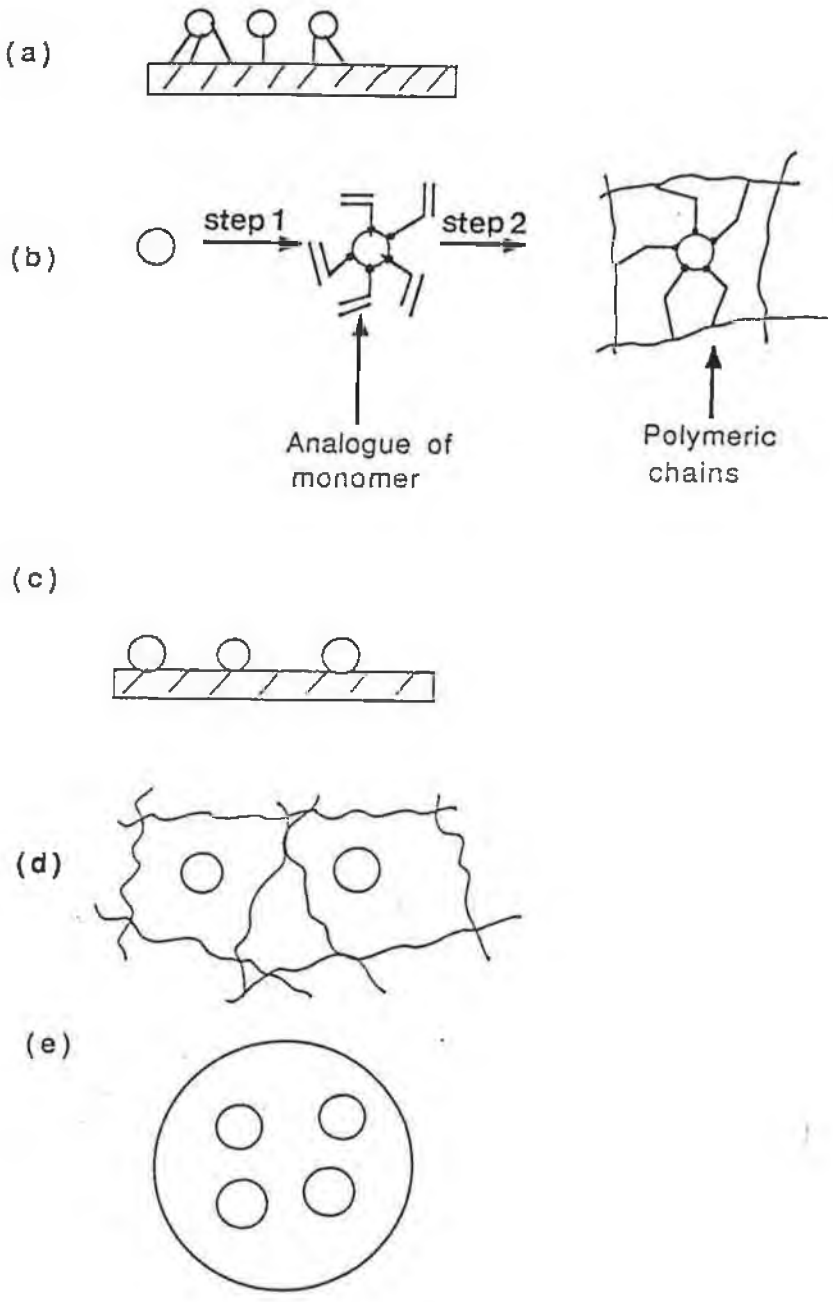


Fig. 1.8.5. Methods of enzyme immobilization.
(a) covalent attachment to solid supports, (b) copolymerization method, (c) adsorption on solid supports, (d) entrapment in polymeric gels, and (e) encapsulation.

1. Covalent attachment of enzymes to solid supports.
2. Adsorption of enzymes on solid supports.
3. Entrapment of enzymes in polymeric gels.
4. Encapsulation of enzymes.

1. Covalent attachment of enzymes to solid supports (Fig.1.8.5a)

Immobilization by covalent attachment means fixing the enzyme molecule with the insoluble matrix by at least one covalent bond (Zaborsky, 1973). A variety of supports have been used, including porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers and metallic oxides (Axen, 1970). Enzymes are usually immobilized through their amino or carboxyl groups. In most instances, the immobilization procedure consists of at least two steps: activation of the support and enzyme attachment.

Among the numerous immobilization procedures aimed at the suppression of protein unfolding, the multipoint attachment of the protein molecule to the surface of a support is the most promising one (Martinek et al., 1977a and b; Otero et al., 1988; Mozhaev and Melik-Nubarov, 1990). This type of immobilization has made it possible to slow down by several hundred to a thousand times both the reversible (Koch-Schmidt and Mosbach, 1977) and irreversible (Martinek et al., 1977a and b) thermoinactivation of enzymes, as well as their reversible unfolding caused by denaturants such as urea (Gabel, 1973) or the dissociation of oligomeric enzymes into

subunits (Baev, 1984).

A combination of enzyme entrapment and covalent bonding has also been used (Mozhaev et al., 1983; Mozhaev, 1990; Mozhaev and Melik-Nubarov, 1990). This has been called the copolymerization method and involves firstly modifying the functional groups of an enzyme with an analogue of monomer and them copolymerizing the modified enzyme with a monomer and bifunctional cross-linker, producing a three-dimensional polymeric network, at the junctions of which are the enzyme molecules (Fig.1.8.5b). This technique has been used to stabilize trypsin and α-chymotrypsin (Mozhaev et al., 1983) and thermitase (Mozhaev, 1990).

2. Adsorption of enzymes on solid supports (Fig 1.8.5c).

Ion-exchangers readily adsorb most proteins, and therefore they have been widely employed for enzyme immobilization (Chibata 1978). Such supports as the anion-exchangers diethylaminoethyl cellulose (DEAE- cellulose) or Sephadex and the cation exchanger carboxymethyl cellulose (CM-cellulose) are used industrially for adsorption of enzymes. The appealing feature of adsorption immobilization is its simplicity: an enzyme solution is added to the support, the system is stirred for a few minutes and then the enzyme remaining in solution is removed by washing.

3. Entrapment of enzymes in polymeric gels (Fig.1.8.5d).

In this approach, an enzyme is added to a solution of monomers before the gel is formed (Weetal, 1974). Then gel formation is initiated by either changing the temperature or adding a gel-inducing chemical. As a result, the enzyme becomes trapped in the gel volume. The gels employed for immobilization of enzymes may be covalent (for example, polyacrylamide crosslinked with N,N^1-methylenebisacrylamide or noncovalent (calcium alginate or kappa carrageenan).

4. Encapsulation of enzymes (Fig.1.8.5e).

In this approach, pioneered by Chang, (1972), enzymes are enveloped within various forms of membranes that are impermeable for enzymes and other macromolecules but permeable for low molecular weight substrates and products. Typical examples include entrapment of enzymes in microcapsules, in liposomes and in hollow fibers. The first two methods are intended for medical applications and the third for industrial ones. For instance, the Snamprogetti Company in Italy has used penicillin acylase, lactase and aminoacylase entrapped in hollow fibres (Marconi and Morisi, 1979).

1.8.6 Chemical modification of enzymes.

While enzyme immobilization has been very successful in stabilizing enzymes, more attention has been paid recently to methods which lead to water-soluble enzyme conjugates. Chemically modified soluble enzymes have a number of potential advantages: they often exhibit higher catalytic activity (Svensson, 1976), they may be more stable in liquid formulations and they might show favorable properties eg. enhanced stability and reduced antigenicity.

Much work has been dedicated to the chemical modification of proteins. A recent review has outlined the history and applications of this subject (Meens and Feeney, 1990). While most efforts have been aimed at the elucidation of amino acid sequences and at the identification of residues at the catalytic and binding sites of proteins, the effects of chemical modification on the stability of proteins is now being more extensively investigated (Snyder et al., 1983; Torchilin et al., 1983; Cupo et al., 1982; Munch and Tritsch, 1990; Hilvert, 1991). Table 1.8.6 contains a list of some of the most commonly used group-selective reagents and brief descriptions of some of their important properties and applications.

Table 1.8.6 Side chain modification reagents useful in studies on stabilizing enzymes.

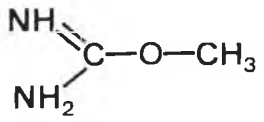
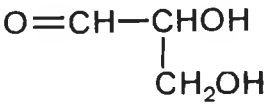
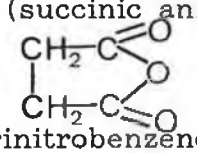
SIDE CHAIN OR GROUP FOR MODIFICATION	REAGENT OR PROCEDURE	OPTIMUM REACTION PH, SIDE CHAIN SELECTIVITY, COMMENTS.
Amino	O-methylisourea 	pH 8.4.reacts with ε-NH_2 groups converting lysine to homoargine; positive charge maintained.
	reductive alkylation (glyoxylic acid + $NaBH_3CN$) 	pH 8.4, carboxymethylates amino groups making them more hydrophilic at neutral or slightly alkaline pH.
	acylation (acetic anhydride) (succinic anhydride) 	pH 8.0 and above, Tyr residues also modified; elimination of positive charge.
	trinitrobenzenesulfonate	pH 8.0 and above, also reacts slowly with thiol groups; eliminates positive charge and introduces large hydrophobic substituent; extent of reaction may be determined spectrophotometrically.
carboxyl (asp + glu)	water-soluble carbodiimide + nucleophile (EDC + glycine ethyl ester.	pH 4.5-5, some side reactions with Tyr and thiol groups; other carbodiimides are available; many other nucleophiles may be used to either maintain or alter the charge.
guanido (arg)	dicarbonyls [2,3-butanedione, phenylglyoxal, and (p-hydroxyphenyl) glyoxal]	pH 7 or higher,reaction promoted by borate buffer; no major side reactions; partially reversible upon dialysis; eliminates positive charge.

Table 1.8.6. (continued).

SIDE CHAIN OR GROUP FOR MODIFICATION	REAGENT OR PROCEDURE	OPTIMUM REACTION PH, SIDE CHAIN SELECTIVITY, COMMENTS.
imidazole (his)	diethylpyrocarbonate (ethoxyformic anhydride)	pH 4.5, side reactions with lys kept to a minimum by low pH.
indole (trp)	N-bromosuccinimide	usually pH 4 or lower, higher pH values can be used; thiol groups are rapidly oxidised; tyr and his react more slowly.
thiol (cys-SH)	carboxymethylation (iodo- and bromo-acetate and iodo- and bromoacetamide)	pH 7 or higher; no effect on other residues under appropiate conditions. Lys his, tyr and met react slowly with excess reagent and long reaction times.
	5,5-dithiobis (2-nitrobenzoic acid) (Ellman's reagent)	pH 7 or higher, no other side chains react; reversible in presence of low MW thiols.

A number of reasons for protein stabilization by covalent modification have been outlined and are as follows:

1. Stabilization may occur as a result of modification of "key functional groups" (Torchilin and Martinek, 1979). However, it should be noted that the chemical modification of proteins very often does not lead to a significant change in stability with the increasing number of modified functional groups until a certain critical value has been reached at which the stability increases abruptly (Torchilin et al., 1979). This can be explained by assuming that at low modification degrees, only those functional groups are modified which are localized on the surface of a protein and whose role in the protein structure is inessential. If an excess of the modifying reagent is used, some functional groups localized inside the protein globule are also modified. The modification of these groups appears to improve the balance of intramolecular interactions in the protein and results in its stabilization.

There are, however, some problems with the modification of the key functional groups. The main problem is to determine which of the functional groups is the key one. This must be established empirically by investigating the dependence of the protein stability on the degree of modification. The empirical search for the key functional groups must be performed very cautiously since after the modification of the key functional groups, some amino acid residues buried more deeply in the protein globule may be

modified as well, effecting the active site. The latter process usually results in destabilization (Torchilin et al., 1979). There is another problem associated with the covalent modification of oligomeric enzymes. Modification of some key functional group in the intersubunit region very often results in destruction of intersubunit contacts and dissociation of enzyme into monomers. An example of the modification of "key" functional groups can be seen by the dependence of α-chymotrypsin thermostability and catalytic activity on the degree of its amino groups modification (Torchilin et al., 1979). Modification was carried out by both alkylation (using acrolein) and acylation (with succinic and acetic anhydrides). It was determined that modification of approximately 80% of the amino groups had only a slight effect on the thermostability of the enzyme. The thermostability, however, sharply increased when the degree of modification was higher than 80% and then became destabilized when complete substitution of all the amino groups occurred.

2. New functional groups introduced into the protein by chemical modification can form additional hydrogen or salt bonds (Muller, 1981), leading to increased stabilization. Polar or charged groups can be introduced into the protein molecule by chemical modification to form new hydrogen bonds or salt bridges. It is very difficult, however, to realize such experiments in practice.

The most suitable way is the following: the three-dimensional structure of the protein is determined and a search is made for noncompensated (e.g. charged) groups that could be involved in new electrostatic interactions. Then an appropriate "anchor" functional group is selected which is not too far from the charged group in the tertiary structure of the protein. Finally, a suitable chemical reagent bearing a group specifically reacting with the anchor group of the protein and having a charged fragment localized at a given distance is chosen or synthesized. These experiments are clearly quite elaborate. However, no information has been available to date to show success with this technique.

3. Chemical modification by nonpolar reagents strengthens hydrophobic interactions in the protein. It has been postulated (Shatsky et al., 1973) that protein stability can be increased by hydrophobization. However, the experimental data are conflicting. In contrast to the observed stabilizing effects (Shatsky et al., 1973), examples can be presented of hydrophobized proteins whose stability is significantly lower than the stability of the native ones (Kagawa and Nukiwa, 1981; Cupa and Pace, 1983). This apparent contradiction can be easily explained. The modification of a hydrophilic residue by a hydrophobic reagent (e.g. of the ε-NH_2 group of Lys by methyl iodide) should destabilize the protein. After such modification of the protein, in fact, there occurs a thermodynamically disadvantageous (destabilizing) contact of the

introduced nonpolar CH_3-groups of the protein with water. There is an exception, however, from this general rule. Many hydrophobic residues localized on the surface of proteins (along with polar and charged amino acids) very often accumulate to form surface hydrophobic clusters (Krigbaum and Kamoriya, 1979; Burley and Petsko, 1985). If a residue to be modified is localized in the vicinity of such a cluster, then a modifier with a suitable chain length could come into contact with it, thus increasing the stability of the protein because of additional hydrophobic interactions (Shatsky et al., 1973).

In terms of hydrophobic stabilization, the most efficient mechanism should be a modification which permits the introduction of nonpolar molecules inside the hydrophobic nucleus of the protein (Mozhaev and Martinek, 1984). This approach is schematically represented in Fig. 1.8.6.3. The protein to be modified has in its initial state an unfolded conformation (random coil) rather than a folded native conformation. After the unfolding, the structure of the protein should be changed by one of the three following procedures. First, the protein can be refolded under "non-native" conditions, i.e. in the presence of concentrated salt solutions, organic solvents, or at elevated temperatures. Under such conditions, the protein may adopt another conformaton which retains catalytic activity and has a higher stability. If the refolding is performed under conditions which favour hydrophobic interactions, the interior of the protein

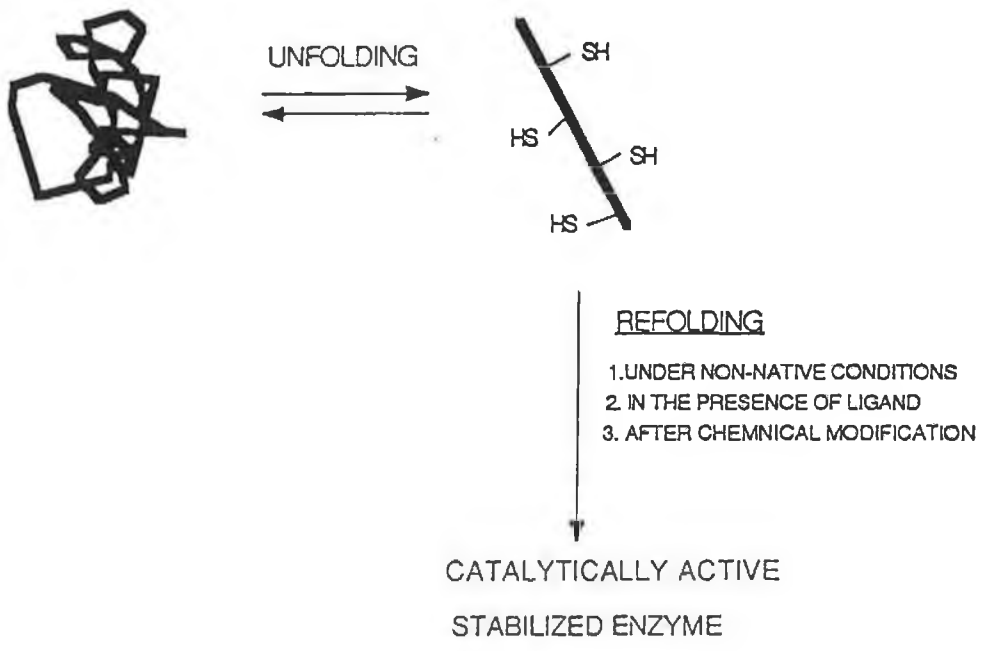


Fig. 1.8.6.3 Schematic representation of hydrophobic stabilization. The protein molecule is first unfolded and then refolded in one of the three ways described above (Mozhaev and Martinek, 1984).

molecule will become more hydrophobic and, the contrary, its surface more hydrophilic; this leads to stabilization. Secondly, the protein can be refolded in the presence of substances (e.g. compounds of nonpolar and diphilic nature) which interact with it noncovalently in a multipoint fashion. The former can be entrapped into the hydrophobic nucleus of the protein in the course of refolding, while the latter can be incorporated into the protein in a manner which permits their nonpolar moiety to contact the hydrophobic areas of the protein, leaving polar or charged fragments exposed to the solvent. Thirdly, the unfolded protein can be modified with a chemical reagent and then refolded.

The above approach has been used successfully on immobilized trypsin (Mozhaev and Martinek, 1984). The enzyme was unfolded and then refolded at elevated temperatures, a condition known to enhance the stability of hydrophobic interactions (Brandts, 1964). It was found that trypsin refolded at 50°C was more stable than the starting enzyme (before unfolding) or the enzyme refolded at normal temperatures (20–35°C). Thus, it seemed that refolding the enzyme at elevated temperatures successfully induced stronger hydrophobic binding in the interior of the protein with no loss of the enzymes catalytic activity.

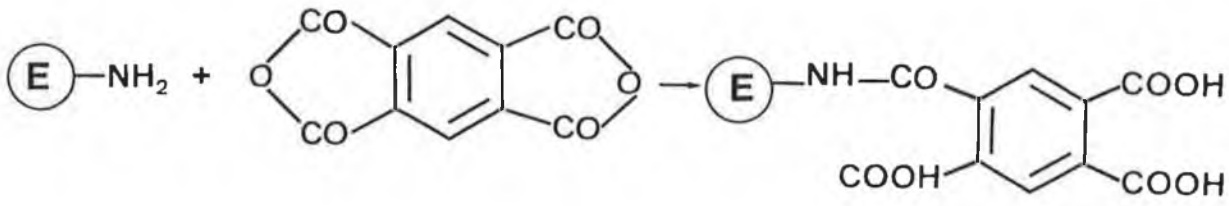
4. Hydrophilization of the protein surface groups diminishes the surface area responsible for unfavorable hydrophobic contact with water (Mozhaev and Martinek, 1984; Mozhaev et al., 1988; Siksnis

et al., 1990). This is based on the assumption (as discussed earlier) that the contact of the hydrophobic surface area of a protein with water is thermodynamically disadvantageous and, thus, destabilizes the protein. Therefore, chemical modification is used to decrease the hydrophobic character of the protein surface (Mozhaev et al., 1986; Siksnis et al., 1986). The modification of the amino groups of proteins by O-methylisourea and its analogs to arginine-like structures is an example of this where the surface hydrophobicity of proteins is decreased (Tuengler and Pfleiderer, 1977; Minotani et al., 1979; Cupo et al., 1980).

A marked stabilizing effect was observed to result from the amination of the tyrosine residues in trypsin (Mozhaev et al., 1988). Four tyrosine residues are localized on the surface of this enzyme and the introduction of an amino group into the aromatic ring of tyrosine decreases its hydrophobicity. The stability of trypsin in which four tyrosine residues have been modified increases more than 100 times (Mozhaev et al., 1986). Such a large stabilization seems to be the result of an essential hydrophilization of the nonpolar surface area of the enzyme molecule.

The stabilization via hydrophilization was very successful in the case of acylation of α-chymotrypsin by pyromellitic dianhydride (Siksnis et al., 1986; Mozhaev et al., 1988). The amino groups of the protein were mainly modified by this reagent, although the dianhydride can also react with the -OH groups of Ser

and Thr. The modification of any group in the enzyme molecule introduces three new carboxylic groups as follows.



At slightly alkaline pH, all the carboxylic groups are ionized and the protein surface is therefore hydrophilized to a higher degree which results in a dramatic stabilization of the enzyme.

1.8.7 Crosslinking with bifunctional reagents.

In the process of evolution, Nature has found that intramolecular crosslinks stabilize enzyme molecules. These crosslinks can be covalent disulphide bonds (Kauzman, 1959; Tanford et al., 1967) or the weaker salt bridges (Hess, 1971) or Ca^{2+} ions incorporated in the protein molecule (Hasegawa and Imahori, 1976). The presence of intramolecular crosslinks hinders the unfolding of the macromolecule thereby making it more stable (Nicdi and Benedeck, 1976). This led to the idea of artificial links via treatment with bifunctional reagents (Wold, 1972; Feeney et al., 1975). Bifunctional reagents can be either homo-bifunctional (where both functional groups are identical) or hetero-bifunctional (where two different functional groups are

located on the same molecule) (Ji, 1983; Wold, 1967 and 1972). A number of commonly used bifunctional reagents are shown in Table 1.8.7.

The interaction of a bifunctional reagent with an enzyme can yield three types of products (Fig.1.8.7).

(1) an intermolecular crosslinked enzyme

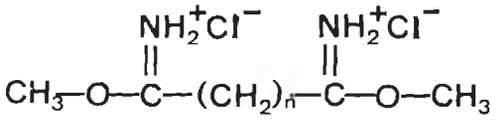
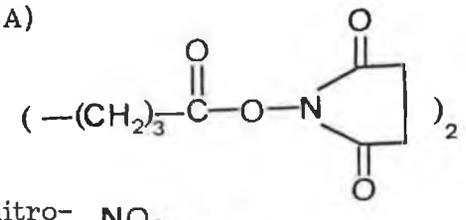
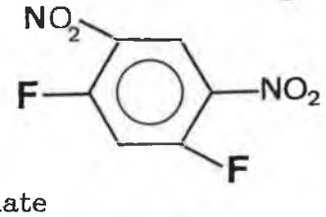
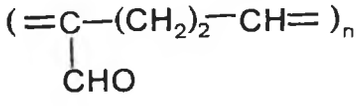
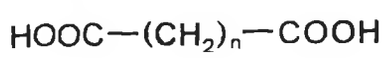
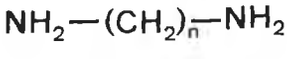
(2) an intramolecular crosslinked enzyme

(3) a one-point modified enzyme

Conditions can be selected such that intramolecular links or intermolecular ones are predominantly formed (Torchilin et al., 1978, 1979, 1983). Sometimes, as in the case of oligomeric enzymes where dissociation leads to loss of activity, intermolecular links are to be preferred i.e. between subunits (Torchilin et al., 1983; Dziember-Gryszkiewicz et al., 1983).

In order to stabilize an enzyme by crosslinking, one has to try out a large number of bifunctional reagents to determine the optimal one for each enzyme. For example, rabbit skeletal muscle glyceraldehyde-3-phosphate dehydrogenase was studied using a range of diimidoesters (Trubetskoy and Torchilin, 1985). It was found that the enzyme was stabilized by intersubunit crosslinking, with dimethyl pimelimidate having the most stabilizing effect. The effect of a range of diacids on the enzyme has also been studied (Torchilin et al., 1983). In this instance, maximal stabilization was observed in the case of treatment with succinic acid. The enzyme, formyltetrahydrofolate synthetase from *Clostridium*

Table 1.8.7. HOMOBIFUNCTIONAL REAGENTS.

NAME	FORMULA	MAX. LINK (Å)	REACTION SPECIFICITY
Bisimidates			Amine
Dimethyl malonimidate (DMM)	n = 1	5	
" succinimidate (DMSc)	n = 2	6	
" adipimidate (DMA)	n = 4	9	
" pimilimidate (DMP)	n = 5	10	
" suberimidate (DMS)	n = 6	11	
N-hydroxysuccinimide ester of suberic acid (NHS-SA)		11	Amine
1,5-Difluoro-2,4-(dinitro-benzene) (FFDNB)			Amine
p-Phenylenediisothiocyanate			Amine
Glutaraldehyde			Nonspecific
Diacids			Amine (following activation with carbodiimide
Diamines			Carboxyl groups following activation with carbodiimide

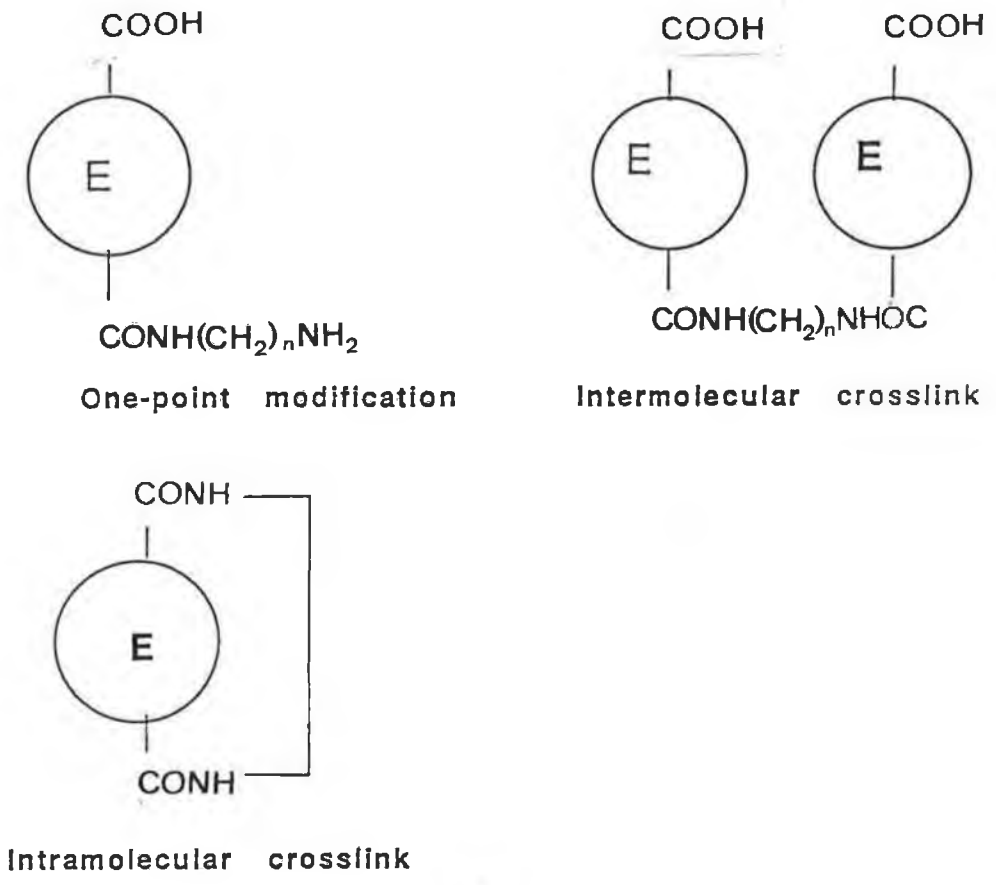


Fig. 1.8.7. Three possible ways for a bifunctional reagent to react with an enzyme. (a) a one-point modification, (b) an intermolecular crosslink and (c) an intramolecular crosslink.

cylindrosporum has been crosslinked with the diimodoester, dimethyl suberimidate (De Renobales and Welch, 1980). Intersubunit crosslinking was observed, and while the thermal stability of this enzyme was not studied, it was found that the crosslinked enzyme remained active in the absence of the monovalent cations normally required for enzymic activity.

A large number of studies, to date, have been devoted to the possibility of stabilizing enzymes with glutaraldehyde. Often, the stabilization of enzymes by treatment with glutaraldehyde appears as a result of inter-subunit crosslinking (Gottschalk and Jaenicke, 1987). Lactate dehydrogenase (Foster and Thomson,1973) and glucose oxidase (Solomon et al., 1977) have been stabilized in this way. It has been difficult, however, to obtain data confirming that intramolecular crosslinking has occurred in enzymes treated with glutaraldehyde. In many cases it has been suggested that a one-point modification and not intramolecular crosslinks are responsible for the observed stabilization of some enzymes (Torchilin and Martinek, 1979). Generally speaking, the absence of a comparison of the effects produced by treatment with a bifunctional reagent with the effects arising from treatment with a monofunctional analogue, hinders the distinction between modification and intramolecular crosslinking. Thus, the wide use of glutaraldehyde to obtain stabilized enzymes by intramolecular crosslinking gives rise to well founded doubts.

A number of reports have described attempts at forming

intramolecular crosslinks with other bifunctional compounds. For example, when modified chymotrypsin was treated with different dithiols, it was found that 1,5-pentamethylene dithiol was able to form intramolecular crosslinks causing a substantial increase in the stabilization of the enzyme (Torchilin et al., 1979).

The effect of intramolecular crosslinkages of different length on the thermostability of α-chymotrypsin has also been studied (Torchilin et al., 1978). The enzyme was first activated by carbodiimide, then the activated protein was treated by diamines of $NH_2(CH_2)_nNH_2$ type with n ranging from 0 to 12. Tetramethylenediamine was found to have the most stabilizing effect, significantly decreasing (by 3-fold) the rate constant of thermoinactivation of the enzyme.

Thus, the use of a series of bifunctional reagents having the same functional group gives an opportunity for a guided choice of a reagent capable of forming crosslinks in a given enzyme and leading to an increase of the enzymes thermostability. At the present time, the limiting factor governing the use of bifunctional reagents, is their commercial availability. When this situation improves, one is sure to see an increase in the amount of data reporting the successful stabilization of enzymes through crosslinking with these and other reagents.

AIMS AND OBJECTIVES

The aim of the present work was to investigate the possibility of stabilizing the clinically important enzyme, creatine kinase, by chemical modification. Having achieved an improved thermal stability, the effects of modification on the enzyme would then be examined.

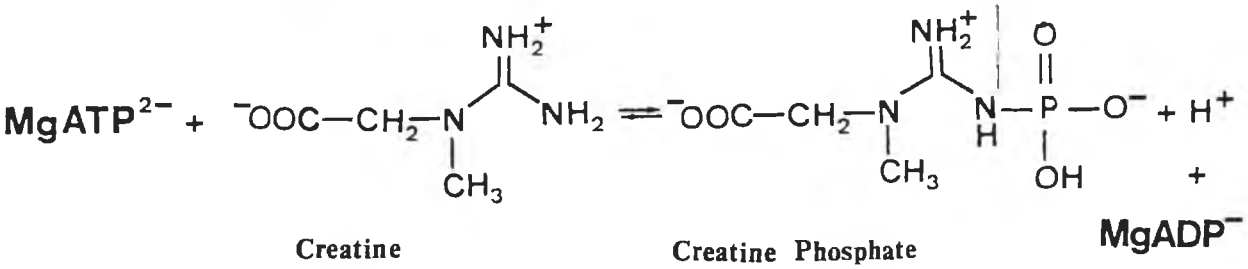
An analysis of the processes causing thermal inactivation of the native enzyme would also be undertaken, as the determination of these processes could provide a basis for future strategies for enhancing the thermostability of the enzyme.

It was hoped that the technology used to stabilize creatine kinase, might also have a more widespread application, and perhaps be used to stabilize two other enzymes, lactate dehydrogenase and aspartate aminotransferase.

CHEMICAL MODIFICATION OF CREATINE KINASE

INTRODUCTION

The enzyme, creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) catalyses the following reaction:



Creatine Creatine Phosphate

with the reaction proceeding from left to right being arbitrarily designated the forward direction. The enzyme has a wide tissue distribution and can generally be associated with the physiological role of ATP regeneration in conjunction with contractile or transport systems. It may represent as much as 10-20% (w/v) of the soluble sarcoplasmic proteins of muscle (Gosselin-Rey and Gerday, 1970).

The enzyme exists as a dimer with a molecular weight of approximately 82,000 daltons (Olson and Kuby, 1964). It occurs as three forms, the (MM) muscle type, the (MB) hybrid, and the (BB) brain type isoenzyme. Chemical investigation has revealed that there are no disulfide bridges between the subunits and that there are two catalytic sites on the enzyme with one cysteine residue at each. Also, there appeared to be two lysine and two histidine groups/molecule which are essential for catalytic activity (Watts, 1973).

Creatine kinase is widely used as a clinical diagnostic marker of myocardial infarction (Konttinen and Somer, 1973). The

following chapter reports the results obtained when this clinically important enzyme was subjected to chemical modification and crosslinking, with a view to increasing the thermal stability of the enzyme.

2.1 MATERIALS:

Bovine heart creatine kinase was purchased from Sigma Chemical Co. Dorset, U.K. Acrylamide, N,N - methylene bisacrylamide, ammonium persulphate and sodium dodecylsulphate were from BDH chemicals, Poole, U.K. DEAE-cellulose was obtained from Whatman Ltd., Kent, U.K. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All crosslinking and chemical modification reagents were purchased from Sigma as were all the reagents and enzymes used in the assay of creatine kinase. All other reagents were Analytical grade reagents. The Fortran programme, Degtest, was kindly supplied by the National Institute for Biological Standards and Control, Potters Bar, Herts, ENG 30G, U.K. The EnzFitter software package of R.J. Leatherbarrow was obtained from Biosoft/Elsevier, Cambridge, CB2 ILA, U.K. The gel densitometer GS300 and computer were obtained from Hoefer Scientific Instruments.

1.2 METHODS:

All pH measurements were performed using a Beckman Zeromatic SS-3 pH meter. Spectrophotometric measurements were carried out using a Pye Unicam SP8-400 Double Beam UV-VIS Spectrophotometer. Electrophoresis was performed using the LKB 2001 Vertical Electrophoresis unit.

2.3 Purification of Creatine Kinase from Bovine Heart.

This was performed as described by Dawson and Eppenburger (1970).

Creatine kinase was dissolved in 0.02M Tris-HCl, pH 8.0 and applied to a DEAE-cellulose column (2.5cm x 10cm) which had been previously equilibrated in the same buffer. A linear concentration gradient, total volume of 400ml, of 0.02M Tris-HCl, pH 8.0, to 0.1M Tris-HCL, pH 8.0, was used to elute the enzyme. The fractions containing enzymatic activity were combined and the enzyme lyophilised.

The purity of the enzyme was established by SDS-polyacrylamide gel electrophoresis which was performed as described in Section 2.4.

2.4 SDS-polyacrylamide slab gel electrophoresis:

Polyacrylamide slab gel electrophoresis, in the presence of SDS, was performed using the discontinous system described by Laemmli (1970). Details for the preparation of gels (7, 10 and 15%) and the stacking gel (3%) are given in Table 2.4. The electrode buffer, pH 8.3, contained Tris (0.025M), glycine (0.192M) and SDS (0.1%, w/v). Samples for electrophoresis were solubilised by heating at 100°C for 3min in 5% (v/v) 2-mercaptoethanol; 2% (w/v) SDS; 0.08M Tris-HCl, pH 6.8; 10% (v/v) glycerol; 0.2% (w/v) bromophenol blue(4μl), and the samples applied to the gel.

The slab gel system described by Laemmli, includes a resolving gel (7, 10 or 15% acrylamide) and a stacking gel (3% acrylamide). The resolving gel is poured to a height of 12cm, overlayed with distilled water and left to polymerize before the stacking gel is prepared. Electrophoresis was performed at a constant current of 100mA per gel.

The gels were stained for 1h with a 0.5% (w/v) solution of Coomassie brilliant blue in acetic acid : water : methanol (1:10:8, v/v/v). The same solution was used for destaining.

Table 2.4. Details of preparation of polyacrylamide slab gels utilising the discontinous system of Laemmli (1970) as described in Section 2.4.

Stock solution	7% gel	10% gel	15% gel	3% gel
[1]Acrylamide/Bisacrylamide (ml)	4.9	7.0	10.15	1.25
Tris-HCl (1.87M, pH 8.8) (ml)	4.2	4.2	4.2	---
Tris-HCl (0.5M, pH 6.8) (ml)	---	---	---	0.602
TEMED (ml)	0.02	0.02	0.02	0.05
SDS (10% w/v) (ml)	0.12	0.12	0.12	0.05
Ammonium persulphate (10%, w/v), (ml)	0.1	0.1	0.1	0.04
Water (ml)	11.5	9.4	6.2	3.75

[1]Acrylamide (30%,w/v), methylene bisacrylamide (0.8%,w/v) in water.

2.5.1 Assay of Creatine Kinase.

Creatine kinase activity was determined in the reverse direction using the method recommended by the Deutsche Gesellschaft fur Klinishe Chemie (Anon, 1977). The assay is based on standard methods that couple creatine kinase with the reactions catalysed by hexokinase and glucose-6-phosphate dehydrogenase and follows the increase in absorbance at 340nm due to the formation of NADPH as follows.

$$\text{creatine phosphate} + \text{ADP} \xrightleftharpoons{CK} \text{creatine} + \text{ATP}$$

$$\text{glucose} + \text{ATP} \xrightleftharpoons{HK} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NADP}^{+} \xrightleftharpoons{G6P\text{-}DH} \text{gluconate-6-P} + \text{NADPH} + \text{H}^{+}$$

The following reagents were required for the assay.

Reagent A: 0.1M Imidazole, 20mM glucose, 10mM mg-acetate, 2mM EDTA, 2mM ADP, 5mM AMP, 10μM AP_5A, 2mM NADP and 30mM creatine phosphate, pH 6.7, (2.38ml).

Reagent B: 20mM N-acetylcysteine, (100μl).

Reagent C: Hexokinase $\geq$ 2.5 U/ml and G6P-DH $\geq$ 1.5 U/ml, (20μl).

Finally, 100μl of creatine kinase sample was pipetted into the cuvette. The contents were mixed and incubated for 2min at 30°C. The mean absorbance change per minute (ΔA/min) at 340nm was then determined and used to calculate creatine kinase activity.

Calculation of activity:

Units/Litre ($30^{\circ}C$) = 4,127 x A_{340nm} /min.

One unit of creatine kinase activity is defined as that amount of enzyme which catalyses the production of 1μmole of NADPH per minute under the above conditions.

2.5.2 Protein measurement

The protein concentration for pure enzyme was determined by measuring the absorbance at 280nm and then using the relationship $A^{1\%}$ = 8.96 (Noda et al., 1960) and a molecular weight of 82,000 daltons for creatine kinase.

2.5.3 Total Activity of Creatine Kinase:

The total activity was defined as the number of units in 1ml of enzyme solution multiplied by the total volume of that solution.

2.5.4 Specific Activity of Creatine Kinase:

The specific activity was defined as the number of units of activity per mg of pure enzyme.

2.6 Thermodeactivation of Creatine Kinase:

Solutions of native or modified enzyme at a concentration of 100-150μg/ml in 0.01M imidazole, pH 6.7, were incubated at 55°C. Samples were taken at appropriate time intervals and catalytic activity measurements were carried out after preliminary cooling on ice. Activity measurements were performed in duplicate as described in Section 2.5.

2.7 Accelerated Degradation Tests:

Accelerated storage studies were performed as described by Kirkwood (1984).

Samples for accelerated storage studies were first sterilised by filtration through a low protein binding 0.22μm membrane filter. Samples were then aliquotted into sterile Sarstedt tubes and stored at a particular temperature. The temperatures used for the accelerated storage studies were 45°C, 37°C and 26°C. Reference samples were also stored at either 4°C or -20°C. At least ten samples were stored at each temperature. At appropriate time intervals, samples were taken, assayed in duplicate and the percentage residual catalytic activity determined. The results were then analysed by the Degtest programme which employs the Arrhenius equation to predict the long term stability of the

enzyme.

2.8 Crosslinking with Diacids:

This was performed as described by Torchilin et al., (1983). Two portions of solid carbodiimide (2x4mg) were added at 45min intervals up to a final concentration of $2x10^{-3}$M to a $5x10^{-4}$M solution of dicarboxylic acid at pH 4.5. The total volume of the reaction mixture was 4ml and the mixture was incubated at room temperature for 1.5hr. The pH was then increased to 8.2 by the addition of NaOH. A 1ml aliquot of the solution was taken and enzyme solution added to this to give a final protein concentration of 0.25mg/ml. The reaction was carried out in the presence of substrates (2mM ADP and 20mM CP). The mixture was incubated for an additional 1.5hr and the reaction stopped by dialysis against distilled water (2x1l)for 24hr.

2.9 Crosslinking with Diamines:

This was carried out as described by Torchilin et al., (1978). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (6mg) was added to 8ml of a 0.25mg/ml enzyme solution and incubated in a bufferless medium at pH 4.5 for 1hr at room temperature. Aliquots (1ml) of the solution obtained after treatment of the enzyme with carbodiimide were supplemented with 1ml of 0.02M phosphate buffer,

pH 8.2, distilled water (0.5ml) and amine solutions (0.5ml) of the following concentrations: 10% (v/v) hydrazine, 10% (v/v)ethylamine, 0.01M hexamethylenediamine, 0.1M pentamethylene, 0.1M diaminopropane, 0.1M ethylenediamine and 0.1M tetramethylenediamine. The pH was adjusted to 8.2 and the mixture incubated at room temperature for 1hr. The reaction was again carried out in the presence of substrates. The reaction was stopped by dialysis against distilled water (2x1l for 24hr).

2.10 Reductive Alkylation with Glyoxylic Acid:

Reductive alkylation was performed as described by Melik-Nubarov et al., (1987).

Glyoxylic acid (10mg) and sodium cyanoborohydride (7mg) were added to 1.2ml of a 1.6mg/ml solution of enzyme. The reaction was performed in 0.1M KH_2PO_4/0.5M H_3BO_3buffer, pH 8.4, for 30min. the solution was then fractionated by passage through a Sephadex G-25 column (1.5 x 7cm) to remove any low molecular weight material using 0.1M imidazole, pH 6.7, as eluting buffer.

2.11 Guanidination with O-methylisourea:

Guanidination was performed as described by Minotani et al., (1979) and Cupo et al., (1980).

Enzyme (1mg/ml) was dissolved in 1ml of 0.1M phosphate buffer,

pH 10. O-methylisourea (17.2mg) was added to the solution and the reaction was allowed to proceed at 4°C for 24hr. The reaction was stopped by dialysis against distilled water or by fractionation through a Sephadex G-25 column using distilled water as eluting buffer.

2.12 Crosslinking with Glutaraldehyde:

This was carried out as described by Klemes and Citri (1979). Enzyme in 0.05M sodium phosphate buffer, pH 7.8, was stirred with glutaraldehyde at room temperature. The reaction was terminated after 18hr by the addition of sodium borohydride to a final concentration of 0.05M and incubated for an additional 20min at 4°C. In this work glutaraldehyde was added to a 0.35mg/ml solution of creatine kinase to give a 0.002%, a 0.01% and a 0.1% (v/v) solution of glutaraldehyde. The enzyme derivative was separated from low molecular weight material by dialysis against distilled water (2x1l) for 24hr or by fractionation through a Sephadex G-25 column as above.

2.13 Attachment of Activated Polyethylene Glycol to Creatine Kinase:

This was performed as described by Abuchowski et al., (1977). Creatine kinase (1.7 mg/ml; 21μM) and activated PEG-2000 (8mg;

3.9mM) were incubated in 0.1M sodium tetraborate, pH 9.2, at 37°C for 1hr. After this time, unattached PEG was removed by passage through a Sephadex G-25 column using 0.1M imidazole, pH 6.7, as eluting buffer. The attachment of PEG to creatine kinase was performed in the presence and absence of substrates (2mM ADP and 20mM CP).

2.14.1 Crosslinking with Bis-imidates:

Crosslinking was carried out according to the methods described by De Renobales and Welch (1980) and Minotani et al., (1979).

To 1mg/ml of creatine kinase in 0.1M phosphate buffer, pH 8.0, was added 5mg of bis-imidate (i.e. DMS, DMA and DMP). The reaction was allowed to proceed for 3hr at r.t. and was then stopped by gel-filtration through a Sephadex G-25 column equilibrated in 0.1M imizadole, pH 6.7.

2.14.2 Crosslinking with dimethyl suberimidate

To 1mg/ml of creatine kinase in 0.1M phosphate buffer (pH 8.0), was added varying amounts of dimethyl suberimidate to give final concentrations of DMS ranging from 0.5 to 10mg/ml. The reaction was allowed to proceed for 3h at room temperature after which time it was stopped by passage through a G-25 column and enzymic activity determined.

2.15 Gradient Gel Electrophoresis:

This was performed according to a modification of the method described by O'Farrell (1975) for producing SDS gradient gels with a 4 to 22.5% exponential gradient.

Details of the preparation of the gels are given in Table 2.15. The electrode and solubilization buffer were the same as that described in Section 2.4. The lower gel buffer was 1.5M Tris/0.4% (w/v) SDS, pH 8.8. The stacking gel was made as described in Section 2.4.

A linear gradient was formed by using equal volumes of the light and dense gel solutions. The dense solution was poured into the mixing chamber of a gradient mixer and the light solution into the reservoir chamber. The solution was then pumped from the mixing chamber between the glass plates so that the denser solution was at the bottom of the gel. The gel was allowed to polymerise and then the stacking gel was poured. Samples were applied to the wells and electrophoresis performed at a constant current of 100mA per gel.

TABLE 2.15 Details of the preparation of light and dense gel solutions for gradient gel electrophoresis according to O'Farrell (1975).

Stock Solution	Light Gel Solution (ml)	Dense Gel Solution (ml)
Lower gel buffer	12.5	12.5
Distilled water	37.5	--
Ammonium persulphate (10%, w/v)	0.012	0.072
TEMED	0.024	0.024
[1]Dense Acrylamide solution	--	37.5

[1]Acrylamide (29.2% w/v), methylene bisacrylamide (1.6% w/v) made up in 75% glycerol.

2.16 Determination of Amino Groups:

This was performed using the method of Fields, (1971). A sample of protein was added to 0.5ml of borate buffer (0.1M $Na_2B_4O_7$ in 0.1M NaOH) and the volume made up to 1ml. Then 20μl of a 1.8M TNP-sulphonic acid solution was added and the solution rapidly mixed. After 5min the reaction was stopped by the addition of 2.0ml of 0.1M NaH_2PO_4/1.5mM sulphite and the extinction at 420nm was determined.

2.17 Construction of Temperature Denaturation Curve

The rates of thermal inactivation of the native and modified enzyme were studied by incubating them at various temperatures in 0.1M imidazole-acetate buffer (pH 6.7) for 10min at a protein concentration of 140μg/ml. Aliquots were taken at 10min, cooled to 0°C and then assayed for residual enzyme activity.

2.18 Stability towards Inactivation by Guanidine-HCl and Urea:

The stability of the native and modified enzyme towards inactivation by Gd-HCl and urea was studied by incubating samples at room temperature in either Gd-HCl (1.0M) or urea (8M) in 0.1M imidazole buffer, pH 6.7. At certain time intervals, a sample was

removed for direct assay of enzyme activity.

2.19 Determination of pH Optimum and Kinetic Parameters:

Enzyme activity was monitored over the pH range 6.0 to 8.0 under standard assay conditions using 0.1M imidazole-acetate buffer.

The kinetic parameters for the substrates (ADP and creatine phosphate) were obtained from the results of assays in which in turn, the concentration of one substrate was varied in the presence of a fixed concentration of the other. The results were analysed using the Enzfitter programme.

2.20 Crosslinking of Creatine Kinase using N-hydroxysuccinimide esters

This was carried out as follows: To 1mg/ml of CK was added 1mg of N-hydroxysuccinimide (dissolved in 10% (v/v) DMSO). The reaction was carried out in 0.1M phosphate buffer, pH 8.0, for 3hr at r.t. and stopped by gel-filtration through a Sephadex G-25 column (1.5cm x 7cm).

RESULTS AND DISCUSSION

2.20 Purification of Creatine Kinase:

The enzyme was purified as described in Section 2.3. Typical results from a purification are shown in Table 2.20 and a typical elution profile is shown in Fig.2.20.1. As can be seen, an 86% recovery of activity was obtained and 45% of the total protein applied to the column was recovered as creatine kinase. The purified protein had a specific activity of 343 units/mg.

SDS-polyacrylamide gel electrophoresis of the lyophilized protein using a 10% resolving gel was performed as described in Section 2.4. This revealed only one protein band (Fig.2.20.2) corresponding to the molecular weight of the monomer of approximately 41,000 daltons. Thus, it appeared that creatine kinase had been succesfully purified to homogeneity from a crude preparation using a single column procedure. This was of importance, since it had been hoped that a fairly rapid purification scheme for the enzyme could be established, enabling time to be spent on stabilizing the enzyme, rather than purifying it.

Table 2.20. **Purification of Bovine Heart Creatine Kinase:**

Purification Step	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)
"Crude" enzyme preparation.	10	1,386	13,867	7.7	77	180	100
After purification on DEAE 52.	121	99	11,991	0.289	35	343	86

Protein was measured by absorbance at 280nm as described in Section 2.5.2 and creatine kinase was assayed as described in Section 2.5.1.

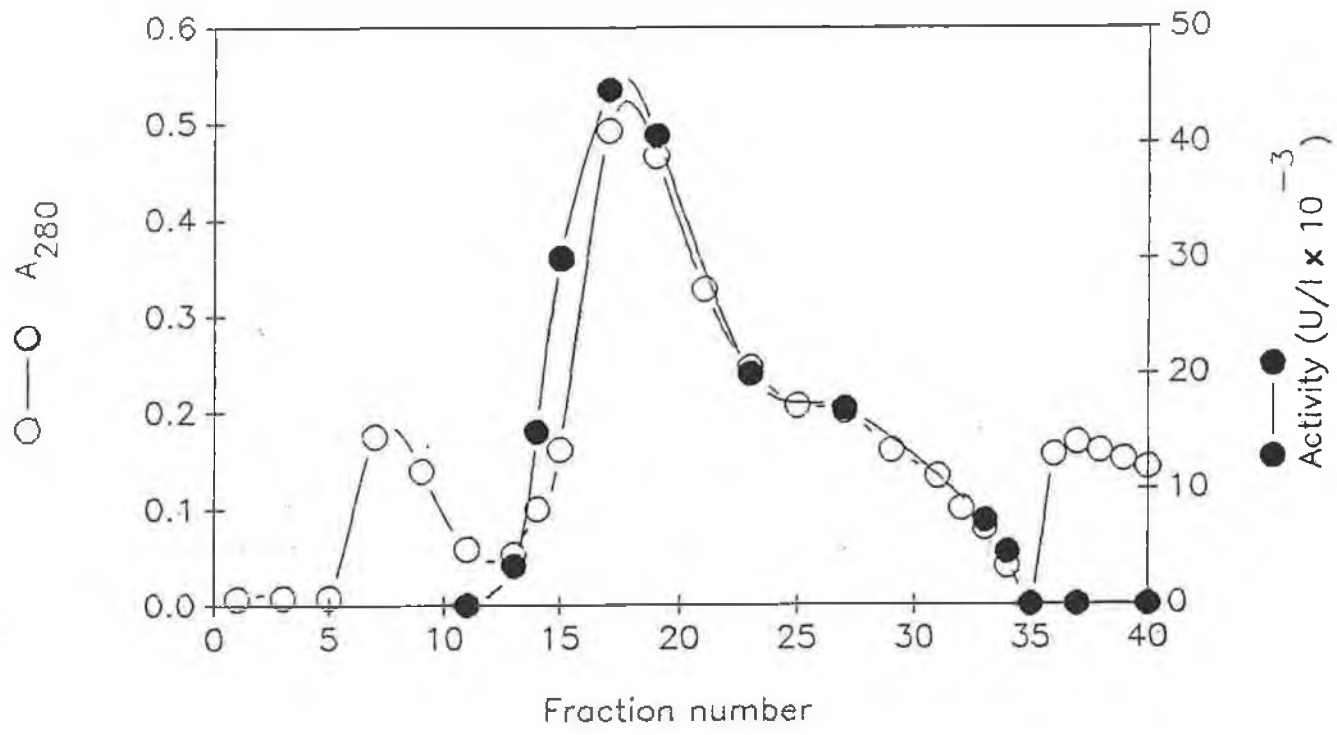


Fig. 2.20.1. Elution profile of creatine kinase purified on a DEAE-cellulose column utilizing a linear concentration gradient as described in Section 2.3. Protein (O — O) was measured as described in Section 2.5.2 and the activity (●—●) was determined as described in Section 2.5.1.

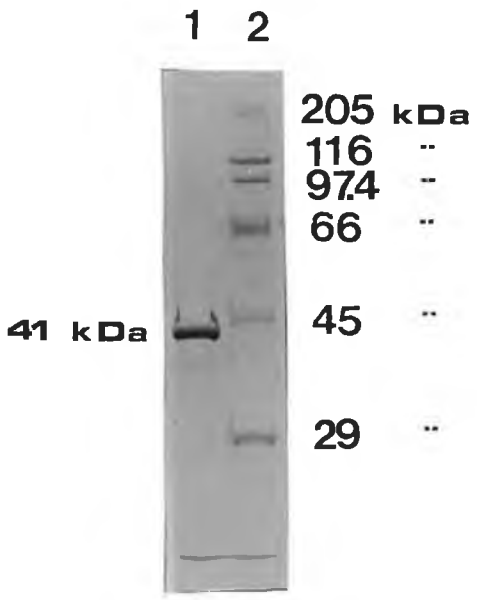


Fig. 2.20.2. SDS-PAGE of creatine kinase following purification on a DEAE-cellulose column. Creatine kinase (15μg) was analysed by SDS-PAGE according to the technique of Laemmli (1970) on a 10% gel. Lane 1 contains purified creatine kinase and lane 2 contains the molecular weight markers; myosin (205,000 Da); β-galactosidase (116,000 Da); phosphorylase B (97,400 Da); bovine albumin (66,000 Da); ovalbumin (45,000 Da); and carbonic anhydrase (29,000 Da).

2.21 Crosslinking with diacids:

This was performed as described in Section 2.8. Thermodeactivation was then carried out. The diacid-treated samples all retained the same amount of activity as the native enzyme after the reaction and all denatured at the same rate when thermodeactivation was performed. This implied that no modification of the enzyme by diacids had occurred.

The experiment was then repeated using oxalic acid, succinic acid and glutaric acid at concentrations of 0.1M and a 4-fold molar excess of carbodiimide over diacid. The results are presented in Fig.2.21. The diacids had no stabilizing effect on creatine kinase. Modification of the enzyme had occurred as the succinic, glutaric and oxalic acid treated samples lost 43%, 30% and 59% activity, respectively, after the reaction, although it was possible that other forms of denaturation may have occurred. However, no stabilization of the enzyme was observed. SDS-PAGE revealed only one band indicating that no intermolecular crosslinking had occurred.

2.22 Crosslinking with Diamines:

This was carried out as described in Section 2.9. Assay of the diamine modified samples revealed a total loss of activity and again only one band was present after SDS-PAGE. Variation of the carbodiimide and diamine concentration had no effect and gave

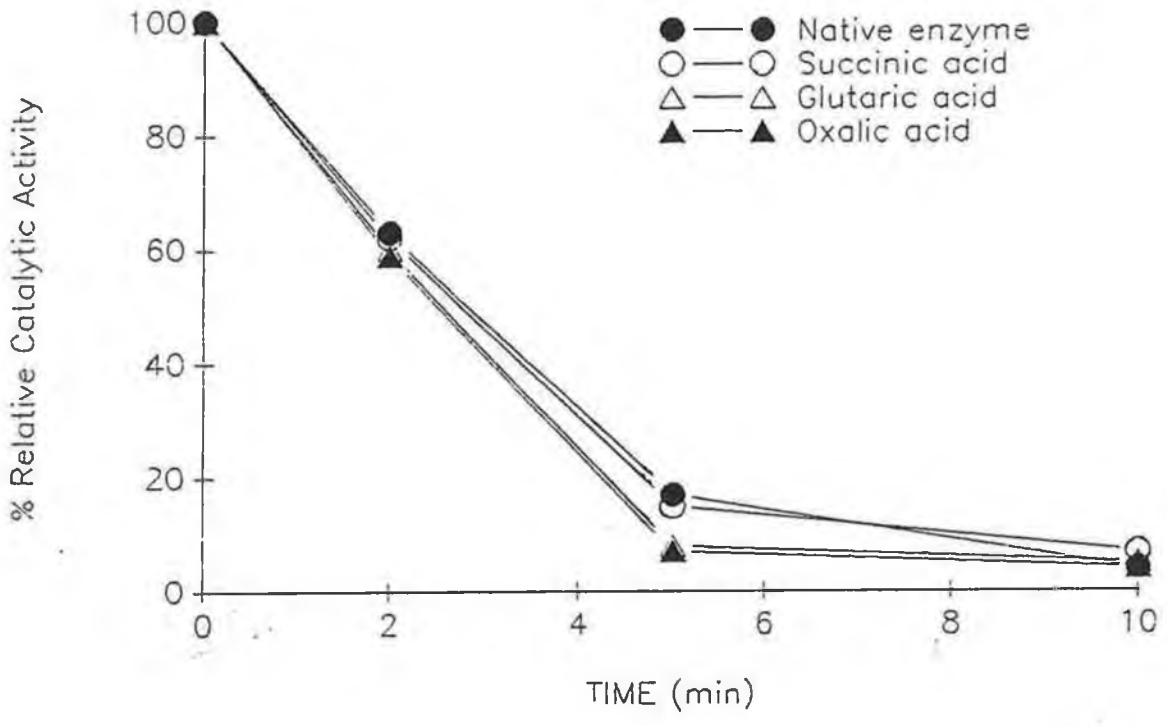


Fig. 2.21. Thermodeactivation at 55°C of native creatine kinase and creatine kinase crosslinked with succinic, glutaric and oxalic acid. Crosslinking was performed as described in Section 2.8 using a 0.1M concentration of diacid, and a 4-fold molar excess of carbodiimide over diacid. The final protein concentration in the reaction mixture was 0.25mg/ml.

similar results as above. In the reaction with diamines, the carboxy groups of the protein were first activated using carbodiimide, then the activated protein was treated with diamines. The results obtained from this experiment, however, indicate that modification of the carboxy groups will cause destabilization of the protein. The carboxyl groups are probably involved in stabilizing interactions eg. salt bridges and, therefore, crosslinking with diamines may not be suitable.

2.23 Reductive Alkylation with Glyoxlic Acid:

This was performed as described in Section 2.10. The native and modified enzyme were then assayed for creatine kinase activity. The modified enzyme had lost 40% of its original activity after the reaction. The results of thermodeactivation are presented in Fig. 2.23. As can be seen, the native enzyme was much more thermostable. The above experiment was performed in the presence of substrates and so a second experiment was performed in the absence of substrates to ascertain whether or not the substrates were protecting the active site. In this instance, reductive alkylation caused a loss of 72% of the enzymes original activity and a very dramatic loss of activity upon heating (see Fig. 2.23). These results demonstrated the need for inclusion of substrates in the above reaction and also indicated that reductive alkylation of creatine kinase in fact destabilized the enzyme at high

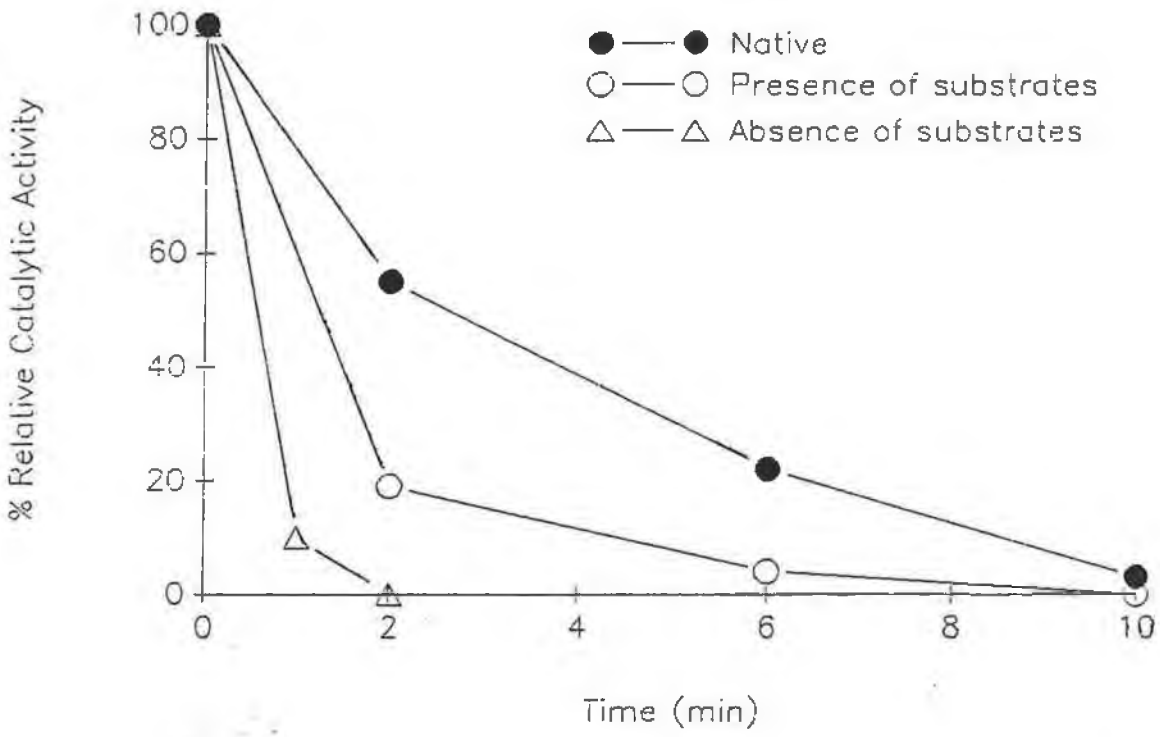


Fig. 2.23. Thermodeactivation at 55°C of native creatine kinase and creatine kinase modified by reductive alkylation with glyoxylic acid. Reductive alkylation was performed according to Melik-Nubarov et al., (1987), and is described in Section 2.10. The reaction was performed in the absence and presence of substrates (2mM ADP and 20mM CP).

temperatures. Carboxymethylation causes the enzyme to become more hydrophilic and in this case, while the substrates do protect the active site, perhaps increased repulsion of like charges on the surface of the protein and the loss of involvement of the modified lysine groups in stabilizing electrostatic interactions resulted in the destabilization of the enzyme.

2.24 Guanidination with O-methylisourea:

This was carried out as described in Section 2.11. Both the native and the modified enzyme denatured at the same rate (data not shown) indicating that guanidination had not conferred additional stability upon the enzyme. O-methylisourea reacts with ε-amino groups converting lysine to homoarginine. The positive charge of the original amino group is retained in the reaction and the importance of this is reflected perhaps by the fact that the enzyme was not destabilized.

2.25 Crosslinking with Glutaraldehyde.

This was performed as described in Section 2.12. After the reaction, no activity was detected in the sample modified with 0.1% (v/v) glutaraldehyde. The enzyme modified with 0.01% and 0.002% lost 13% and 4%, respectively, of original activity. A

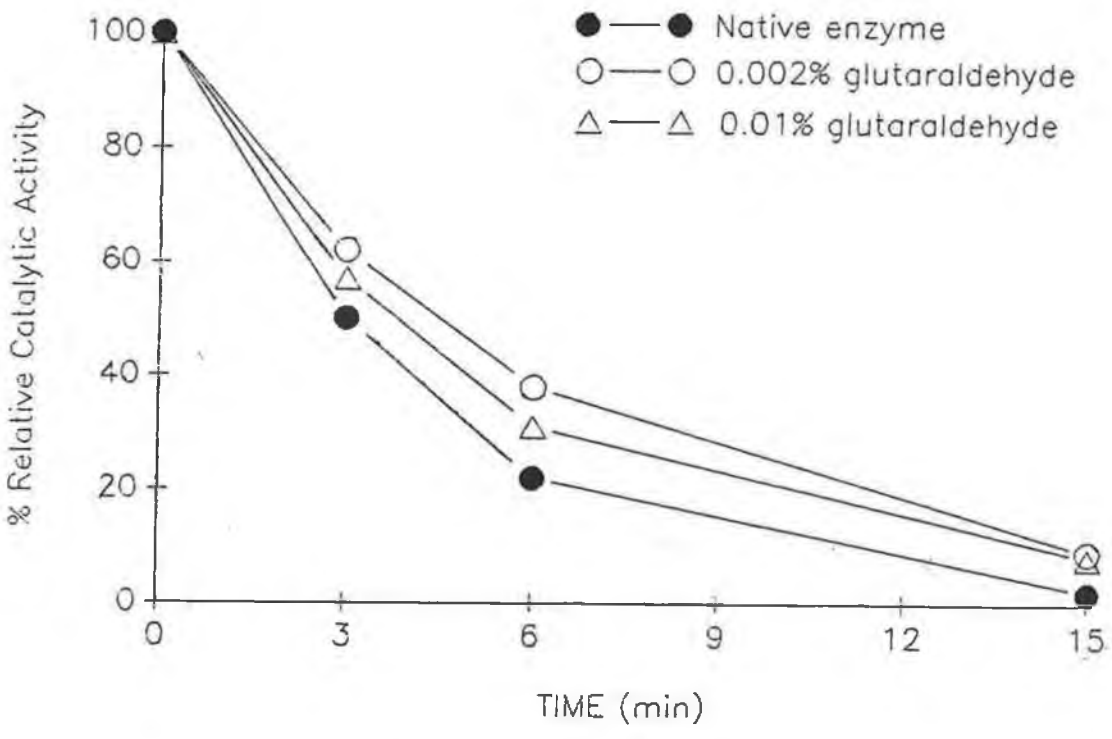


Fig. 2.25. Thermodeactivation at 55°C of native creatine kinase and creatine kinase crosslinked with varying concentrations of glutaraldehyde. The reaction was performed as described in Section 2.12 using 0.002%, 0.01% and 0.1% (v/v) solutions of glutaraldehyde and a protein concentration of 0.35mg/ml.

thermodeactivation study was performed and the results are shown in Fig.2.25. The modified enzymes were both slightly more stable than the native enzyme. It appeared from the above results that if a less concentrated solution of glutaraldehyde were used, then possibly a greater degree of stabilization might be achieved. However, when this was performed using 0.001% (v/v) glutaraldehyde solution, the modified enzyme displayed the same rate of denaturation as the native enzyme, indicating that the stabilization achieved using the 0.002% solution was probably the best that could be obtained using glutaraldehyde.

2.26 Attachment of activated PEG_2 to Creatine Kinase:

This was carried out as described in Section 2.13. The reaction scheme for the synthesis of activated PEG_2 and its subsequent reaction with proteins is shown in Fig.2.26.1. The residual chlorine atom of the activated PEG_2 can react with N-terminal and/or lysine residual amino groups on the surface of an enzyme molecule which means that two chains of PEG can be attached to each amino group through the triazine ring. The hydrophilic nature of PEG makes it possible to modify enzymes in aqueous solution, and its hydrophobic nature enables the modified enzymes to function in a hydrophobic environment. This procedure was normally performed with a view to using enzymes in organic solvents but it was also noticed that some modified enzymes were

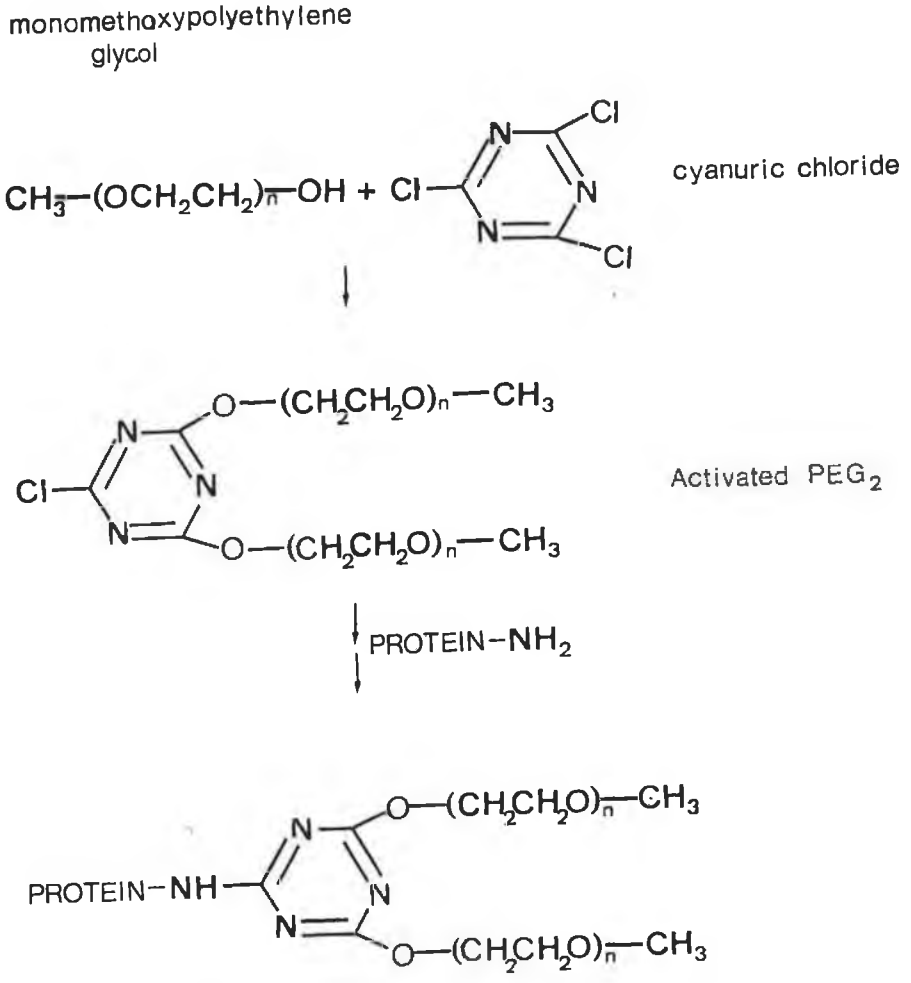


Fig. 2.26.1. Reaction scheme for the synthesis of activated PEG_2 and its subsequent reaction with proteins.

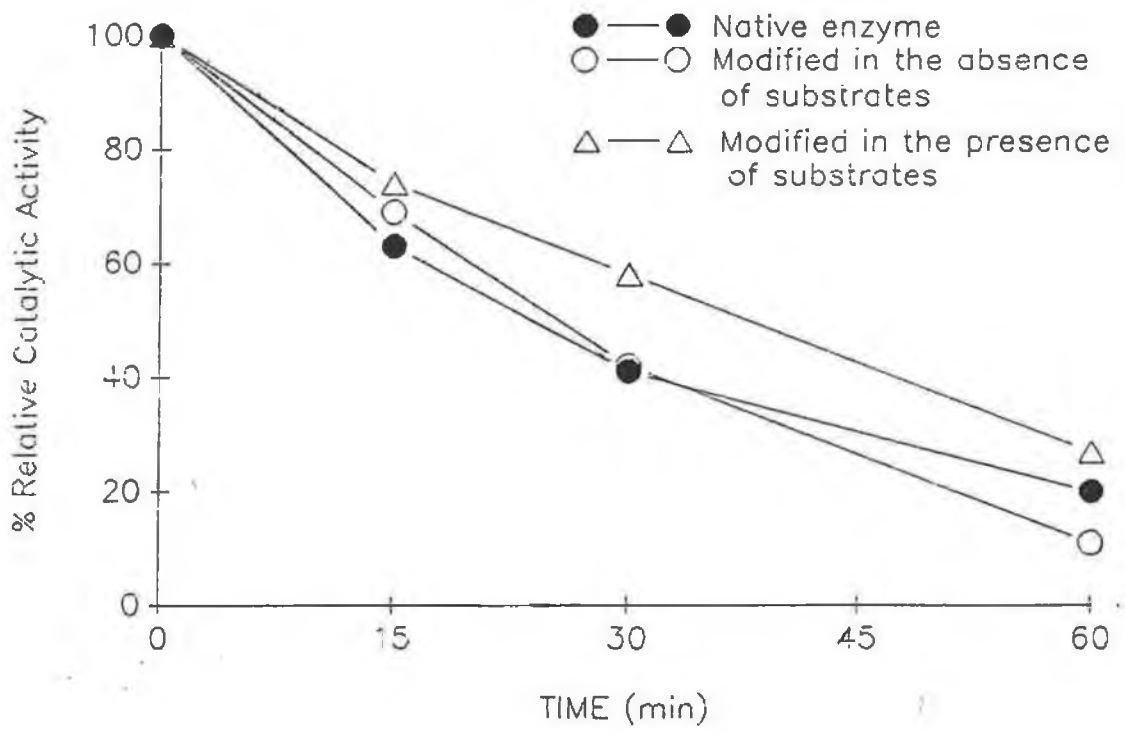


Fig. 2.26.2. Thermodeactivation at 50°C of native creatine kinase and creatine kinase modified with activated PEG_2. The reaction with activated PEG_2 was performed according to the method of Abuchowski et al., (1977), using a protein concentration of 21μM and an activated PEG_2 concentration of 3.9mM. The reaction was performed in the absence and presence of substrates (2mM ADP and 20mM CP).

considerably more stable in an aqueous solution than were the native forms (Inada et al., 1986). The results of modification of creatine kinase with activated PEG_2 are shown in Fig.2.26.2. As can be seen, the attachment of PEG to creatine kinase resulted in very little change in the denaturation rate of the enzyme in aqueous solution when the reaction was performed in the absence of substrates. However, when performed in the presence of substrates, a slight degree of stabilisation was observed, although not very significant.

2.27 Crosslinking with Bis-Imidates:

This was performed as described in Section 2.14.1. The following bis-imidates were used: dimethyl adipimidate, dimethyl pimelimidate and dimethyl suberimidate which have maximum linkage distances of 9, 10 and 11Å, respectively (see Chapter 1, Table 1.8.7). When the modified samples were assayed after the reaction, it was found that the DMS treated enzyme had lost 28% of its original activity and the DMA and DMP modified enzyme had lost 40% and 50%, respectively, of their activities.

However, the results of thermodeactivation (see Fig.2.27), revealed that the DMS and DMP-modified enzymes were significantly more stable than the native enzyme.

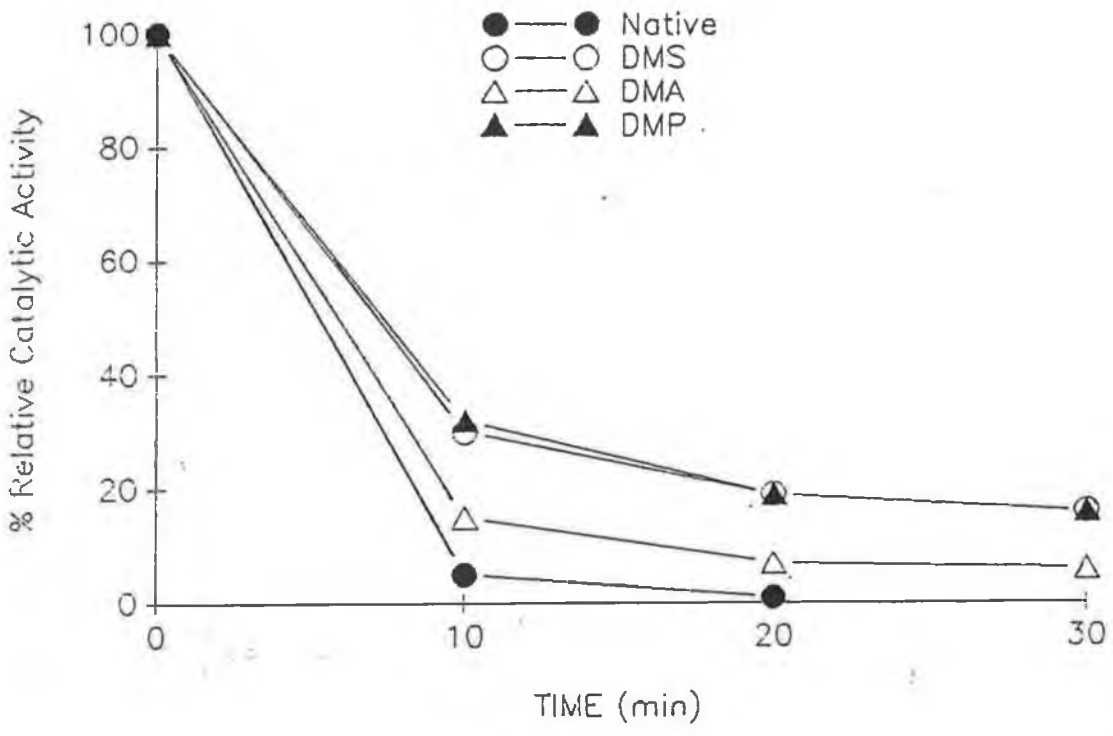


Fig. 2.27. Thermodeactivation at 55°C of native creatine kinase and creatine kinase crosslinked with bis-imidates. 5mg of bis-imidates (i.e. DMS, DMA and DMP) were added to a 1mg/ml solution of enzyme in 0.1M phosphate buffer, pH 8.0, and the reaction allowed to proceed as described in Section 2.14.1.

2.28 Accelerated Storage Studies of Creatine Kinase Crosslinked with Bis-Imidates:

Due to the positive nature of the results of thermodeactivation in the above section, it was decided to proceed with accelerated storage studies which were carried out as described in Section 2.7. The results are presented in Tables 2.28.1a to 2.28.4b. The predicted degradation rates for the native enzyme are twice as high as those predicted for creatine kinase crosslinked with DMS. However, the enzyme that was crosslinked with DMA and DMP had approximately the same predicted degradation rates as the native enzyme. Thus, it appeared as if no stabilisation of the enzyme was occurring at those temperatures when the enzyme was crosslinked with DMA and DMP. When the enzyme was modified with DMS, however, the predicted degradation rates indicated that the modified protein would be twice as stable as the native enzyme at 4°C and 20°C, and at 37°C it would be one and a half times more stable.

2.29 Crosslinking of Creatine Kinase with Dimethyl Suberimidate:

The results presented in Section 2.27 and Section 2.28 indicated that of the bis-imidates used to crosslink creatine kinase, DMS resulted in the least loss of activity after modification and also gave rise to the most stable enzyme at the

Table 2.28.1a. Accelerated Degradation Test data for native creatine kinase.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	0.46	8.00	7.27
37	0.46	48.00	54.95
37	0.70	26.00	40.20
26	3	52.00	63.00
26	8	40.00	30.06
4	14	74.00	98.34
4	21	44.00	97.52

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 2.7.

Table2.28.1b. Predicted Degradation Rates for native creatine kinase at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	0.0012	0.005	0.120
20	0.0432	0.100	4.229
37	1.3018	1.073	72.795

Degradation rates were calculated using the predicted remaining activity values given in Table 2.28.1a which were obtained by use of the Degtest programme as described by Kirkwood (1984) in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 2.28.2a. Accelerated degradation test data for creatine kinase crosslinked with dimethyl suberimidate.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	0.46	34.00	27.55
37	0.46	61.00	74.56
37	0.70	25.00	63.97
26	3	62.00	80.25
26	8	44.00	55.61
26	21	28.00	21.43
4	14	75.00	99.19
4	21	52.00	98.79

(*) Relative to samples stored at -20°C.

Creatine kinase was crosslinked as described in Section 2.14 and accelerated storage studies were performed as described in Section 2.7.

Table 2.28.2b. Predicted degradation rates for creatine kinase crosslinked with dimethyl suberimidate at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E. (K)	% LOSS PER DAY
4	5.8×10^{-4}	0.002	0.058
20	0.021	0.041	2.083
37	0.638	0.738	47.18

Degradation rates were calculated using the predicted remaining activity values given in Table 2.28.2a using the Degtest programme as described by Kirkwood (1984) in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 2.28.3a. Accelerated degradation test data for creatine kinase crosslinked with dimethyl adipimidate.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	0.46	17.00	15.12
37	0.46	42.00	56.39
37	0.70	28.00	41.82
26	3	54.00	52.08
26	5	46.00	33.00
4	14	73.00	94.09
4	21	53.00	91.27

(*) Relative to samples stored at -20°C.

Creatine kinase was crosslinked as described in Section 2.14 and accelerated storage studies were performed as described in Section 2.7.

Table 2.28.3b. Predicted degradation rates for creatine kinase crosslinked with dimethyl adipimidate at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E. (K)	% LOSS PER DAY
4	0.0044	0.019	0.437
20	0.079	0.19	7.637
37	1.25	1.16	71.219

Degradation rates were calculated using the predicted remaining activity values given in Table 2.28.3a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 2.28.4a. Accelerated degradation test data for creatine kinase crosslinked with dimethyl pimilimidate.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	0.46	20.00	16.87
37	0.46	42.00	61.36
37	0.70	28.00	47.56
26	3	72.00	61.84
26	5	60.00	44.88
4	14	74.00	96.00
4	21	61.00	95.25

Relative to samples stored at -20°C.

Creatine kinase was crosslinked as described in Section 2.14 and accelerated storage studies were performed as described in Section 2.7.

Table 2.28.4b. Predicted degradation rates for creatine kinase crosslinked with dimethyl pimilimidate at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	0.002	0.013	0.232
20	0.054	0.169	5.238
37	1.062	1.20	65.416

Degradation rates were calculated using the predicted remaining activity values given in Table 2.28.4a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

temperatures studied. Therefore, it was decided to optimise the procedure for crosslinking creatine kinase with DMS in order to maximise stabilization of the enzyme.

The crosslinking reaction was carried out as described in Section 2.14.2 in the presence of concentrations of DMS ranging from 0.5 mg/ml to 10 mg/ml. The results of thermodeactivation are shown in Fig.2.29. As can be seen, as the concentration of DMS was increased, additional thermal stability was conferred upon the modified enzyme with optimum stabilization occurring when the enzyme was crosslinked with 2 mg and 5 mg of DMS. Both of these modified enzymes had an identical rate of denaturation. At 10mg DMS, the rate began to increase. A 3h incubation period was chosen as optimum, as longer incubation periods led to no further increases in stabilization.

Further investigation showed that when using 2, 3, 4 and 5mg DMS in the crosslinking reaction, the percentage residual catalytic activity (% RCA) after 30min at 55°C was almost the same for each (Table 2.29). However, the specific activity of these samples decreased with increasing DMS concentration (Table 2.29). For this reason, subsequent crosslinking of creatine kinase was carried out using 2mg DMS / mg of creatine kinase. The inclusion of substrates in the reaction did not alter the denaturation curve or the specific activity of the modified protein indicating that the substrates were not imparting any additional protection to the active site of the enzyme.

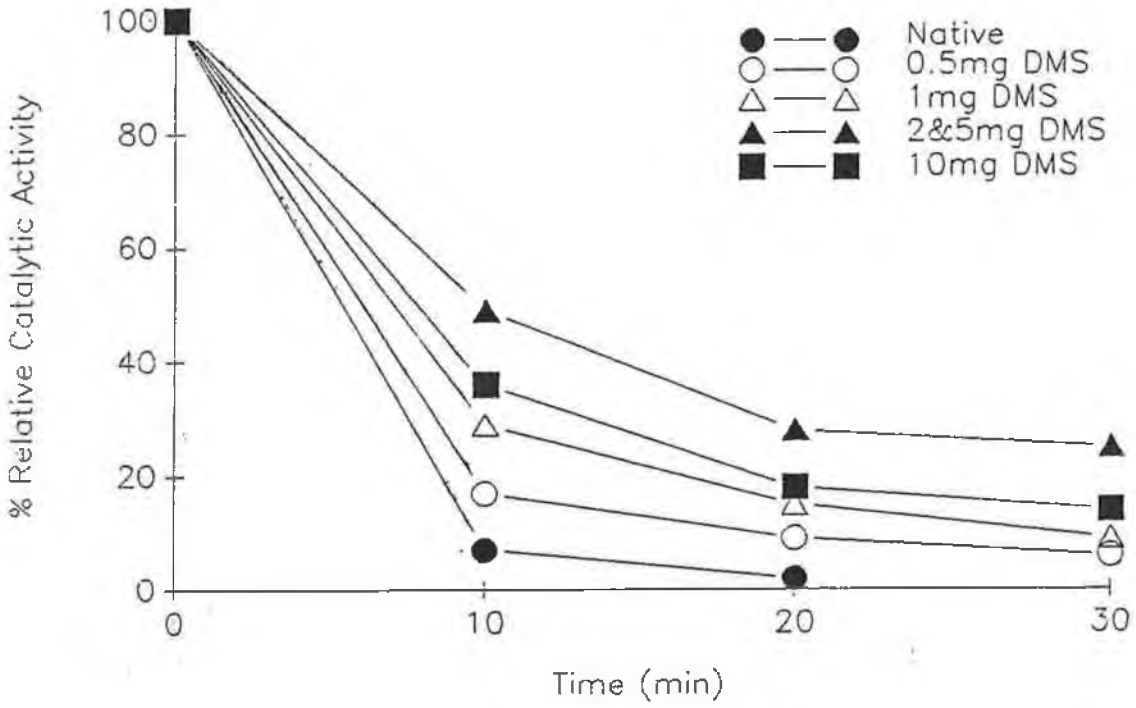


Fig. 2.29. The thermal stability observed when native enzyme and enzyme crosslinked with varying amounts of DMS were heated at 55 C. DMS was added to a 1mg/ml solution of creatine kinase in 0.1M phosphate buffer, pH 8, to give final concentrations of DMS ranging from 0.5 to 10mg/ml as described in Section 2.14.2. Thermodeactivation was performed at a protein concentration of 140μg/ml as described in Section 2.6.

Table 2.29. **Thermodeactivation at 55°C of creatine kinase crosslinked with varying amounts of DMS.**

SAMPLE	SPECIFIC ACTIVITY (units/mg)	% RCA 0 Time	% RCA 10min	% RCA 20min	% RCA 30min
Native	165	100	6	1	0
2mg DMS	121	100	33	17	10
3mg DMS	105	100	35	17	13
4mg DMS	96	100	37	22	10
5mg DMS	92	100	35	19	12

Crosslinking was performed as described in Section 2.14.2 and 2.29 and thermodeactivation was carried out as described in Section 2.6. % RCA refers to the % relative catalytic activity.

2.30 Physical Evidence for Crosslinking:

After the crosslinking reaction (Section 2.14.2), samples from reaction mixtures were subjected to gradient SDS-PAGE as described in Section 2.15. SDS-polyacrylamide gel electrophoretic profiles showed the differing degrees of intermolecular crosslinking when the DMS concentration in the reaction mixture was varied (Fig.2.30). The fastest migrating band exhibited the same mobility as the original enzyme subunit with a molecular mass of 41,000 daltons. With increasing DMS concentration, the intensity of the four minor bands increased relative to that of the monomeric subunit. The next fastest migrating band had a molecular weight corresponding to that of the dimer. These other components appear to be oligomers, probably trimers, tetramers and pentamers of the enzyme.

The unfolding of protein globules is an essential step in denaturation (Joly, 1965) and it does appear from the results shown above, that DMS rigidified the structure of creatine kinase, hindering the unfolding of the protein, and as a consequence the protein was stabilised. There seems to be a direct correlation between the degree of thermal stability and the degree of crosslinking.

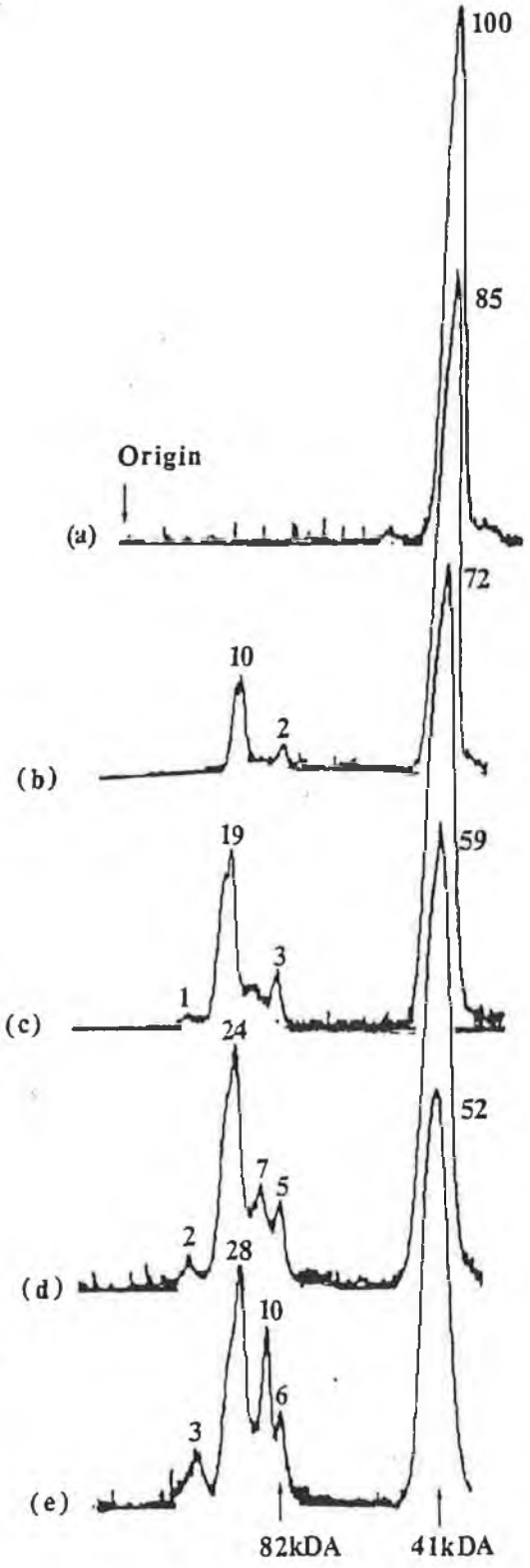


Fig. 2.30. The SDS-polyacrylamide gel electrophoretic profiles of (a) native enzyme (b) enzyme crosslinked with 0.5mg DMS (c) 1mg DMS (d) 2mg DMS and (e) 5mg DMS. The figures over the peaks represents the area of the peak relative to the total integrated area of the plot. The molecular weights are also indicated.

2.31 Amino Group Determination in Native and Modified creatine kinase:

This was carried out as described in Section 2.16. This method is used for determining amino groups of proteins and peptides with 2,4,6-trinitrobenzene sulphonic acid, in which the absorbance at 420nm of sulphite complexes of the trinitrophenylated amino groups is measured. In this work the method was used to check the extent of blocking of amino groups by DMS in creatine kinase. The results are shown in Table 2.31.1. The absorbance at 420nm of the native enzyme was taken as representing all available unblocked amino groups i.e. 100%. The absorbance at 420nm of the modified enzyme was expressed as a percentage of this to give the % blocked amino groups.

As can be seen, when using 0.5mg of DMS, 22% of the amino groups were blocked. The percentage of blocked amino groups increased with DMS concentration where up to 5 and 10mg DMS gave rise to 90 and 100%, respectively, of all available amino groups blocked on creatine kinase. Table 2.31.2 also shows the percentage of monomer found in the crosslinked samples (results taken from Fig.2.30). One can see that the % of monomer present in each of the fractions correlated reasonably well with the % of unblocked amino groups. At a concentration of 5 and 10mg of DMS, the percent monomer was 52 and the % amino groups available was 0 and 10, indicating that above 5mg DMS, no further intermolecular

Table 2.31.1. Effect of DMS on the activity of creatine kinase and determination of the % of amino groups blocked by the reagent.

SAMPLE	% LOSS OF ACTIVITY AFTER MODIFICATION	% BLOCKED AMINO GROUPS
Native	0	0
0.5mg DMS	28	22
1mg DMS	37	36
2mg DMS	50	59
5mg DMS	57	90
10mg DMS	57	100

Amino group determination was performed as described by Fields (1971) on native and crosslinked creatine kinase (see Section 2.16).

Table 2.31.2. **Determination of the percentage of unblocked amino groups and the percentage of enzyme present as monomer after crosslinking as shown by SDS-PAGE (see Fig. 2.30).**

SAMPLE	% UNBLOCKED AMINO GROUPS	% MONOMER
Native	100	100
0.5mg DMS	78	85
1mg DMS	64	72
2mg DMS	41	59
5mg DMS	10	52
10mg DMS	0	52

crosslinking of the monomer would occur, although small changes in the relative percentages of the oligomers did occur.

2.32 Stability Towards Thermal Inactivation and Accelerated Storage Studies:

A temperature denaturation curve was constructed as described in Section 2.17 and is shown in Fig.2.32. This demonstrates clearly the increased ability of the modified enzyme to withstand denaturation at higher temperatures. This was as expected since the introduction of covalent bridges between the subunits of a protein should act as "braces" and prevent both the unfolding of the tertiary structure and the dissociation of oligomers into subunits (Wold, 1972).

To investigate whether the stabilization observed at the higher temperatures would also be evident at lower temperatures, accelerated storage studies were performed as described in Section 2.7. The data from these studies were first analysed using the Enzfitter programme to ensure that the degradative reaction took place with first-order kinetics as this is a prequisite to use of the Degtest programme and then the predicted degradation rates were estimated using the Degtest programme. The results are presented in Table 2.32.1a to 2.32.2b. At 37°C, the native enzyme lost nearly 4 times more activity per day than the modified enzyme. At 20°C, the degradation rate was three times that of the

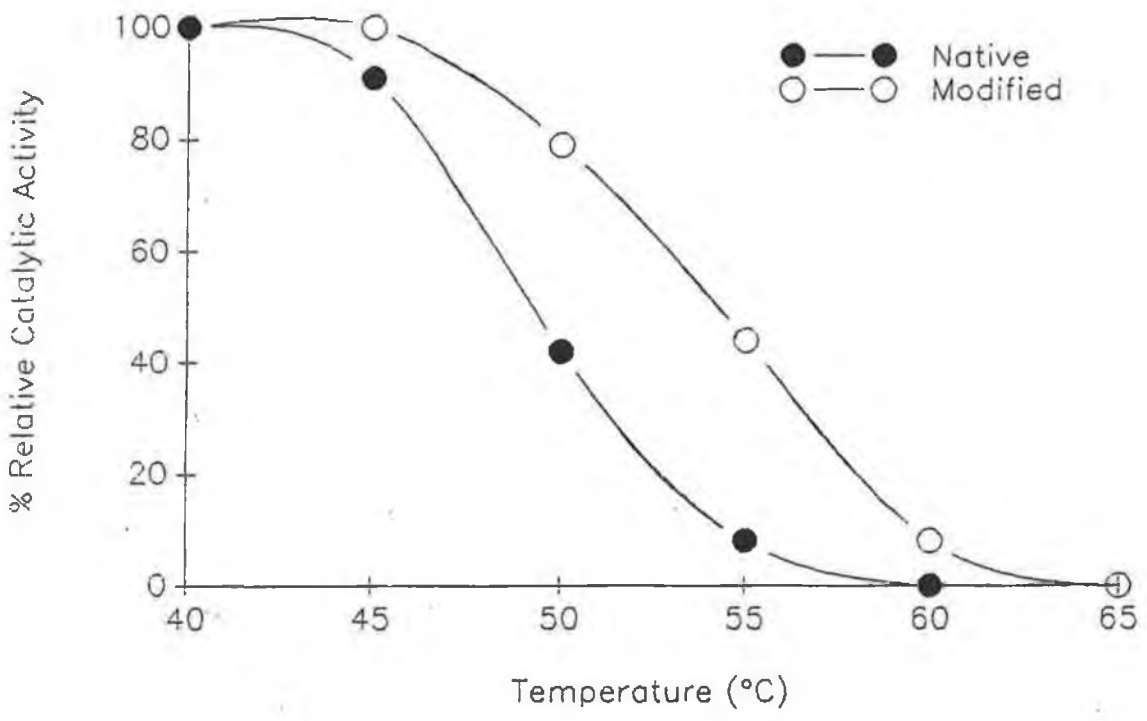


Fig. 2.32. The temperature denaturation curve for native enzyme and enzyme crosslinked with 2mg of DMS. The rates of thermal inactivation were studied by incubating the enzyme (140μg/ml in 0.1M imidazole-acetate buffer, pH 6.7), at various temperatures for 10min. After this time, aliquots were taken, cooled to 0 °C, and assayed for residual enzyme activity.

Table 2.32.1a. Accelerated degradation test data for native creatine kinase.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	0.14	73.00	67.20
45	0.31	50.00	42.51
45	1	12.00	6.45
45	1.5	1.00	1.64
45	2	0.50	0.42
37	1	69.00	32.77
37	2	30.00	10.74
37	3	19.00	3.52
37	4	1.00	1.15
26	3	63.00	40.74
26	8	23.00	9.12

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 2.7.

Table 2.32.1b. Predicted degradation rates for native creatine kinase at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E. (K)	% LOSS PER DAY
4	0.016	0.005	1.568
20	0.140	0.024	13.095
37	1.120	0.055	67.240

Degradation rates were calculated using the predicted remaining activity values from Table 2.32.1a which were obtained using the Degtest programme as described by Kirkwood (1984) in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 2.32.2a. Accelerated degradation test data for creatine kinase crosslinked with dimethyl suberimidate.

TEMP. (°C)	TIME (days)	% ACTIVITY REMAINING	% ACTIVITY REMAINING (*) PREDICTED
45	0.14	75.00	94.90
45	0.31	61.00	89.00
45	1	40.00	69.00
45	1.5	42.00	58.17
45	2	22.00	48.56
45	6	15.00	11.45
37	1	82.00	82.79
37	2	78.00	68.55
37	3	71.00	56.75
37	6	27.00	32.21
26	3	90.00	80.34
26	6	81.00	64.55
26	8	59.00	55.79

(*) Relative to samples at -20°C.

Creatine kinase was crosslinked as described in Section 2.14 and 2.29 and accelerated storage studies were performed as described in Section 2.7.

Table 2.32.2b. Predicted degradation rates for creatine kinase crosslinked with dimethyl suberimidate at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E. (K)	% LOSS PER DAY
4	0.008	0.038	0.880
20	0.042	0.104	4.169
37	0.189	0.139	17.255

Degradation rates were calculated using the predicted remaining activity values from Table 2.32.2a which were obtained using the Degtest programme as described by Kirkwood (1984) in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

modified protein and at 4°C, the native enzyme lost activity at twice the rate of the modified protein. Therefore, it would appear that stabilization of modified creatine kinase was occurring at the lower temperatures. It was evident, however, that as the temperature was lowered, the stabilization effect was reduced. This would seem to indicate some difference in the mechanism of denaturation occurring at these temperatures.

This may be explained by the fact that at the higher temperatures, the unfolding of the tertiary structure caused by thermal stress was probably the single most important event in denaturation. However, at the lower temperatures, because the enzyme was stored for longer periods of time and no reducing agent was present, other factors eg. oxidation are probably implicated in the loss of activity of the enzyme. While the DMS can prevent to a large extent the unfolding of the protein and dissociation of subunits, it will have no influence over other factors which may lead to a loss in enzyme activity at the lower temperatures.

2.33 Stability towards Inactivation by Guanidine-HCl and Urea:

This was performed as described in Section 2.18. The rates of inactivation of the native enzyme and its derivative in 8M urea and 1M Gd-HCl were compared. The modified enzyme was significantly more resistant to denaturation by 8M urea than the native enzyme (Fig.2.33). An even more marked resistance to denaturation by 1M

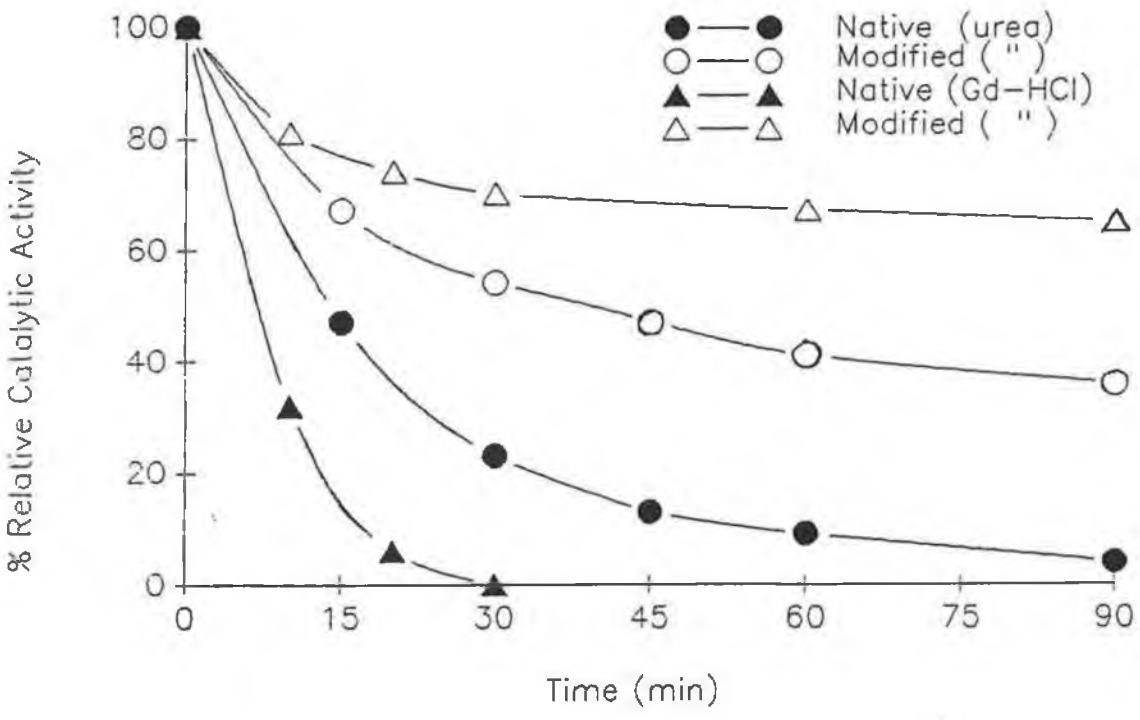


Fig. 2.33. The rates of denaturation of native and modified creatine kinase in 1M guanidine-HCl and 8M urea. The stability of the enzyme towards denaturation was studied by incubating the enzyme in either Gd-HCl or urea in 0.1M imidazole buffer, pH 6.7, at room temperature and removing them at certain time intervals for direct assay of enzyme activity.

Gd-HCl was observed with the modified enzyme (Fig.2.33). When incubated in 1M Gd-HCl, the native protein was completely denatured within 30min, whereas after a period of 21hr, the modified enzyme had retained 44% of its activity.

It is known that strong denaturants such as Gd-HCl, disrupt non-covalent interactions in proteins which lead to their unfolding (Creighton, 1983). However, the covalent bridges formed by DMS in the enzyme, should be resistant to this action, maintaining the enzyme structure when the non-covalent bonds have been broken, thus stabilising the enzyme against denaturation.

2.34 Determination of pH Optimum and Kinetic Parameters:

This was carried out as described in Section 2.19. The pH profile of the native and modified enzyme was examined to determine whether it had been altered as a result of crosslinking (Fig.2.34.1). The modified enzyme exhibited a much broader optimum profile ranging from pH 6.8 to pH 7.4, with optimum activity at pH 7.3. The optimum pH for the native enzyme was 7.1. The shift in pH optimum for the modified enzyme toward the alkaline region was pobably due to the fact that the amidines formed in the reaction between DMS and the primary amines have a higher pK_a than that of the ε-amino groups of lysine (Dawson et al., 1986).

The Michaelis constants of the native and crosslinked enzyme were determined for creatine phosphate (CP) and ADP (see

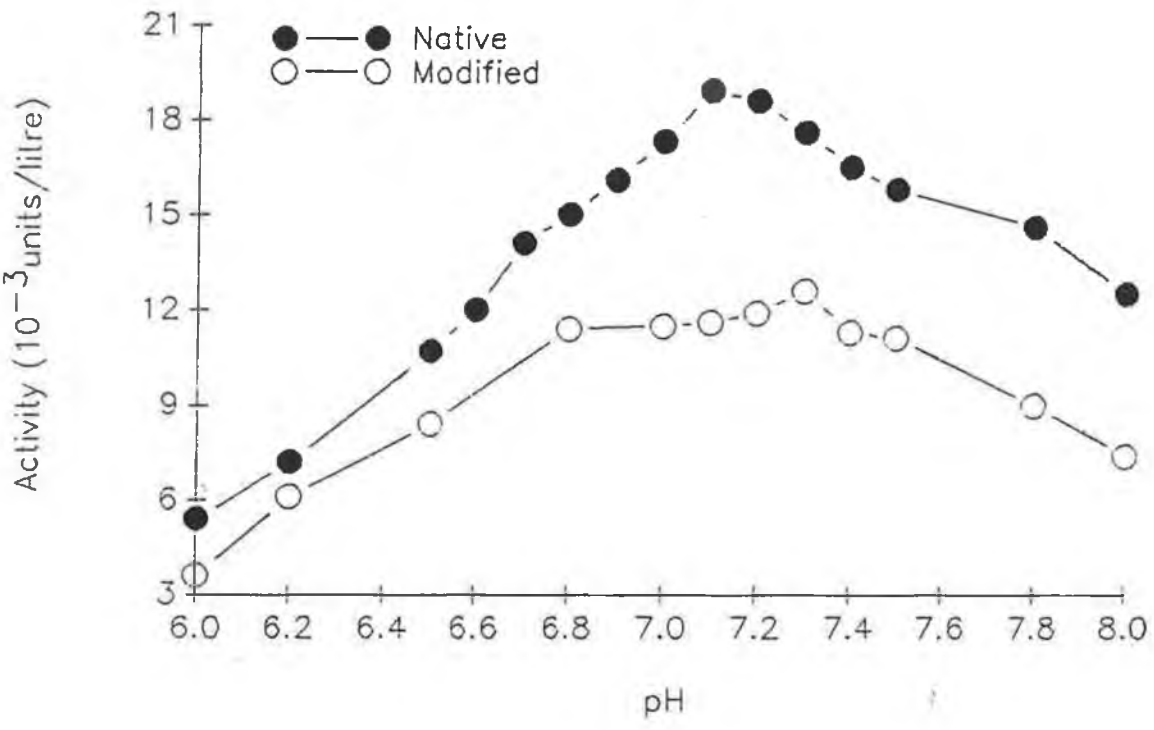


Fig. 2.34.1. The pH profiles of native and crosslinked creatine kinase. Enzyme activity was monitored over the pH range 6.0 to 8.0 under standard assay conditions using 0.1M imidazole-acetate buffer.

Fig.2.34.2 and Fig.2.34.3). The Km's obtained for the native enzyme and its crosslinked derivative for CP (in the presence of 2mM ADP) were 2.35mM and 2.47mM, respectively. For ADP in the presence of 30mM CP, the Km for the native enzyme was 0.23mM and 0.19mM for the modified enzyme. At a concentration of 1.5mM ADP, the substrate became limiting for both native and modified enzymes. The difference in the Km values obtained was small and within experimental error, indicating that the Michaelis constants of the enzyme had not been significantly altered by crosslinking with DMS.

2.35 Crosslinking of Creatine kinase with N-hydroxysuccinimide esters:

This was performed as described in Section 2.20 and the results are presented in Fig.2.35. As can be seen, crosslinking with ethylene glycol bis(succinic acid N-hydroxysuccinimide ester) and suberic acid bis(N-hydroxysuccinimide ester) resulted in a vastly decreased thermal stability in creatine kinase. In the above reaction, the positive charge of the original amino group is lost (Ji, 1983). The loss of thermal stability would therefore seem to be due to the loss of the positive charge on the amino groups. This would imply that the lysines in creatine kinase play an important role in maintaining the enzyme structure through electrostatic interactions.

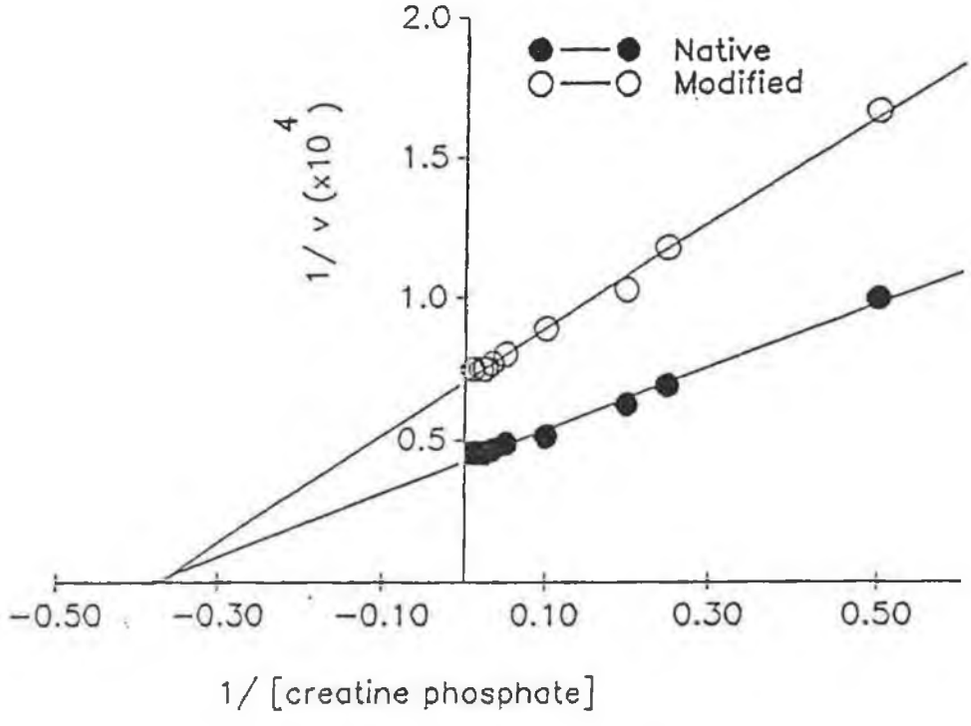


Fig. 2.34.2. Determination of K_m values for creatine phosphate in the presence of 2mM ADP, for native and crosslinked creatine kinase.

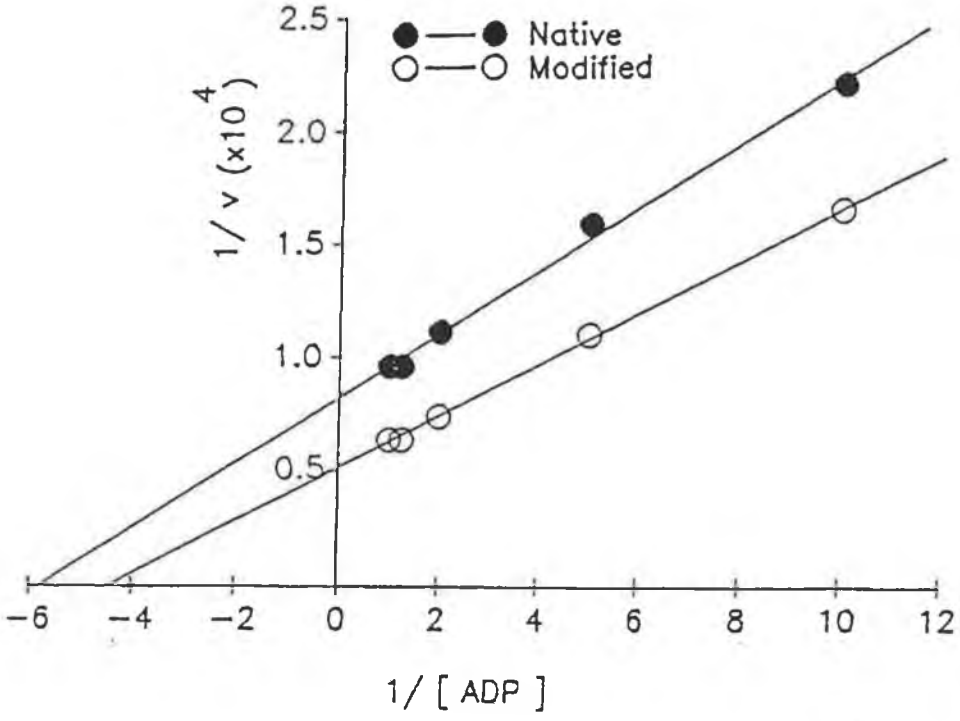


Fig. 2.34.3. Determination of K_m values for ADP in the presence of 30mM creatine phosphate, for native and crosslinked creatine kinase.

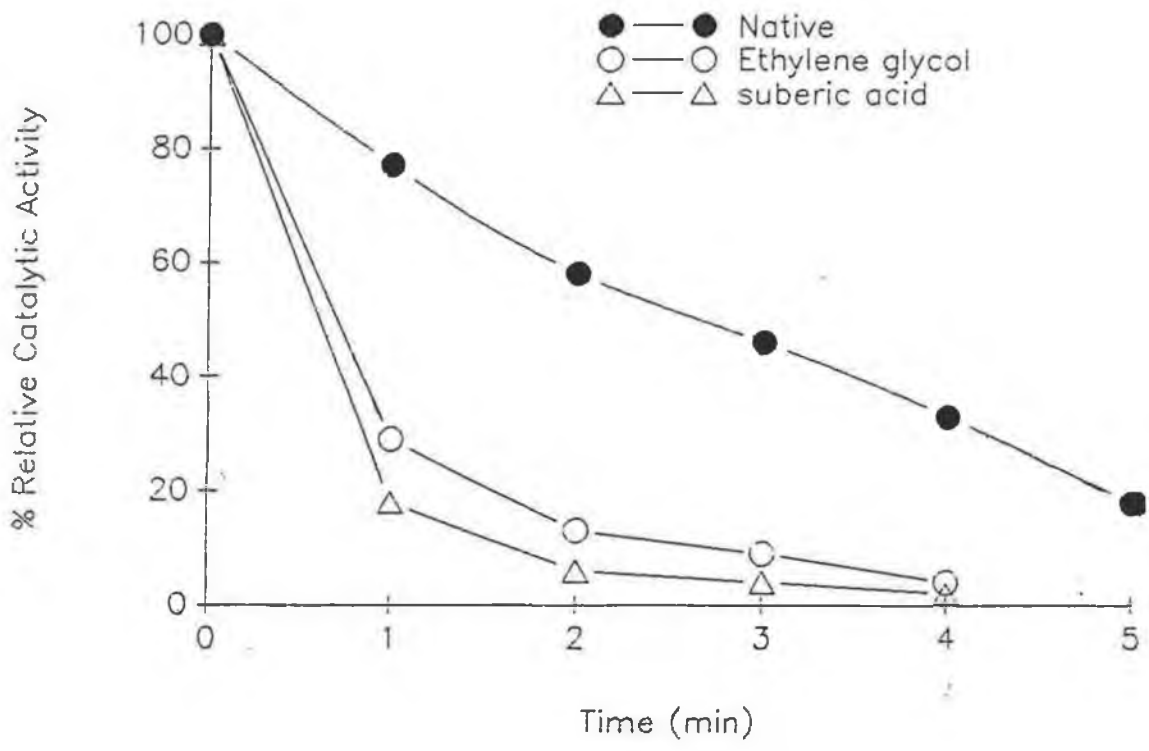


Fig. 2.35. Thermodeactivation at 55°C of native creatine kinase and creatine kinase crosslinked with N-hydroxysuccinimide esters. 1mg of N-hydroxysuccinimide (i.e. ethylene glycol and suberic acid) esters was added to a 1mg/ml solution of creatine kinase in 0.1M phosphate buffer, pH 8.0, as described in Section 2.20 and thermodeactivation performed as described in Section 2.6.

2.36 Investigation of the role of salt bridges in maintaining the structure of creatine kinase:

From the results presented in the above sections, it was obvious that the type of reagent used to modify creatine kinase was extremely important and could give valuable clues as to the identity of groups needed to maintain enzyme structure and to stabilize the protein. An example of this can be seen where the introduction of crosslinks by DMS to amino groups in the protein, resulted in the retention of the positive charge of the original amino group (Creighton, 1983) and increased thermal stability of the enzyme. However, crosslinking with N-hydrosuccinimide esters in which the positive charge of the original amino group is lost resulted in destabilization of creatine kinase. Similarly, when reductive alkylation was performed, the carboxymethylated protein became more hydrophilic and the thermal stability of the protein was again dramatically lowered (see Section 2.23).

As stated in Section 2.35, the role of lysine residues was therefore thought to play an important part in stabilizing native creatine kinase through electrostatic interactions i.e. salt bridges. To test whether salt bridges were responsible for maintaining some degree of enzyme structure in creatine kinase, the effect of concentrated inorganic salts (which are known to weaken electrostatic forces (Hill, 1956), on the thermal

inactivation of creatine kinase was examined. Thermodeactivation was performed as described in Section 2.6 and was carried out in the presence and absence of 1M KCl. The results are presented in Fig.2.36. The rates of denaturation of the enzyme in the presence and absence of 1M KCl were $1.82min^{-1}$ and $6x10^{-1}min^{-1}$, respectively. This 3-fold difference in the rates implies that salt bridges are involved as stabilizing forces in native creatine kinase.

Tomazic and Klibanov (1988b) devised an experimental strategy to distinguish between two possible distinct mechanisms as to how salt bridges stabilised B. licheniformis α-amylase. This strategy was used here to investigate the type of effect salt bridges had in creatine kinase.

According to Klibanov (1983), the simplest scheme describing irreversible thermoinactivation of enzymes involves reversible unfolding of enzyme molecules, followed by a conformational or covalent step:

$$N \longleftrightarrow U \longrightarrow I \qquad (1)$$

where N is the catalytically active enzyme;

U is the partially unfolded enzyme;

and I is the irreversibly inactivated enzyme.

According to the first possible mechanism, the salt bridges rigidify the structure, hindering the unfolding of the enzyme at

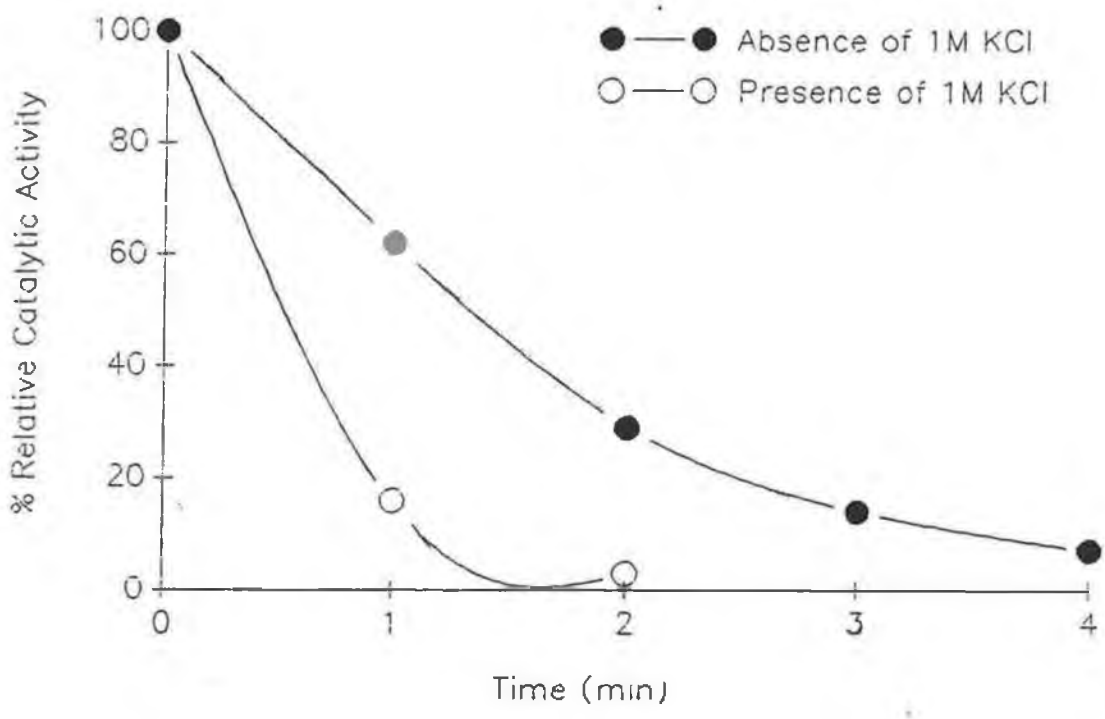


Fig. 2.36.1. Thermodeactivation at 55°C of creatine kinase in the presence and absence of 1M KCl. Thermodeactivation was performed as described in Section 2.6.

high temperatures (ie. suppressing the first step in reaction 1). According to the second mechanism, the stabilizing electrostatic interactions reduce the rate constant of formation of incorrect structures (the second step in reaction 1) via electrostatic repulsion.

If the first mechanism was correct, it was predicted that the ratio of half-lives ($T_{0.5}$) of enzyme in buffer alone to that in the presence of 1M KCl would decrease when the thermoinactivation temperature was raised. This is because an increase in temperature would disrupt some of the remaining non-covalent forces maintaining the tertiary structure and consequently lead to a more unfolded macromolecule. Hence, the potential for an additional salt-induced unfolding should be lower at a higher temperature. If the second mechanism was valid, then the ratio of half-lives, if anything, should be greater at a higher temperature.

Using the above strategy, creatine kinase was subjected to thermodeactivation in the presence and absence of 1M KCl at 50°C and 55°C (see Figs.2.36.1 and 2). Table 2.36.1 shows the rates and half-lives obtained. The ratio of half-lives did decrease when the temperature was raised, suggesting that the salt bridges rigidify the structure of native creatine kinase and hinder the unfolding of the enzyme. Therefore, the first mechanism would apply to creatine kinase.

It was of interest to see if this same mechanism would apply to creatine kinase crosslinked with DMS, and so the same experiments

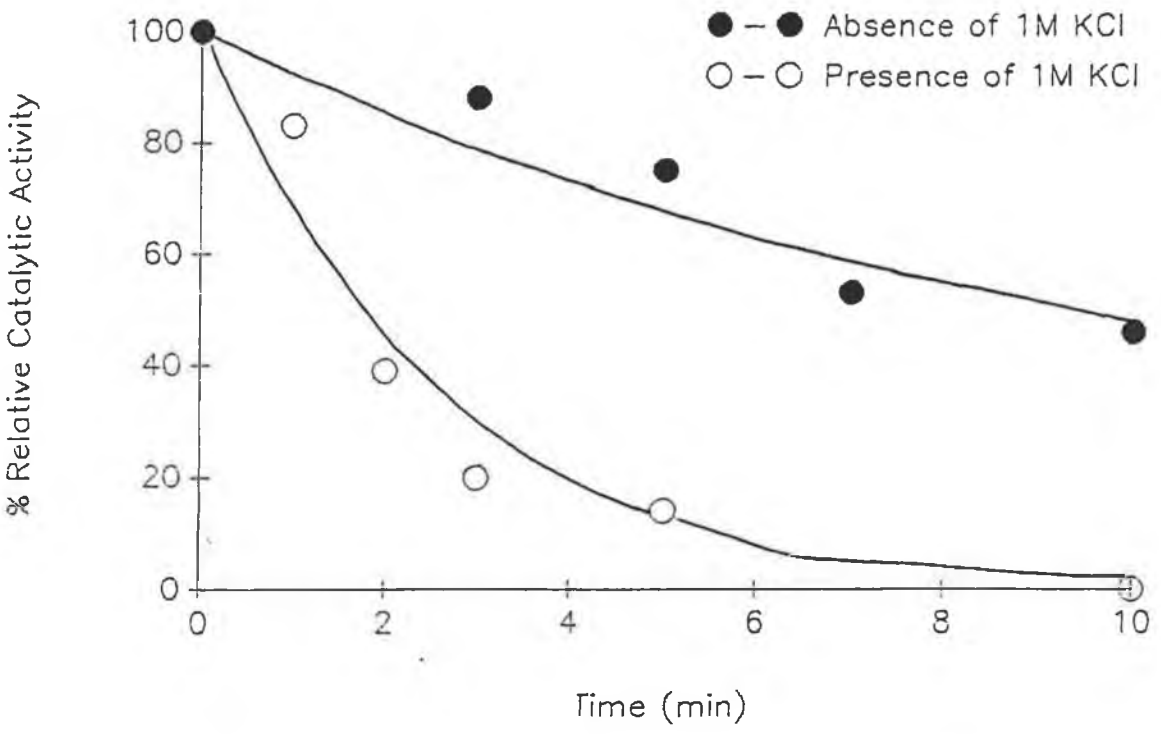


Fig. 2.36.2. Thermodeactivation at 50°C of creatine kinase in the presence and absence of 1M KCl. Thermodeactivation was performed as described in Section 2.6.

as were carried out above, were performed on modified creatine kinase. The results of thermodeactivation at 50°C and 55°C in the presence and absence of 1M KCl are shown in Figs.2.36.3 and 4. As was expected, salt bridges while still involved in maintaining the tertiary structure of the modified enzyme, showed a decreased dependence on these ionic interactions in stabilizing the enzyme structure. However, in this instance, the ratio of half-lives increased with an increase in temperature (see Table 2.36.2). Since the introduction of crosslinks is known to prevent the unfolding of the tertiary structure and the dissociation of oligomeric proteins into subunits (ie. the second step in reaction 1), the above results verify that DMS prevents the complete unfolding of CK to an irreversibly inactivated enzyme.

An understanding of the groups required to maintain enzyme structure, and also the factors which cause irreversible thermoinactivation is needed if suitable reagents are to be used to stabilise enzymes. From the above results it can be concluded that lysines are involved in electrostatic interactions that rigidify the structure of creatine kinase. Reagents such as N-hydrosuccinimide esters which remove the positive charge on the amino groups are therefore unsuitable for stabilizing the protein.

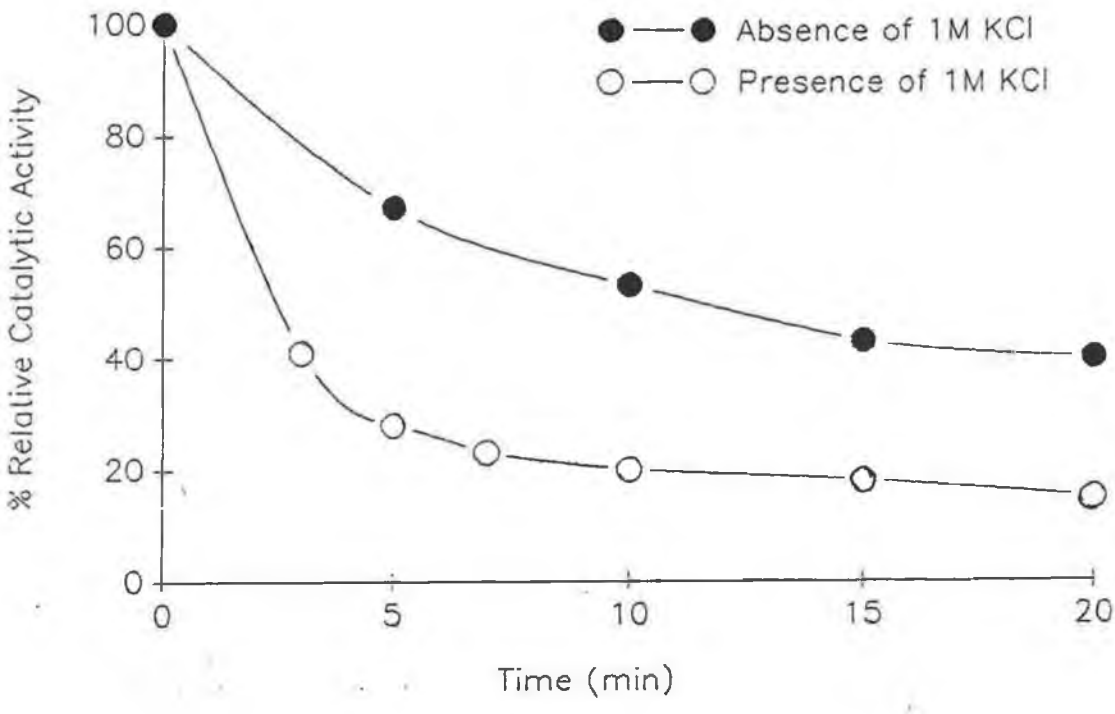


Fig. 2.36.3. Thermodeactivation at 50°C of crosslinked creatine kinase in the presence and absence of 1M KCl. Crosslinking was performed as described in Section 2.14.2. using 2mg of DMS, and thermodeactivation was performed as described in Section 2.6.

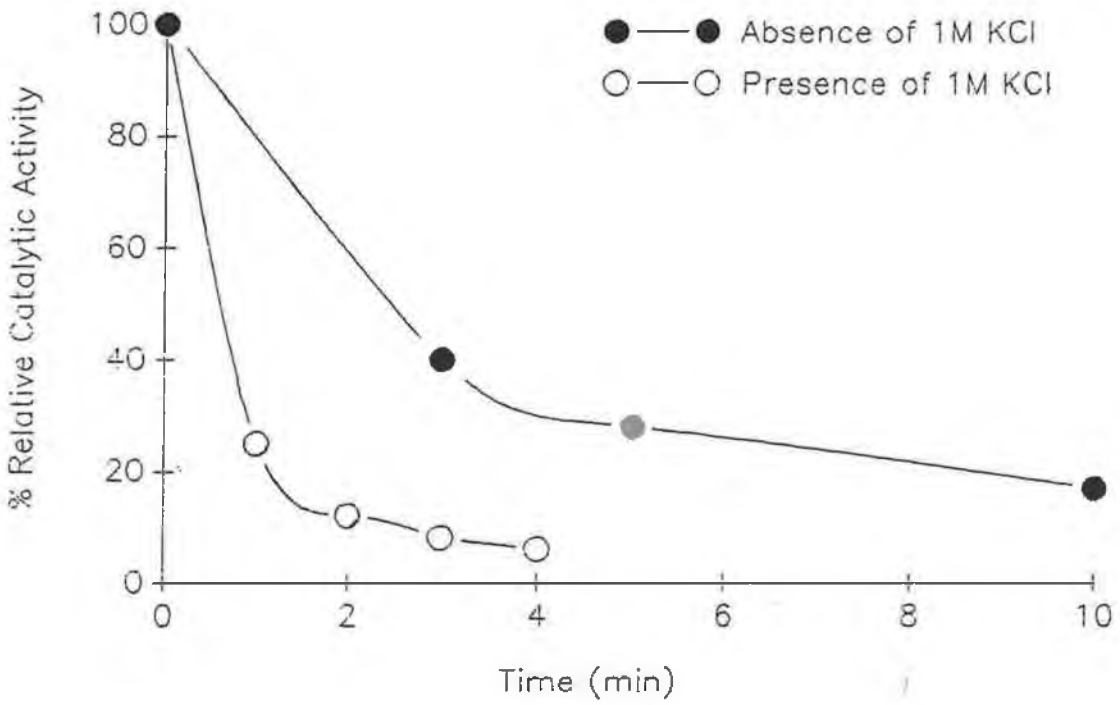


Fig. 2.36.4. Thermodeactivation at 55°C of crosslinked creatine kinase in the presence and absence of 1M KCl. Crosslinking was performed as described in Section 2.14.2. using 2mg DMS and thermodeactivation was performed as described in Section 2.6.

Table 2.36.1. The half-lives obtained when creatine kinase was thermoinactivated at 50°C and 55°C, in the presence and absence of 1M KCl.

SAMPLE	TEMP. (°C)	K (min)	$T_{0.5}$ (min)	$T_{0.5(buffer)}/T_{0.5(salt)}$
Absence of 1M KCl	50	7.93×10^{-2}	9.23	5.41
Presence of 1M KCl	50	4.38×10^{-1}	1.71	
Absence of 1M KCl	55	6.0×10^{-1}	1.19	3.31
Presence of 1M KCl	55	1.82	0.38	

Table 2.36.2. The half-lives obtained when creatine kinase crosslinked with DMS was thermoinactivated at 50°C and 55°C, in the presence and absence of 1M KCl.

SAMPLE	TEMP. (°C)	K (min)	$T_{0.5}$ (min)	$T_{0.5(buffer)}/T_{0.5(salt)}$
Absence of 1M KCl	50	4.63×10^{-2}	12.27	4.27
Presence of 1M KCl	50	2.31×10^{-1}	2.87	
Absence of 1M KCl	55	2.54×10^{-1}	2.65	4.80
Presence of 1M KCl	55	1.24	0.55	

SUMMARY

A number of different modification reagents were used to increase the stability of creatine kinase. Of these, the crosslinking reagent, dimethyl suberimidate, appeared to be the most successful. The degree of thermal stability was dependant on the degree of crosslinking, with optimal stabilization occurring when approximately half of all the available amino groups were modified. Accelerated storage studies were performed and the results were used to predict the storage time of the native and modified enzyme at lower temperatures. The crosslinked derivative was predicted to have a longer shelf-life at 37°C, 20°C and 4°C than the native enzyme. Modification caused a reduction in the specific activity of the enzyme. The pH profile was altered following crosslinking, but the Michaelis constants were not changed. The modified enzyme also exhibited a marked resistance to the action of some denaturing agents.

It appeared as if salt-bridges were involved in maintaining the structure of the native enzyme, and that lysine residues play an important role in this intrinsic stabilization.

ANALYSIS OF THE STABILITY OF NATIVE CREATINE KINASE

INTRODUCTION

When the temperature rises above a certain level, enzymes in aqueous solutions undergo partial unfolding caused by heat induced disruption of the balance of noncovalent interactions (Creighton, 1983) maintaining the native conformation. This process, which results in enzyme inactivation due to disintegration of the active center, is fully reversible: the enzymatic activity returns if the temperature is promptly lowered to the ambient (Tanford, 1968; Ahern and Klibanov, 1987). Upon longer heating, however, only a diminishing fraction of the enzymatic activity is regained upon cooling, thus reflecting another process, irreversible inactivation (Klibanov, 1983).

While mechanisms of thermal denaturation of enzymes are well understood (Tanford, 1968; Lapanje, 1978;), the reasons for irreversible enzyme thermoinactivation, until recently, remained obscure. It is, however, the latter process which is responsible for the gradual loss of enzymatic activity with time at elevated temperatures, a very common, widespread, and undesirable phenomenon.

Therefore, in order to stabilise an enzyme against irreversible thermoinactivation, it is important to know what mechanisms actually cause the inactivation of the enzyme. It was to this end that a mechanistic investigation of irreversible thermoinactivation of bovine heart creatine kinase was undertaken.

3.0 General Methods:

Creatine kinase was assayed by adaptation of the existing assay (Section 2.5.1) to a microplate reader and 96-well microtitre plates. This assay was developed during the course of this work and is described fully in Chapter 4. Electrophoresis was performed using the LKB 2050 Midget Electrophoresis Unit.

3.1 Kinetics of Thermoinactivation of Creatine Kinase:

The time course of irreversible thermoinactivation of creatine kinase was measured by incubating aqueous solutions of the enzyme (0.05–0.2 mg/ml in a buffered system i.e. 0.1M acetate, pH 4.0, 0.1M imidazole, pH 6.7, and 0.1M phosphate, pH 8.0, that had been adjusted to the desired pH at room temperature) at 50°C, and periodically removing samples and assaying them for enzymatic activity as described in Section 3.0. First-order rate constants of irreversible thermoinactivation were obtained by linear regression in semilogarithmic coordinates.

3.2 "Irreversible" Thermoinactivation in the presence of Guanidine-hydrochloride and subsequent Reactivation of creatine kinase:

The enzyme (0.2 mg/ml) was thermoinactivated in the presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in a buffered system) at

55°C and 100°C. Reactivation of creatine kinase was as described by Bickerstaff and Price (1976), by dilution in a buffered system containing 12.5mM DTE (normally a 100-fold dilution) at room temperature.

3.3 Reactivation of "Inactivated" Creatine Kinase:

In order to determine whether thermodeactivated creatine kinase could be reactivated, the heat-treated enzyme was allowed to unfold completely in a denaturing agent and then refold correctly in a renaturing buffer as follows. Creatine kinase (0.2mg/ml in 0.1M imidazole-acetate, pH 6.7) was heated at 55°C for 10min. The enzyme was then cooled on ice and assayed for activity. The enzyme solution was supplemented with 6.4M Gd-HCl containing 12.5mM DTE and incubated at 37°C for 1h. Following this, the enzyme was diluted (a 1:100 dilution) into renaturing buffer (0.1M imidazole/12.5mM DTE, pH 6.7). After 1h, the enzyme was again assayed for activity.

The same procedure was also performed on creatine kinase which had not been heated to 55°C.

3.4 Titration of Tryptophan:

Tryptophan residues in creatine kinase were titrated with N-bromosuccinimide (NBS) using the method of Spande and Witkop

(1967). 1ml of a 1mg/ml solution of creatine kinase in 0.1M acetate buffer, pH 4, was placed in a quartz cuvette and the absorbance at 280nm was recorded. 10μl of a 10mM aqueous solution of NBS was then added to the contents of the cuvette. After several minutes the absorbance at 280nm was again recorded. Additions were continued in this stepwise fashion until further increments led to no further decrease in absorbance. The minimum absorbance was recorded and corrected for the volume increase due to the added reagent.

For a protein of known molecular weight and absorptivity (at 280nm), the number of tryptophan residues per mole was calculated by the following expression.

$$n = \frac{\mu\text{moles Trp}}{\mu\text{moles of protein}} = \frac{\Delta A \times 1.31 \times V/5.50}{A \times a.f. \times V \times 1000/M.W.}$$

where n = number of tryptophan residues per mole of protein, A = initial absorbance at 280nm, ΔA = corrected absorbance decrease at 280nm, a.f. = absorptivity factor to convert absorbance at 280nm to mg/ml of protein, V = initial volume of titrated solution (ml), M.W. = molecular weight of protein and 5500 = molar extinction coefficient at 280nm for tryptophan.

3.5 Determination of the Difference Spectrum between Native and Heated Creatine Kinase:

The difference spectra for creatine kinase (100μg/ml in 0.1M imidazole-acetate, pH 6.7) thermoinactivated at 55°C for various periods of time was performed as follows.

The sample cuvette of the spectrophotometer (thermostatted at 25°C) was filled with native protein and the reference cuvette filled with buffer. The spectrum was recorded from 320nm to 200nm. Then the reference cuvette was filled with native protein and the sample cuvette with buffer and the absorbance was recorded again. This was used as the reference baseline for the measurement of the difference spectrum.

The native protein in the reference cuvette was then replaced by protein which had been heated for various periods of time at 55°C (and allowed to cool) and the difference spectrum between 320nm and 200nm was scanned.

3.6 Production of Ascitic Fluid containing Polyclonal Antibodies to Creatine Kinase:

A modified method to induce antibody containing ascites fluid in Balb/c mice using Sp2/0-Ag14 cells according to Lacy and Voss (1986) was performed.

Balb/c mice (8-12 weeks old) were used. On day 0, Freunds

incomplete adjuvant (2 ml) was injected intraperitoneally (i.p.).

On day 2, a primary immunization with a 4:1 (v/v) emulsion of Freunds complete adjuvant and immunogen (CK dissolved in PBS (1.2mg/ml)) was given. A volume of 2ml was injected i.p. with a 23 guage needle.

A secondary immunization was given on day 9 as on day 2. On day 23, mice were injected with 10^6 viable Sp2/0-Ag14 cells. On day 26, a tertiary immunization was given as on day 9.

On day 29, the ascitic fluid was tapped using a 23 guage needle. The needle was inserted into the peritoneal cavity after sacrificing the animal. The ascitic fluid was then spun down (12,000g x 5min) to remove cells. Fatty deposits were also discarded.

The mice were also bled and the blood samples were incubated at 37°C for 1 hour. The clot which formed was removed from the walls of the container and allowed to retract. It was then incubated at 4°C. Serum was separated from the clot by centrifugation at 1,100g for 10min and then stored in aliquots at -20°C.

3.7 Dot Blotting of Creatine Kinase:

The procedure was as follows: Nitrocellulose strips were wetted in PBS and allowed to dry thoroughly. Samples of creatine kinase from 1-10μl, and ranging in concentration from approximately

2.5-25μg were applied and allowed to dry. The nitrocellulose strips were blocked in 1% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS for 1 hour at 37°C , shaking constantly.

The strips were incubated with first antibody (ascitic fluid or serum, diluted 1:400 with 0.25% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS for 1 hour at 37°C, with constant shaking. The first antibody was then decanted and the strips washed 3 times in 1 hour with wash buffer containing 10mM Tris, 150mM NaCl and 0.5% (v/v) Tween 20, pH 7.5. The second antibody, alkaline phosphatase-conjugated anti-mouse IgG, diluted 1:1,000 with blocking buffer was added and left for 2 hours at 37°C with shaking. When the second antibody was removed, the strips were washed 5 times in 1 hour with washing buffer as above. After washing, the pH of the strips was optimised for subsequent reaction with the substrate by the addition of 10mM Tris/HCl, pH 8.9. The substrate solution contained the following:

(a) 1ml of a 0.1% (w/v) solution of nitroblue tetrazolium in 10mM Tris-HCl, pH 8.9.

(b) 0.4ml of a 5mg/ml solution of 5-bromo-4-chloro-3-indoyl phosphate in N,N-dimethyl formamide.

(c) 40μl of an aqueous 1M solution of $MgCl_2$ and

(d) 8.86ml of 0.5M Tris-HCl, pH 8.9.

The addition of substrate gave rise to the development of blue/purple dots for positive antigen recognition after a few minutes at room temperature. Serum from a normal mouse was used as a control to measure non-specific binding.

3.8 Peptide Chain Integrity:

Heated samples of creatine kinase (0.2mg/ml at 55°C) were subjected to SDS-PAGE as described in Section 2.4 and then the gels were visualised by silver staining as described below.

3.9 Silver Staining of SDS-polyacrylamide gels:

Silver staining was performed as described by Merril et al., (1984).

Gels were fixed in 40% (v/v) methanol / 10% (v/v) acetic acid for 30min. or overnight. The fixative was then removed and oxidising solution (containing 3.4mM potassium dichromate and 0.0032N nitric acid in ultrapure water) was added and left for 5min at room temperature. The gels were washed three times, for 5min each time, in ultrapure water. A 12mM silver nitrate solution was then added and left for 20min at room temperature with constant shaking.

After washing for 1min with ultrapure water, developer (containing 0.28M sodium carbonate and 0.019% (v/v) formaldehyde) was added for 30sec and then decanted. More developer was added and shaking continued for approximately 5min, until bands started to become visible, whilst the background remained clear.

The reaction was stopped by the addition of 5% (v/v) acetic acid. When the stopping solution was removed the gels could be

stored in sealed plastic bags with a small amount of water present for several months.

3.10 Determination of Ammonia:

Deamidation of Asn and Gln residues gives rise to the release of free ammonia. In order to determine whether deamidation was occurring in thermodeactivated creatine kinase, the release of ammonia from the heated enzyme was detected as follows. The time course of evolution of ammonia during thermoinactivation of creatine kinase was determined by incubating samples of the enzyme (0.1mg/ml in a buffered system) in sealed ampules at 55°C for various periods of time. All of the ampules were then inverted slowly for 9-10 times and then opened. The amount of dissolved ammonia was measured enzymatically with glutamate dehydrogenase as described by Kun and Kearney, (1974). This was performed as follows. The protein sample (0.5ml) was pipetted into a 1ml cuvette. Then 0.5M Tris buffer, pH 8 (0.2ml), 0.1M 2-oxoglutarate, pH 7.4 (0.1ml), 8mM β-NADH in 1% (w/v) $KHCO_3$, (30μl)and distilled water (0.15ml) were added to the cuvette, the contents mixed and the absorbance at 340nm determined (E_1). Then glutamate dehydrogenase (ca. 10mg/ml) was added to the assay mixture. The contents of the cuvette were mixed and after 90min the absorbance was again determined (E_2). The μmoles of NH_4^+/ml of sample was calculated using the following expression.

$$c = \Delta E \times \frac{1}{6.22 \times v} = \mu\text{moles of } NH_4^+/\text{ml}$$

v = volume of sample taken for assay.

ΔE = the change in absorbance at 340nm = $E_1 - E_2$.

6,220 = the extinction coefficient for NADH at 340nm.

3.11 Determination of the Presence of Disulphide Bonds in Creatine Kinase:

This was performed as described by Creighton, (1990). Creatine kinase (1.3mg/ml) was dissolved in 6M guanidinium chloride (GdmCl)/0.1M phosphate buffer, pH 7.3, and dithiothreitol (DTT) added to a final concentration of 10mM. After 1hr at room temperature, the protein was separated from excess DTT by gel filtration through a Sephadex G-25 column using 6M GdmCl/0.1M phosphate, pH 7.3, as eluting buffer to prevent precipitation of the protein.

The Ellman assay (Ellman, 1959) was then used to determine the number of thiol groups in creatine kinase according to the method described by Riddles et al., (1983). Samples (3ml) of the gel-filtered protein solution and the 6M GdmCl buffered solution were equilibrated at 25°C in the thermostatted sample and reference compartments of the spectrophotometer. The absorbance difference at 412nm was set to zero. A 100µl aliquot of 3mM

5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1M phosphate, pH 7.3, was added to each cuvette followed by thorough mixing. The increase in absorbance at 412nm of the protein solution was then followed. When the absorbance had stopped increasing, the absorbance difference between the protein and reference samples was measured. (The above procedure was also performed on creatine kinase (1.3mg/ml) in 6M GdmCl/0.1M phosphate, pH 7.3 to which no reducing agent (DTT) had been added). From the increased absorbance in the protein sample caused by the DTNB, the molar concentration of thiols present from the molar absorbance of the TNB anion [ε_{412}= /M cm in 6M GdmCl] was calculated.

3.12 Effect of reducing agent on the activity of creatine kinase

To creatine kinase (0.2mg/ml) was added varying amounts of DTE (as a dry powder), ranging from 1 to 6 mg after which the enzyme was assayed for activity as described in Section 3.0.

3.13 Oxidation of cysteine residues:

In order to determine whether autooxidation of cysteine residues was occurring during thermoinactivation, creatine kinase (0.2mg/ml) in 0.1M imidazole, pH 6.7, was thermoinactivated at 55°C in the absence and presence of 10μM $CuCl_2$, which is known to catalyse O_2 oxidation of thiols.

3.14 Accelerated storage studies:

Native creatine kinase and creatine kinase which had been crosslinked with DMS as described in Section 2.29 were supplemented with various additives as follows and then accelerated storage studies were performed as described in Section 2.7, in order to determine whether any of the additives would result in stabilization of the enzyme.

1. Native creatine kinase (control).
2. Crosslinked creatine kinase stored in the presence of BSA (80g/l).
3. Native creatine kinase stored in the presence of 1mM EDTA.
4. Native creatine kinase stored in the presence of 10mM EDTA.
5. Native creatine kinase stored in the presence of 5mM DTE.
6. Native creatine kinase stored in the presence of BSA (80g/l).
7. Crosslinked creatine kinase stored in the presence of BSA (80g/l) and 5mM DTE.
8. Native creatine kinase stored in the presence of BSA (80g/l) and 5mM DTE.

RESULTS AND DISCUSSION:

3.15 Kinetics of Thermoinactivation of Creatine Kinase:

Polymolecular processes such as aggregation have often been proposed as the sole mechanism of irreversible thermoinactivation of enzymes (Joly, 1965; Baldwin, 1975). It was of interest, therefore, to identify whether this process played a part in the thermoinactivation of creatine kinase.

The time course of irreversible inactivation of creatine kinase at 50°C as a function of pH and enzyme concentration was studied as described in Section 3.1. The enzyme thermoinactivation was investigated in the pH range from 4 to 8, which covers the range relevant to enzyme catalysis, and yet is sufficiently broad so that different mechanisms may contribute to irreversible thermoinactivation. The above thermoinactivation experiments were carried out at 50°C, where the enzyme inactivates at a conveniently measurable rate.

At pH 4.0, at the four different enzyme concentrations examined, thermoinactivation proceeded so rapidly it was not possible to measure the rate. However, thermoinactivation proceeded more slowly at pH 6.7 and 8.0 and the results are shown in Fig.3.15.1 and Fig.3.15.2, respectively. While the thermodeactivation process was accompanied by aggregation at the higher enzyme concentrations, this did not appear to be the cause

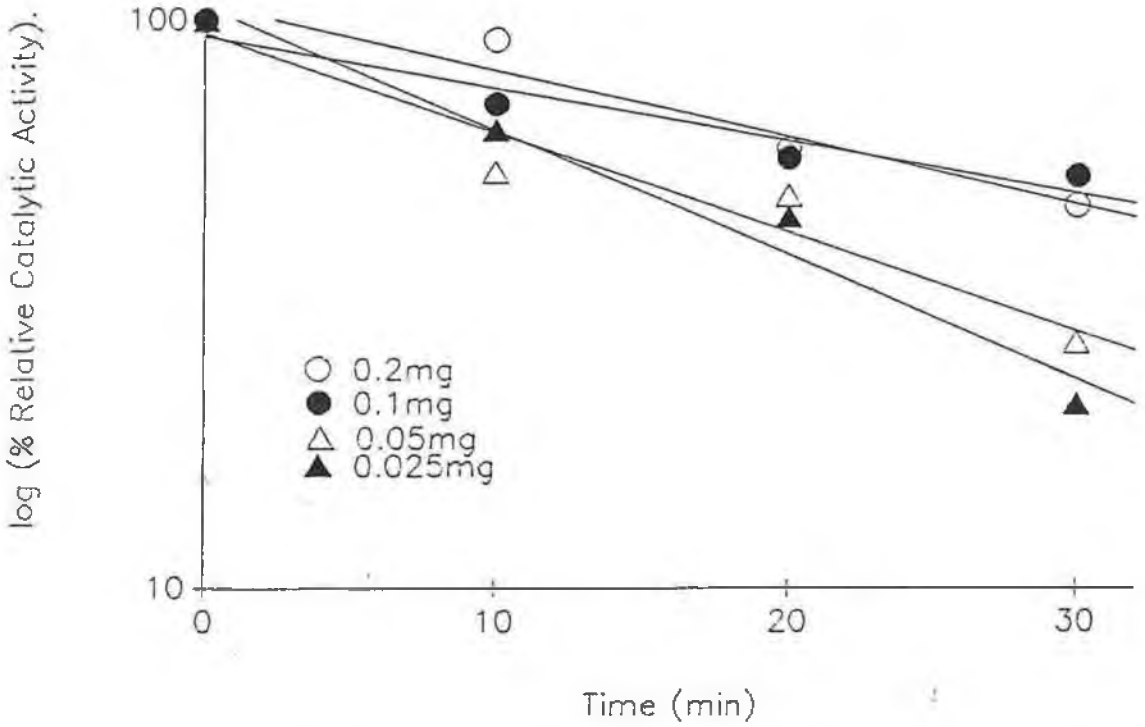


Fig. 3.15.1. Thermoinactivation of creatine kinase at pH 6.7 and 55 C at four different enzyme concentrations and performed as described in Section 3.1.

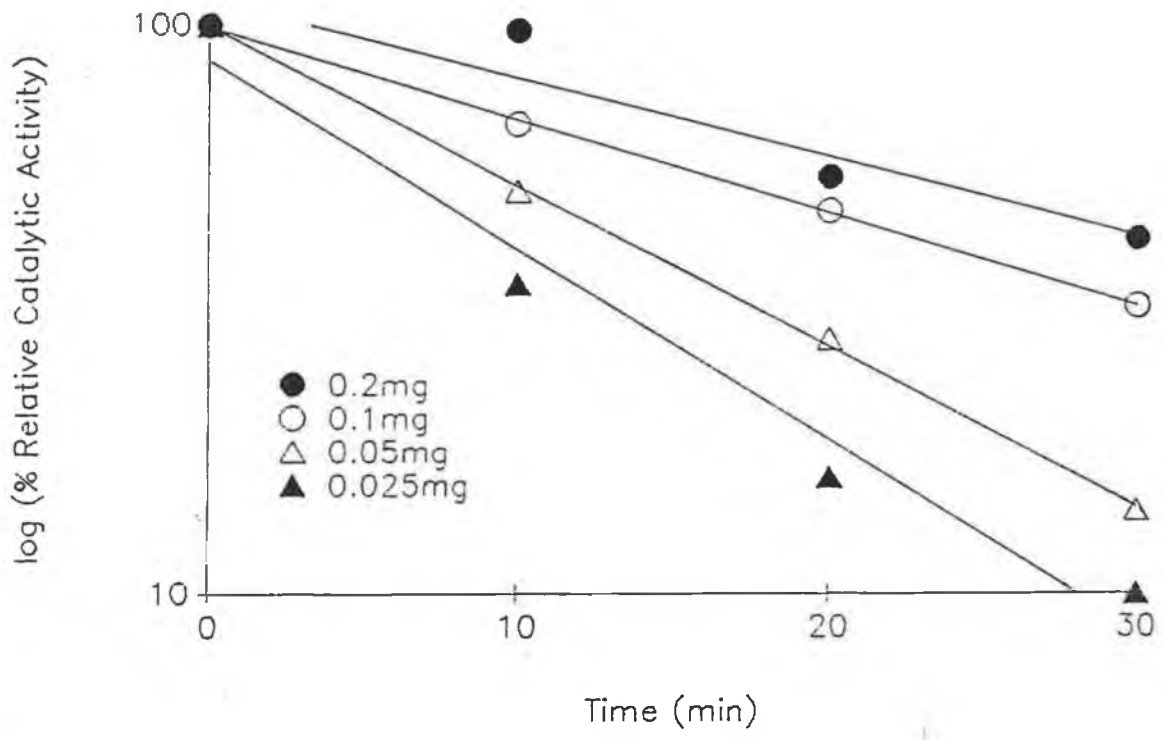


Fig. 3.15.2. Thermoinactivation of creatine kinase at pH 8.0 and 55 C at four different enzyme concentrations and performed as described in Section 3.1.

of thermodeactivation as the higher concentration of enzyme actually thermoinactivated more slowly.

Thus, while it appeared that the inactivation of the enzyme at a particular enzyme concentration followed first-order kinetics, the actual time course of inactivation did not appear to be independant of the initial enzyme concentration. This can be clearly seen at pH 8.0 (Fig.3.15.2). At pH 6.7, (which is close to the optimum for enzyme activity), the effect was not as pronounced. It is possible that in the more dilute enzyme concentrations, dissociation of the dimer into monomers may give rise to the increased rate of thermoinactivation.

However, from the above results, it can be concluded that irreversible thermoinactivation of creatine kinase in the above ranges of pH and concentration is not due to polymolecular processes such as aggregation.

3.16 Irreversible thermoinactivation in the presence of guanidine-hydrochloride and subsequent reactivation of creatine kinase.

It was important to determine whether irreversible thermoinactivation of creatine kinase was caused by conformational or covalent processes. In the absence of aggregation, conformational processes result in incorrectly folded and enzymatically inactive protein molecules that are kinetically or thermodynamically stable at elevated temperatures (Klibanov and

Mozhaev, 1978). These structures are so "scrambled" that they persist even after cooling, because a high kinetic barrier prevents spontaneous refolding to the native conformation. To distinguish between such conformational mechanisms of thermoinactivation and those of a covalent nature, the approach of Ahern and Klibanov (1985) was adopted.

Concentrated solutions of strong denaturants disrupt non-covalent interactions in proteins (Tanford, 1968; Lapanje, 1978; Creighton, 1983). Such agents, especially at high temperatures, should maintain the enzyme molecules in a highly unfolded form and thereby prevent formation of incorrectly folded structures. Hence, heating enzymes in the presence of denaturants should stabilize them against irreversible thermoinactivation due to conformational processes (Martinek et al., 1975). Conversely, denaturing agents are not expected to affect the rates of most covalent reactions.

Therefore, if addition of a denaturant stabilizes an enzyme against irreversible thermal inactivation, then conformational processes are involved; if there is no effect, the rate-limiting step consists exclusively of covalent processes. This criterion was used to try to determine the exact mechanism of irreversible thermoinactivation of creatine kinase at pH 4, 6.7 and 8.

A control experiment was first performed in order to determine whether creatine kinase could be reactivated after treatment with 6M guanidine-HCl (Gd-HCl) at pH 6.7. The results are shown in Fig

3.16.1. It can be seen that a 100-fold dilution into renaturing buffer could reactivate the enzyme by 66% after a period of 110 min. This figure represented the best reactivation obtained in this work and compares reasonably well with the value of 80% obtained by Bickerstaff and Price (1976) using creatine kinase from rabbit skeletal muscle. A 1:10 dilution of the enzyme could only reactivate approximately 30% of the enzymes original activity. Thereafter, in subsequent experiments, renaturation was allowed to proceed for 110 minutes after the enzyme had been diluted 100-fold.

Thermodeactivation at pH 4 and the subsequent renaturation of the enzyme were performed as described in Section 3.2. The results are shown in Fig.3.16.2. In the absence of Gd-HCl, the enzyme had lost all activity after 5 min. However, in the presence of the denaturant, this process was slowed down considerably, with the enzyme retaining 31% activity after 30 min at 55°C. A 40% recovery of activity (in the absence of heating) was the optimum activity recovered at pH 4. Thermodeactivation was also performed at 100°C (Fig.3.16.3). Once again, the presence of the denaturant slowed down the process of inactivation.

At pH 6.7, the slowing down of thermoinactivaton in the presence of 6M Gd-HCl was again observed (Fig.3.16.4 and 3.16.5) at 55°C and 100°C. A 46% recovery of activity was the maximum amount of activity recovered in the absence of heating.

At pH 8, 63% of the enzymes original activity was recovered

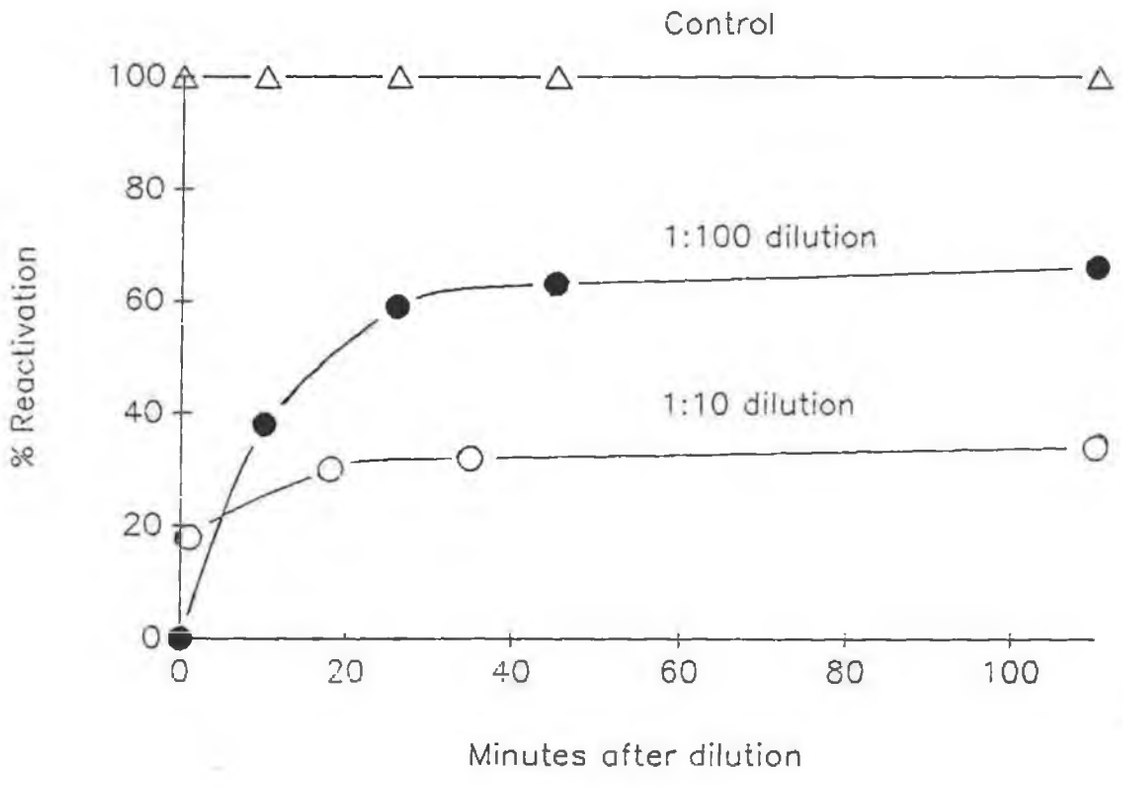


Fig. 3.16.1. Reactivation of creatine kinase after treatment with 6M Gd-HCl/12.5mM DTE in 0.1M imidazole, pH 6.7. The enzyme was thermoinactivated at 55 °C in the presence of denaturing buffer. At zero time, the denatured enzyme solution was diluted 10- and 100-fold into renaturing buffer without Gd-HCl and assayed at the stated times. A parallel control experiment in which the enzyme was incubated in 0.1M imidazole, pH 6.7 containing 12.5mM DTE showed no loss of activity when diluted into renaturing buffer as described in Section 3.2.

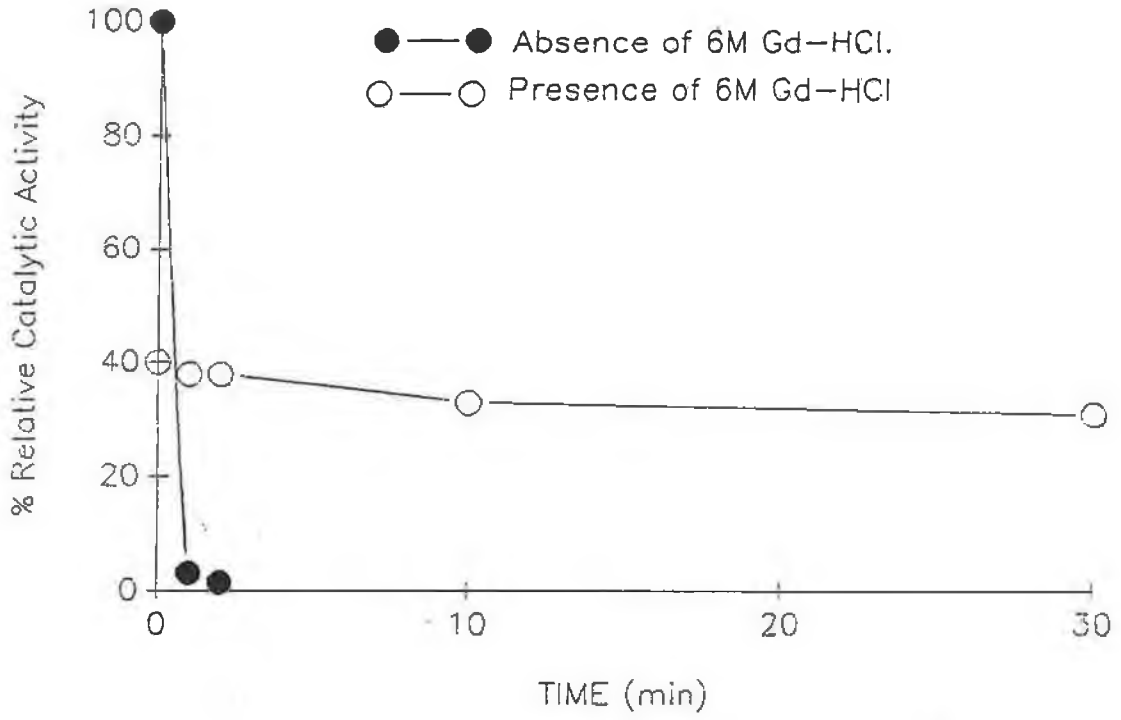


Fig. 3.16.2. Thermodeactivation of creatine kinase at pH 4.0 and 55 °C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M acetate, pH 4.0). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

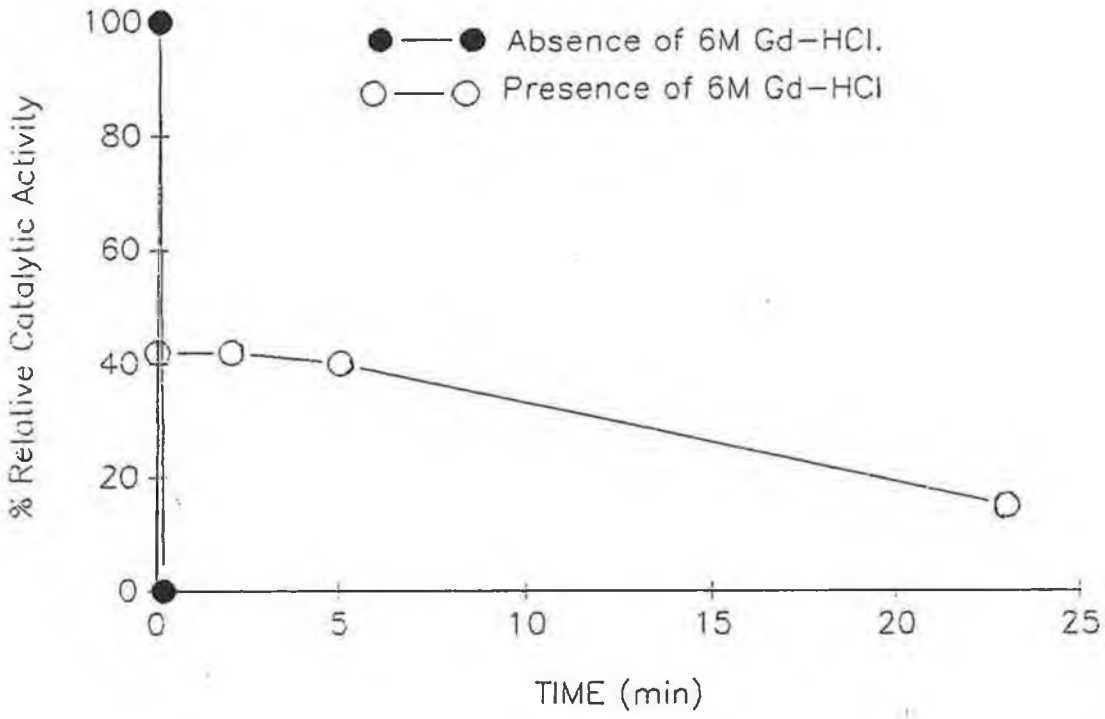


Fig. 3.16.3. Thermodeactivation of creatine kinase at pH 4.0 and 100°C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M acetate, pH 4.0). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

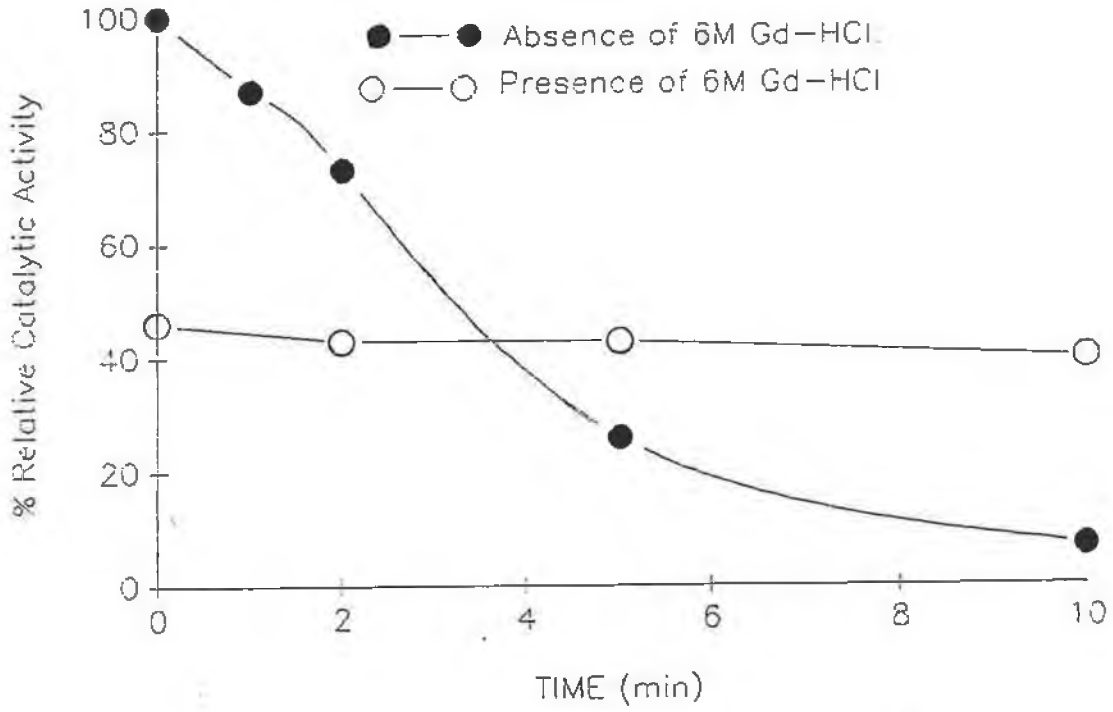


Fig. 3.16.4. Thermodeactivation of creatine kinase at pH 6.7 and 55° C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M imidazole, pH 6.7). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

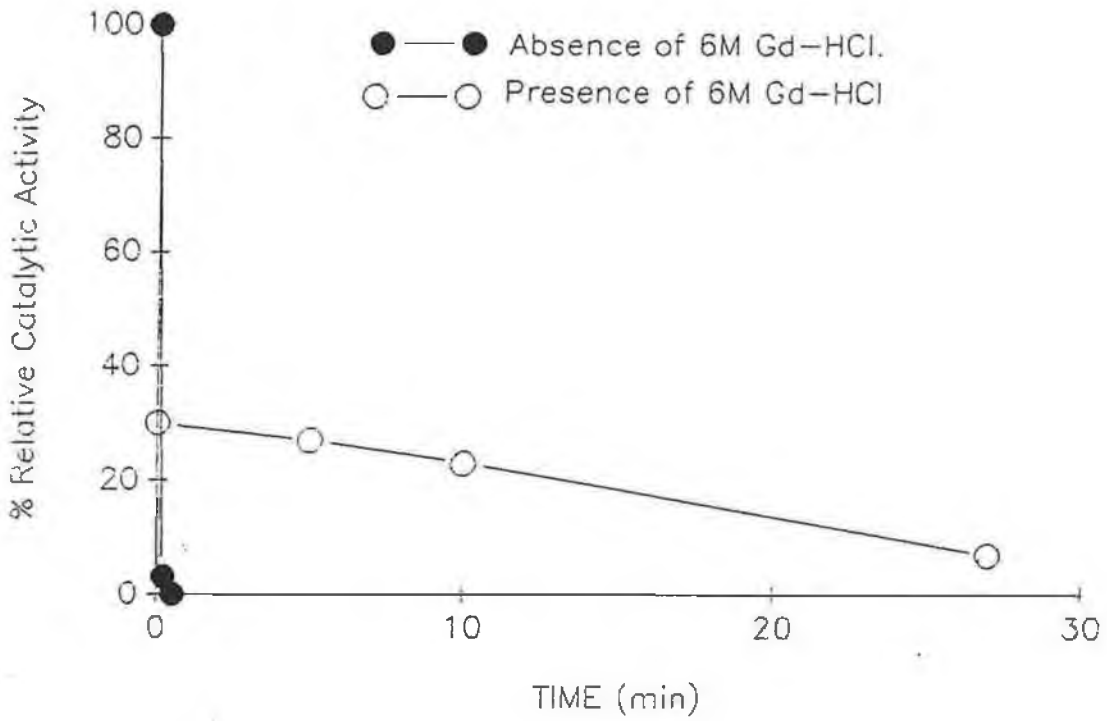


Fig. 3.16.5. Thermodeactivation of creatine kinase at pH 6.7 and 100 °C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M imidazole, pH 6.7). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

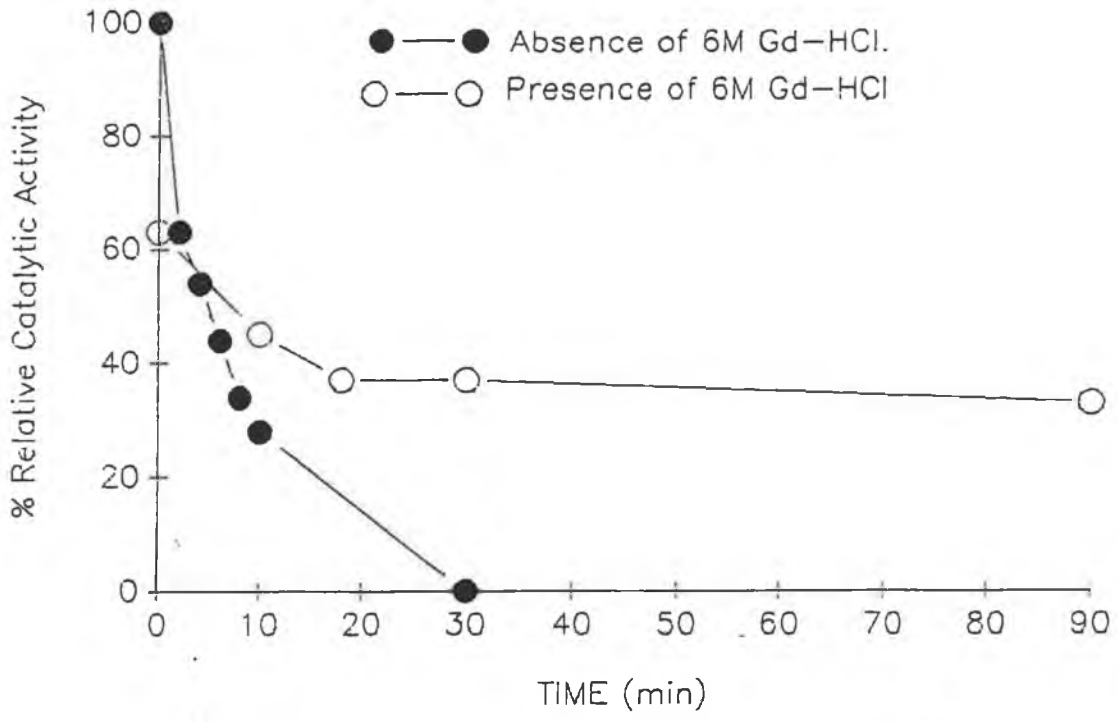


Fig. 3.16.6. Thermodeactivation of creatine kinase at pH 8.0 and 55 C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M phosphate, pH 8.0). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

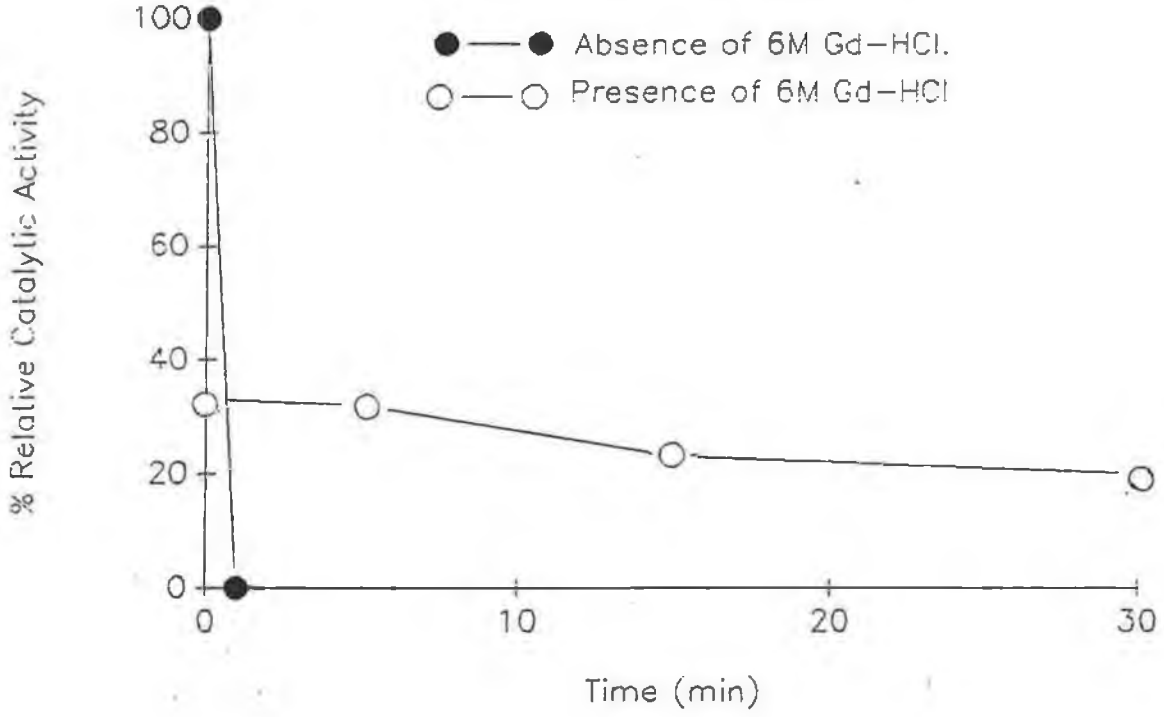


Fig. 3.16.7. Thermodeactivation of creatine kinase at pH 8.0 and 100°C and subsequent reactivation of the enzyme. The enzyme (0.2mg/ml) was thermoinactivated in the absence and presence of denaturing buffer (6M Gd-HCl/12.5mM DTE in 0.1M phosphate, pH 8.0). Reactivation was performed by a 1:100 dilution into renaturing buffer as described in Section 3.2. After 110 minutes, the enzyme was assayed for residual enzyme activity.

after dilution into renaturing buffer. Once again, at this pH, the process of thermoinactivation was again slowed down considerably (Fig.3.16.6 and 3.16.7) in the presence of 6M Gd-HCl.

The results presented above suggest that at pH 4, 6.7 and 8, conformational processes are involved in the "irreversible" thermoinactivation of creatine kinase. The formation of incorrect structures would seem to play a major role in the loss of activity of the enzyme upon heating. This process can be retarded by the presence of a denaturing agent which maintains the molecules in an unfolded form and thereby prevents the formation of incorrect structures.

3.17 Reactivation of "inactivated" creatine kinase

A second approach was used to confirm that incorrect structure formation had occurred. An attempt was made to recover activity which had been lost by incorrect structure formation. This was performed as described in Section 3.3. The enzyme was heated at 55°C at pH 6.7 until 92% of the enzymes original activity had been lost. Following incubation with 6M Gd-HCl and subsequent dilution into renaturing buffer, 34% of the initial total activity was recovered. A control experiment in which creatine kinase was not heated (denaturation and renaturation only was performed) resulted in 39% of the enzymes original activity being recovered. Therefore, a recovery of 34% activity represents nearly a 90%

recovery of the "recoverable" activity. The value of 34% compares favourably with the value of 35% obtained by Tomazic and Klibanov (1988) in their reactivation of "irreversibly" thermoinactivated *B. stearothermophilus* α-amylase.

At pH 8, creatine kinase was heated at 55°C until only 11% of the enzymes original activity was left. Following unfolding in 6M Gd-HCl and then dilution into renaturing buffer, the heated enzyme regained 26% of its initial activity. Protein which had not been heated was able to regain 45% of initial total activity. Therefore, the heated enzyme was able to regain 58% of the total recoverable activity.

At pH 4, creatine kinase was heated until none of the initial activity remained. In this instance the enzyme was able to regain 25% of its initial activity. Unheated enzyme was able to regain 36% of the initial activity. The heated enzyme was thus able to regain nearly 70% of the total recoverable activity.

The above results serves to confirm the role of incorrect structure formation at the 3 different pH values studied, since refolding of the thermoinactivated enzyme would not be able to recover activity lost if the thermoinactivation process was due solely to covalent processes (Ahern and Klibanov, 1987). Only incorrect structures which were allowed to unfold and refold correctly could give rise to the recovery of activity observed.

3.18 Titration of Tryptophan

In order to directly demonstrate the formation of new conformations upon thermoinactivation of creatine kinase, certain functional groups on the surface of the enzyme were titrated before and after heating as described in Section 3.4. To prevent aggregation (which would have made such quantitative studies impossible), the experiment was carried out in the presence of 6M Gd-HCl. In native creatine kinase at pH 4, 3 out of 4 tryptophan residues were found to be titratable with N-bromosuccinimide. When the enzyme was thermoinactivated by approximately 80%, the number of accessible tryptophan residues increased to 4. This reflects a more unfolded conformation of the irreversibly thermoinactivated enzyme compared to its native predecessor. This experiment was only performed at pH 4 due to a scarcity of protein.

3.19 Determination of difference spectra

The difference spectra of creatine kinase after thermoinactivation at 55°C and pH 6.7 was determined as described in Section 3.5. The spectra is shown in Fig.3.19. As can be seen, the major difference in the spectra occurred between 220 and 200nm. This would imply that peptide bonds (which absorb strongly below 230nm, (Schmid, 1989)) are giving rise to the difference in spectra. It was surprising that no differences were observed at

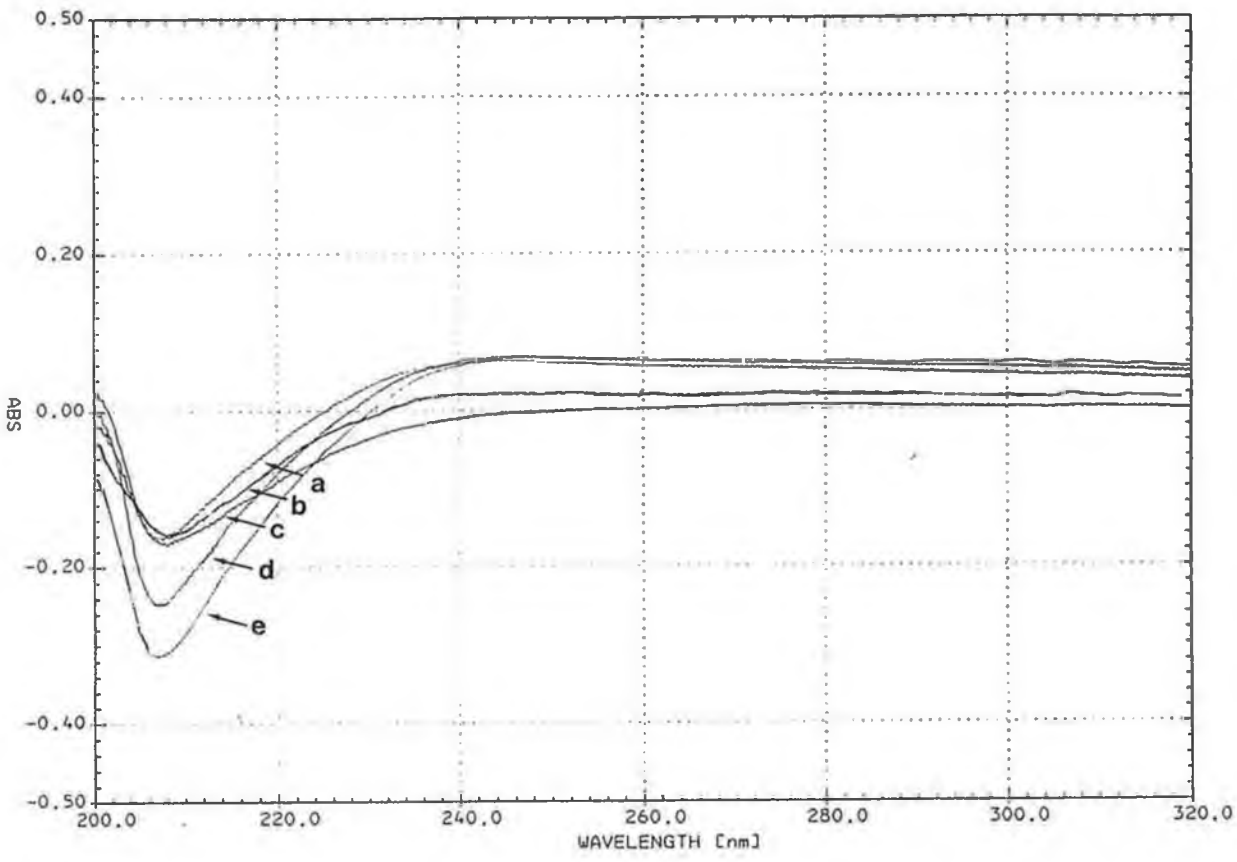


Fig. 3.19. The difference spectra of creatine kinase thermoinactivated at 55 °C. This was determined as described in Section 3.5. The reference cuvette contained native creatine kinase at 25 °C. (a) 1 min (b) 2 min (c) 5 min (d) 10 min and (e) 30 min.

280-320nm (the wavelength characteristic for absorption of aromatic residues) in view of the fact that titration of tryptophan residues at pH 4 had revealed a difference. One would have expected this to have occurred at pH 6.7 also, but perhaps the difference was too small to have been detected. However, changes in chain conformation dramatically affect the absorbance of the peptide bond in the region of 190-230nm (Ahern and Klibanov, 1987). This would, therefore, support the evidence in the preceeding sections of conformational processes being responsible for the inactivation of creatine kinase.

3.20 Production of polyclonal antibodies to creatine kinase

Polyclonal antibodies to creatine kinase were produced as described in Section 3.6 . In order to test whether the antibodies would bind to creatine kinase, dot blotting was performed as described in Section 3.7 . The ascitic fluid and the antiserum from the two balb/c mice were tested and the results are shown in Fig. 3.20.1. The results were positive in all cases with the antiserum from mouse 1 giving the strongest reaction. This antiserum was therefore used in subsequent work.

The aim of raising polyclonal antibodies against creatine kinase was to test whether they could be used to measure the extent of unfolding of creatine kinase due to thermodeactivation.

Creatine kinase was heated at 55°C and 100°C and aliquots

(taken at various time intervals and equivalent to 1μg) were then dot blotted onto nitrocellulose as described in Section 3.7. The blots are shown in Fig.3.20.2. The times that the enzyme was removed from the heat were chosen to ensure that little or no activity remained. However, it can be seen that at both temperatures, the anti-creatine kinase antibodies were able to recognise the inactivated protein equally as well as the protein which had not been exposed to heat treatment. Even in the presence of 8M urea, the antibodies were capable of recognising unfolded creatine kinase.

The results demonstrate that these polyclonal antibodies could not adequately distinguish changes in protein conformation and therefore could not be used to determine the extent of unfolding and refolding of creatine kinase. The heterogeneity in terms of the antigenic sites they recognise on creatine kinase probably precludes the observation of a precise region of the molecule, thus masking local changes. It would seem therefore, that monoclonal antibodies would probably prove more effective in the analysis of protein conformation as they should be more sensitive to perturbations in the tertiary structure (Clausen et al., 1988), and consequently could be used to select appropriately folded molecules.

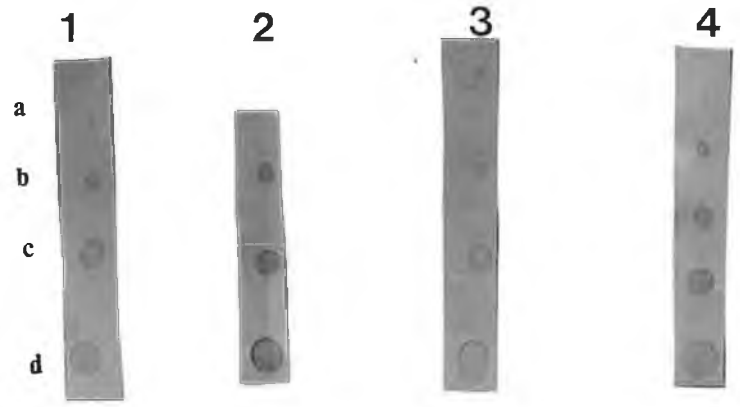


Fig. 3.20.1. Ascitic fluid and antiserum were tested for the capacity of the polyclonal antibodies to bind to creatine kinase by dot blotting as described in Section 3.7. 1 is the ascitic fluid from mouse 1, and 2 is the corresponding antiserum. 3 is the ascitic fluid from mouse 2, and 4 is the corresponding antiserum. a-d represents the amount of creatine kinase dot blotted onto the nitrocellulose strips and is as follows; a = 2.5μg; b = 5μg; c = 12.5μg; and d = 25μg.

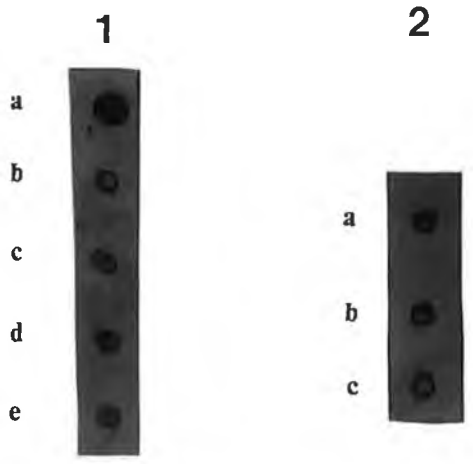


Fig. 3.20.2. Dot blotting of creatine kinase which had been thermoinactivated at 55 °C (strip 1) and 100 °C (strip 2). On strip 1, a is creatine kinase (10μg) in the presence of 8M urea; b is 1μg CK at t = 0; c is 1μg CK at t = 20min; d is 1μg CK at t = 30min, and e is 1μg CK at t = 40min. On strip 2, a is 1μg CK at t = 0; b is 1μg CK at t = 20min, and c is 1μg CK at t = 30min.

3.21 Investigation of the role of covalent processes in the thermal inactivation of creatine kinase

While accepting that conformational processes play a major role in the thermoinactivation of creatine kinase, the possible involvement of covalent processes in thermoinactivation was also investigated as described in the following sections.

3.22 Peptide chain integrity

The possibility that peptide bond hydrolysis might contribute to creatine kinase thermoinactivation at the three pH values studied, was ruled out on the basis of SDS-polyacrylamide gel electrophoresis and quantitative assessment by gel scanning densitometry of native and thermoinactivated enzyme samples carried out under reducing conditions (Section 3.8). No noticeable quantities of low molecular weight protein products were found in samples thermoinactivated at either pH 4, 6.7 or 8. It was thought that this process might play a role in thermodeactivation at pH 4 in particular as hydrolysis of proteins in dilute acid solutions (Inglis, 1983) have indicated that the Asp-x bond (where x is the amino acid residue bonded to the α-carboxyl group of Asp) is a very labile peptide bond under those conditions. However, this was not the case with creatine kinase.

3.23 Deamidation of asparagine and glutamine residues

Deamidation of Asn and Gln residues has been identified as contributing to the thermodeactivation of several enzymes (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). The deamidation of these residues can be quantified by a colorimetric method sensitive to the release of free NH_3 and is described in Section 3.10. The effectiveness and range of this assay was first checked using ammonium chloride as a source of NH_4^+. A linear relationship was evident up to a concentration of 0.227 μmole/ml of NH_4^+.

Creatine kinase (0.2 mg/ml) was thermoinactivated at 55°C at pH 4, 6.7 and 8. However, assay of the thermoinactivated samples did not detect the presence of ammonia. Since the rate of deamidation of Asn residues increases with pH above 6 (Robinson and Rudd, 1974), the enzyme was then thermoinactivated at pH 10. The results are shown in Fig.3.23.1 and indicate that deamidation does occur at this high pH value. The effect of deamidation at 100°C was also investigated (Fig.3.23.2). As can be seen, the release of ammonia was evident at pH 6.7 and 8, but not pH 4. However, since the enzyme is completely inactivated in less than 2 min at 100°C (see Section 3.16) this process cannot be said to be responsible for the inactivation of the enzyme at this temperature. Hence, while deamidation of Asn and Gln residues has been found to contribute to the irreversible inactivation of some enzymes (Tomazic and

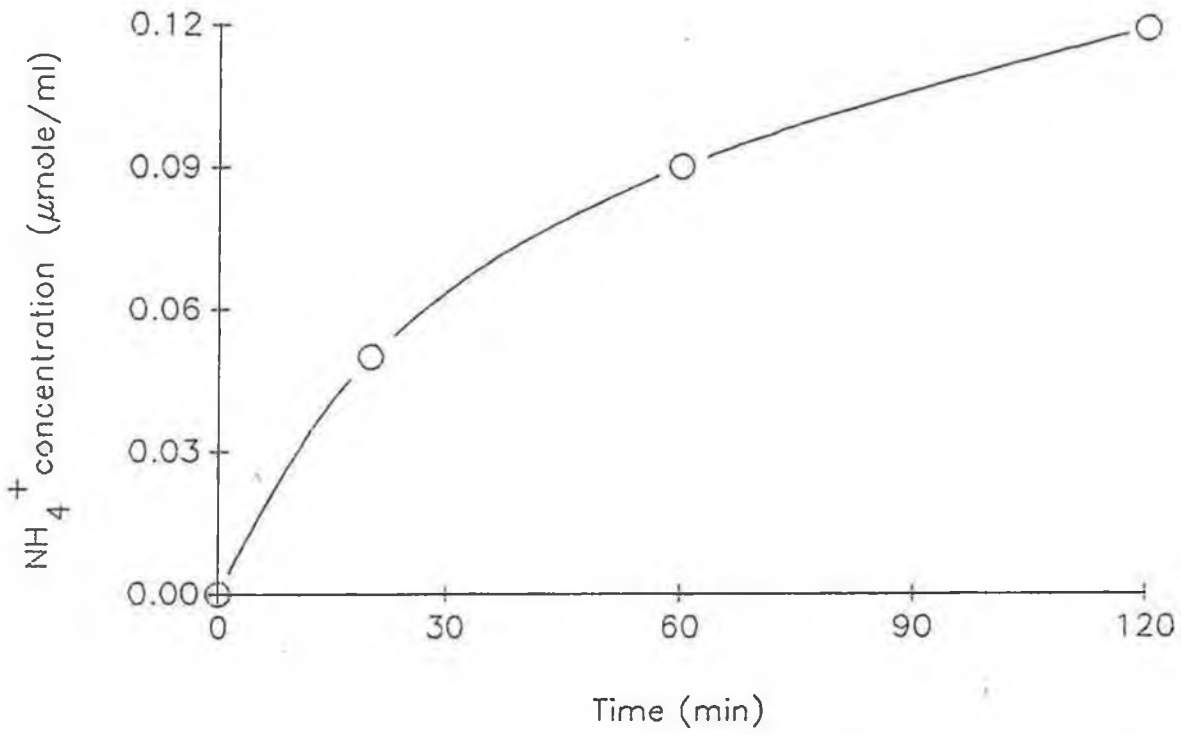


Fig. 3.23.1. Determination of the release of ammonia from creatine kinase thermoinactivated at 55° C and pH 10. A 0.1mg/ml solution of creatine kinase in 0.1M phosphate buffer, pH 10, was thermoinactivated at 55° C and the release of free ammonia then determined as described in Section 3.10.

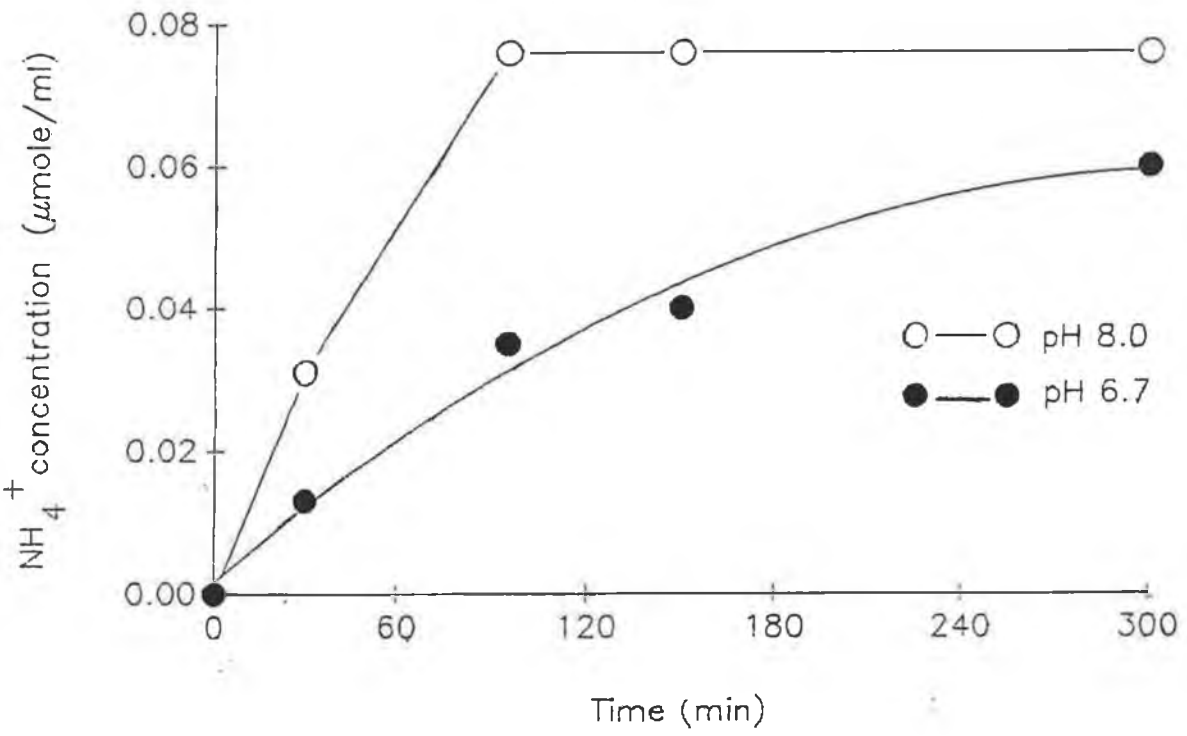


Fig. 3.23.1. Determination of the release of ammonia from creatine kinase thermoinactivated at 100° C and pH 6.7 and 8.0. A 0.1mg/ml solution of creatine kinase in 0.1M imidazole, pH 6.7, and 0.1M phosphate buffer, pH 8.0, was thermoinactivated at 100° C and the release of free ammonia then determined as described in Section 3.10.

Klibanov, 1988), this process does not appear to be involved in the thermoinactivation of creatine kinase.

3.24 Determination of free sulfhydryl groups

The destruction of free sulfhydryl groups has also been found to be involved in the covalent inactivation of several enzymes (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). It is known that creatine kinase contains two thiol groups per molecule (one thiol group per subunit) which are essential for catalytic activity (Boyer, 1973; Bickerstaff and Price, 1978) and destruction of these residues could certainly play a role in the thermal inactivation of the enzyme.

The number of free sulfhydryl groups was first determined as described in Section 3.11 and gave a value of 2 reactive thiol groups. However, under reducing conditions this value increased to 4. This result was unexpected in that it implied the presence of a disulfide bridge. It has been reported in fact that there are no disulfide bridges present in creatine kinase (Bayley and Thomson, 1967). The simplest way to determine whether or not a protein contains any disulfide bonds is to compare its electrophoretic mobility in acrylamide gels in the unfolded form, with and without reduction (Creighton, 1990). This was performed as described in Section 2.4. The result is shown in Fig. 3.24. As can be seen, under reducing conditions, only one band (corresponding to the

Fig. 3.24. SDS-PAGE of creatine kinase electrophoresed under reducing and non-reducing conditions. Creatine kinase (15μg) was assayed by SDS-PAGE according to the technique of Laemmli (1970) on a 10% gel. Lane 1 shows creatine kinase electrophoresed under reducing conditions i.e. in the presence of 5% (v/v) 2-mercaptoethanol. Lane 2 shows creatine kinase electrophoresed under non-reducing conditions i.e. in the absence of 2-mercaptoethanol.

molecular weight of the monomer) was evident. However, under non-reducing conditions, this band decreases in intensity and another band of apparent lower molecular weight appears to be the predominant species. Bands of higher molecular weight are also evident in the non-reduced sample.

A reduction in apparent molecular weight upon passing from the reduced to the non-reduced state is highly indicative of intrachain disulfide bonding (Allore and Barber, 1984). This is because the non-reduced form will have a greater mobility if intra molecular disulfides are present, because the flexibility of the unfolded polypeptide chain is restricted and the hydrodynamic volume is decreased (Creighton, 1990).

Hence, from the results shown in Fig.3.24 it would appear that in the non-reduced state, intra-chain disulfide bonding is occurring to a large extent, while to a lesser extent there is also evidence of intermolecular disulfide bonding. The higher molecular weight bands should not be attributed to oligomers of the enzyme (in the absence of disulfide bonding), as the presence of SDS is known to dissociate the subunits (Boyer, 1973).

3.25 Effect of reducing agent on the activity of creatine kinase

From the results presented above, it seemed logical that the native enzyme might possess more activity if no intra- or interchain disulfide bonds were present. Therefore, the activity

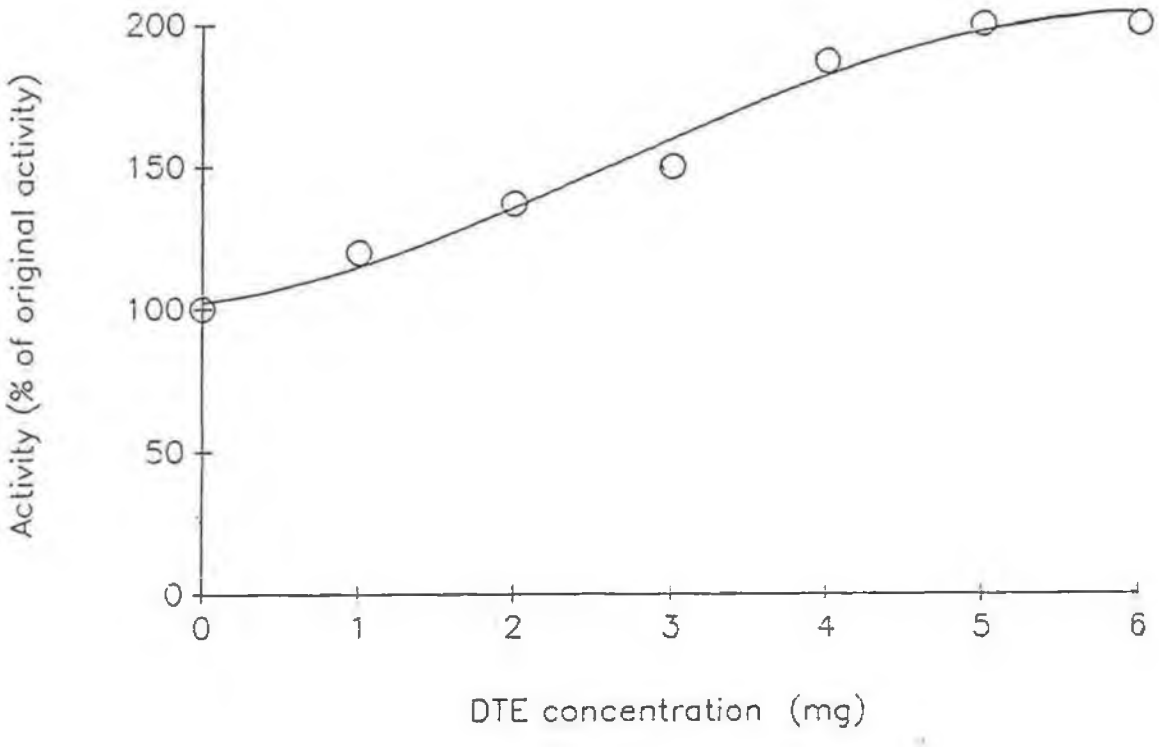


Fig. 3.25. Effect of the addition of DTE on the activity of creatine kinase. The enzyme was assayed before the addition of DTE, and this value was taken as 100% activity (see y-axis). Further increases in activity due to the addition of DTE are expressed as values between 100% and 200%.

of the enzyme in the presence of the reducing agent, dithioerythritol (DTE) was examined as described in Section 3.12. The results are shown in Fig.3.25. With increasing DTE concentration, the activity of creatine kinase increased. The maximum increase (a 100% increase) occurred when approximately a 25-fold excess of DTE (in mgs) over creatine kinase was added.

It has been commonly accepted for some time that thiol activated creatine kinase assay systems measure creatine kinase activity more accurately and more reproducibly than non-activated systems (Miyada et al., 1975). The enzyme, as it occurs in serum, is largely inactive and must be activated by sulfhydryl reagents (Warren, 1972; Bishop et al., 1971; Szasz et al., 1976). The nature of the inactive enzyme and the mechanism of reactivation were unknown until now.

3.26 Oxidation of cysteine residues

Since autoxidation of cysteine residues is known to occur at neutral pH (Cecil and McPhee, 1959), the possible role of this process in creatine kinase thermoinativation was investigated. Copper ions are known to catalyse O_2 oxidation of thiols (Friedman, 1973) and so thermodeactivation in the presence and absence of Cu^{2+} was performed as described in Section 3.13. It was found that Cu^{2+} did in fact accelerate the thermoinactivation of the enzyme (Fig.3.26.1) implying that cysteine residues were

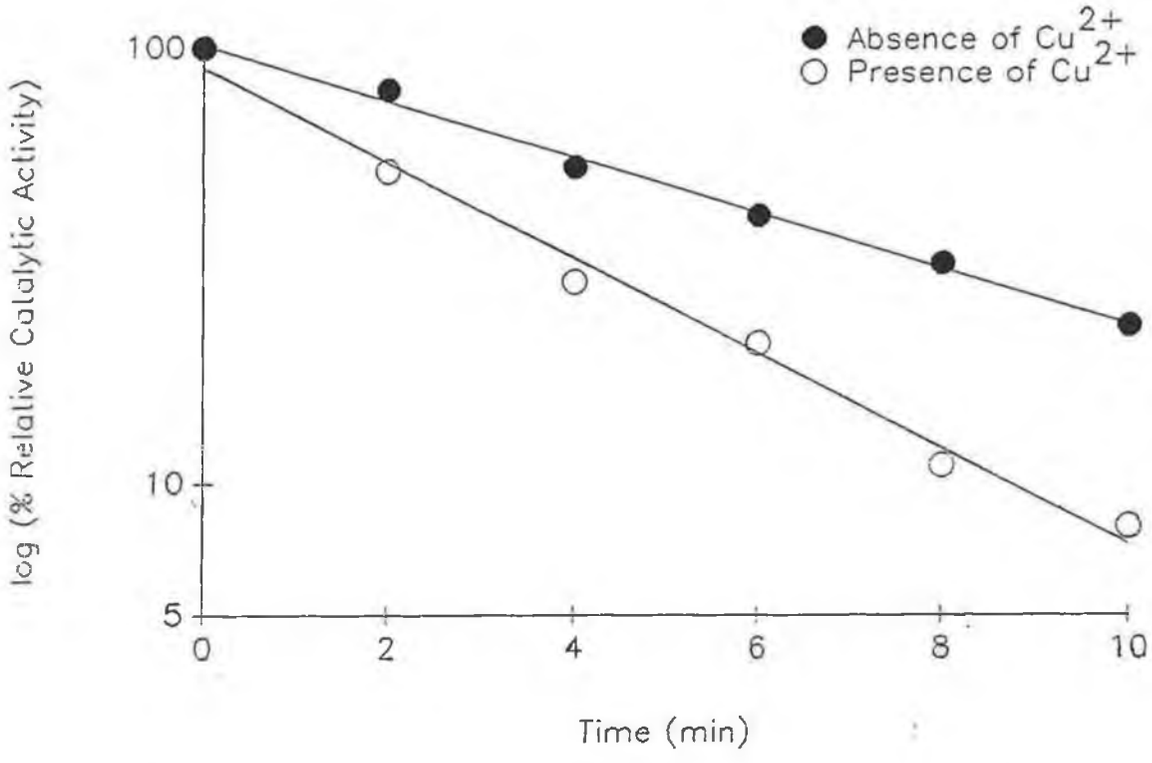


Fig. 3.26.1. Thermodeactivation of creatine kinase at pH 6.7 and 55 C in the presence and absence of copper ions. Enzyme (0.2mg/ml) in 0.1M imidazole, pH 6.7, was thermoinactivated at 55 C in the presence and absence of 10μM $CuCl_2$.

capable of undergoing O_2 oxidation. This result was consistent with that observed in Section 3.24.

Therefore, it was of interest to identify the pathway of heat-induced oxidation of the cysteine residue(s). Three distinct processes have been described in the literature (Torchinsky, 1981): intramolecular oxidations to (1) sulfonic acid or (2) sulfenic acid and (3) intermolecular formation of disulfide bonds. In view of the results presented in Section 3.24 and 3.25 it was thought that the formation of disulfide bonds was the most likely candidate.

Creatine kinase was thermoinactivated, electrophoresis was performed under non-reducing conditions and the protein silver stained as described in Section 3.9. The results are shown in Fig.3.26.2. In lane 2, it can be seen that the bands designated 1 and 4 are present in roughly equal amounts in the native unheated enzyme. However, as the enzyme is progressively heated and activity is lost, band 1, (which corresponds to the position of the native enzyme) and band 2 gradually disappear, while bands 3 and 4 become more intense. The higher molecular weight bands also increased upon heating. This clearly reflects changes in the conformation of creatine kinase during thermal inactivation. These results would seem to be in agreement with those obtained by Rudge and Bickerstaff (1984). These workers noted that the number of reactive thiol groups per subunit of creatine kinase increased from one to two upon heating at 45°C, and then fell gradually back

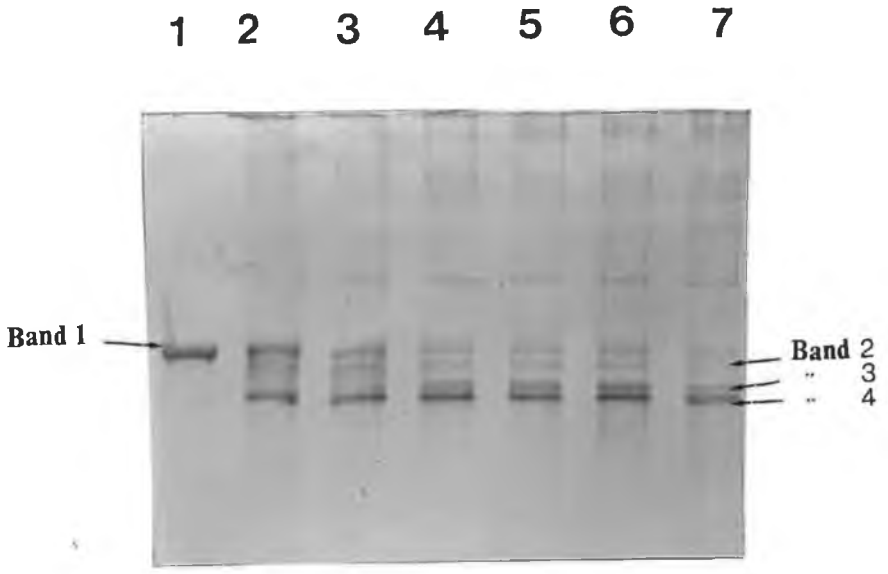


Fig. 3.26.2. The SDS-PAGE profiles of (1) the native enzyme in the presence of 2-mercaptoethanol, (2) the native enzyme in the absence of 2-mercaptoethanol, and thermoinactivated enzyme in the absence of 2-mercaptoethanol after (3) 2min at 55 °C (4) 4min (5) 6min (6) 8min and (7) 10min.

to one thiol group per subunit when the enzyme was returned to room temperature. They attributed this observation to changes in the conformation of the enzyme during thermal inactivation.

Since Cu^{2+} (10μM), was shown to be capable of increasing the rate of inactivation of creatine kinase via O_2 oxidation of thiols, it was decided to include EDTA (1mM), in the thermodeactivation mixture to determine whether it could slow down the rate of inactivation by chelating any Cu^{2+} that might be present. The result is shown in Fig.3.26.3 . EDTA appeared to have only a very marginal stabilising effect on the enzyme.

In order to examine the effect of Cu^{2+} on the conformation of creatine kinase, the enzyme thermoinactivated in the presence of Cu^{2+} (10μM) was run on an SDS-polyacrylamide gel in the absence of a reducing agent and then silver stained. This was also performed on creatine kinase thermoinactivated in the presence of EDTA (1mM). Fig.3.26.4 shows the banding patterns obtained. There was a high level of background in the gel. For reasons of clarity, these results are presented again in Fig.3.26.5 a,b and c. Unfortunately, in this instance the bands are rather diffuse. However, it is hoped that from the combined figures, a clear picture of the different banding patterns can be perceived. In contrast to the native enzyme at time = 0, (see also Fig.3.26.2), creatine kinase in the presence of Cu^{2+} displays a different banding pattern. The lower molecular weight band (band 4) is the predominant species, and this becomes more intense as the time of

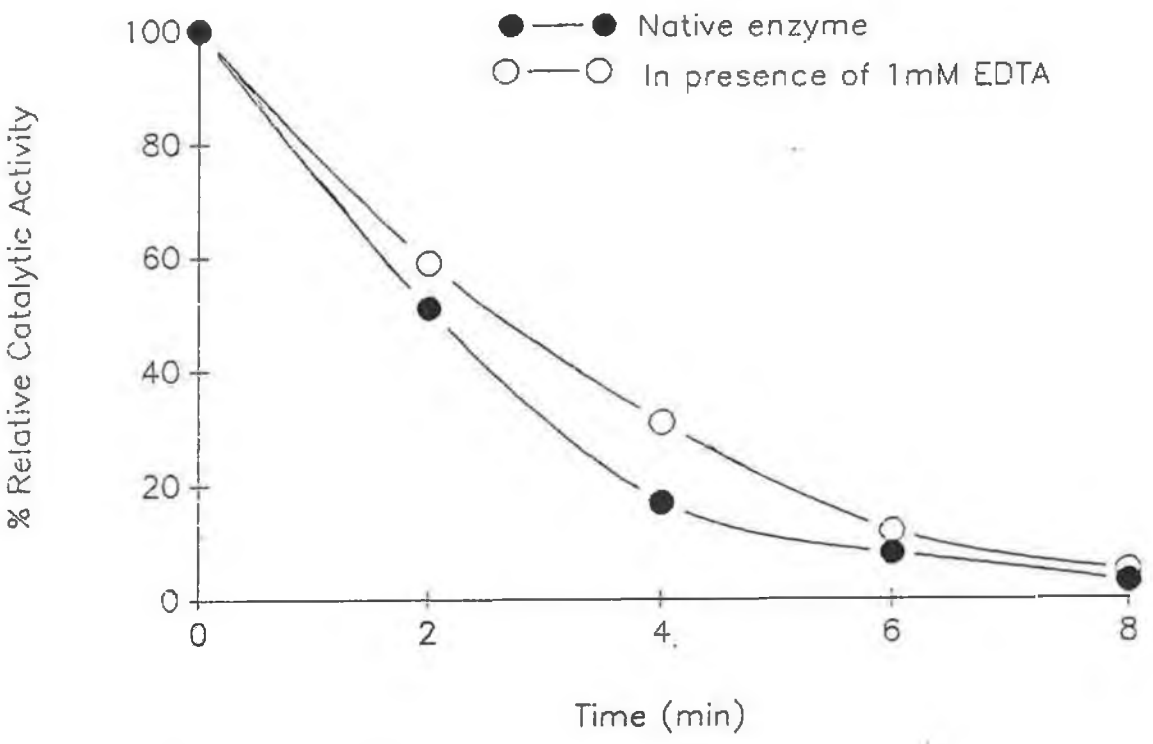


Fig. 3.26.3. Thermodeactivation of creatine kinase at 55°C and pH 6.7 in the absence and presence of 1mM EDTA. The enzyme concentration was 0.2mg/ml in 0.1M imidazole, pH 6.7.

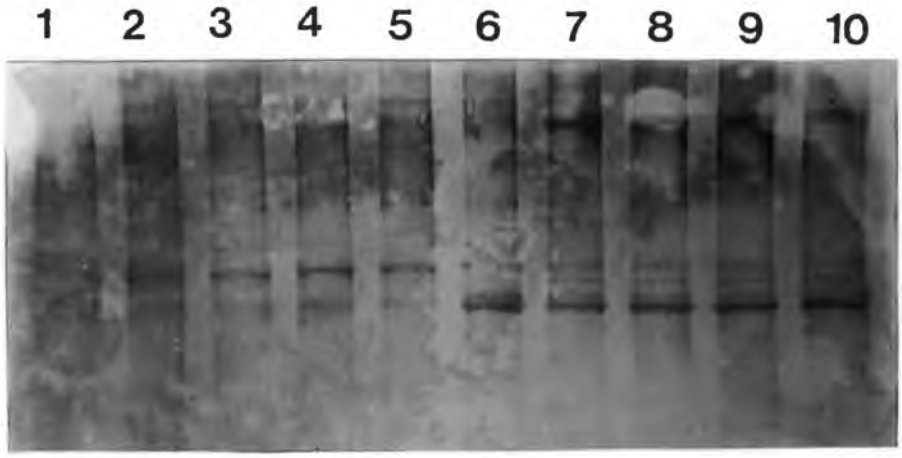


Fig. 3.26.4. SDS-PAGE profiles performed in the absence of 2-mercaptoethanol of creatine kinase thermoinactivated at 55°C in the presence of 1mM EDTA at (1) 0min (2) 2min (3) 4min (4) 6min and (5) 8min; and in the presence of 10μM $CuCl_2$ at (6) 0min (7) 2min (8) 4min (9) 6min and (10) 8min.

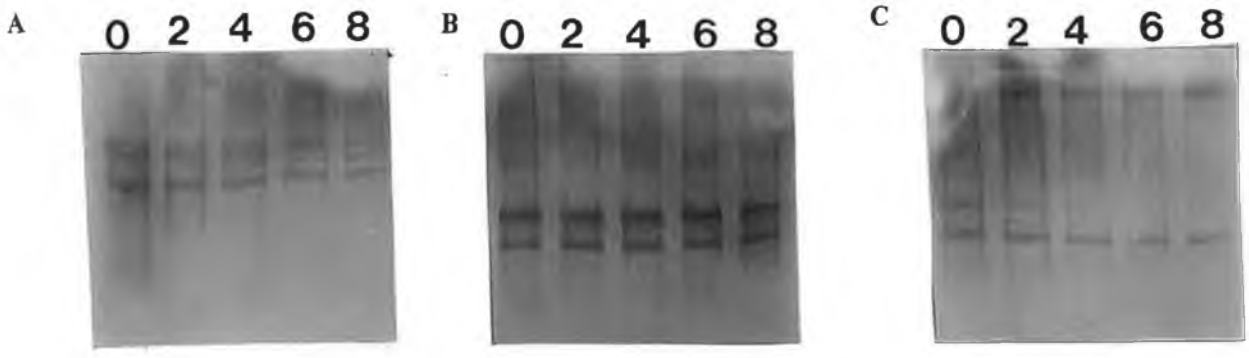


Fig. 3.26.5a,b and c. SDS-PAGE profiles performed in the absence of 2-mercaptoethanol of (a) native enzyme thermoinactivated at 55°C over an 8min time period and (b) enzyme thermoinactivated in the presence of 1mM EDTA and (c) enzyme thermoinactivated in the presence of 10μM $CuCl_2$. The numbers above the lanes denote the time (in minutes) of thermoinactivation .

thermoinactivation is increased. An intense higher molecular weight band is also obvious. It would appear that Cu^{2+} is accelerating a process that is already taking place in the thermoinactivation of the native enzyme.

In the presence of EDTA, band 1 (which corresponds to the monomeric weight of the enzyme) is retained to a higher degree. As stated above, EDTA had a marginal stabilizing effect on the enzyme and this effect is probably reflected in the banding pattern.

It would appear however, that at pH 6.7, heat-induced oxidation of thiol groups in creatine kinase allows disulfide formation to occur, giving rise to intra- and intermolecular disulfides. These incorrect structures are responsible for the loss of activity of creatine kinase.

3.27 Accelerated storage studies:

Accelerated storage studies were performed on creatine kinase in the presence of a number of different additives as described in Section 3.14. It was hoped that some of the reagents added might prevent the heat-induced oxidation described above. The results of this study are shown in Tables 3.27.1-3.27.8 . In general, it appeared that the goodness of fit of the data to the Arrhenius equation was rather poor. In particular, it was noticed with all the data that at 4°C and 26°C, the enzyme suddenly appeared to

lose most of its activity by day 34. Up until day 18-22, the enzyme had been degrading at a much slower rate. This could indicate that the nature of the degradative reaction was qualitatively different at the higher temperatures (Kirkwood and Tydeman, 1984). In fact, some of the data could not even be analysed by the Degtest programme. Of the data that could be analysed, the % activity observed was usually quite different to the % activity predicted by the programme. The Arrhenius equation uses the predicted values to predict the degradation rates at the different temperatures. Therefore, the results of this analysis should be treated with caution.

However, it is worth noting that when the enzyme was crosslinked with DMS and stored in the presence of BSA (Table 3.27.2), the % activity observed was much higher than that of the native enzyme. Enzyme stored in the presence of BSA only (Table 3.27.6), also exhibited a much slower degradation rate compared to the native enzyme. In the presence of EDTA (Table 3.27.3 and 3.27.4), the enzyme was only marginally stabilised. This seems to be in agreement with the results found in Section 3.26. In all of the experiments in which DTE was added (Tables 3.27.5, 7 and 8), the original activity of the enzyme was doubled in its presence. When BSA was added, at the higher temperatures (45°C and 37°C), the enzyme became insoluble and could not be assayed. The DTE probably caused the BSA and creatine kinase to precipitate. However, assays could be performed on the solutions

stored at the lower temperatures. In general though, in these instances, the enzyme seemed to degrade even more quickly than the native enzyme.

It appears from the results presented in this section that the nature of the degradative reaction is different at the higher temperatures. Previous accelerated storage studies (Section 2.28), were not carried out over the same time period as the present study, and therefore did not observe the rapid drop in activity at the lower temperatures. The mechanism of inactivation was not studied at low temperatures in the present work and it is difficult to know what mechanism causes this sudden loss of activity. This problem is worthy of further study and might enable modification of the enzyme to be performed with a view to preventing this inactivation at low temperatures.

Table 3.27.1a. Accelerated degradation test data for native creatine kinase.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	1	6.0	6.00
37	1	40.0	51.85
26	1	75.0	92.48
37	2	17.0	26.89
37	6	14.0	1.94
26	4	46.0	73.14
26	13	35.0	36.18
4	13	70.0	99.34
26	18	26.0	24.47
26	34	7.0	7.00
4	34	12.0	98.27

(*) Relative to samples at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.1b. Predicted degradation rates for creatine kinase at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	6.7×10^{-3}	1×10^{-4}	0.067
20	0.022	1.3×10^{-4}	2.272
37	0.657	1.8×10^{-3}	48.157

Degradation rates were calculated using the predicted remaining activity values given in Table 3.27.1a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 3.27.2a. Accelerated degradation test data for creatine kinase crosslinked with dimethyl suberimidate and stored in the presence of BSA. This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	1	63.0	81.64
37	1	92.0	90.04
45	2	40.0	66.66
37	13	71.0	25.57
45	6	24.0	29.62
37	22	63.0	9.95
26	22	89.0	41.53
26	34	21.0	25.72

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.2b. Predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	0.0047	1.1×10^{-3}	0.469
20	0.0231	2.7×10^{-3}	2.286
37	0.1052	6.2×10^{-3}	9.985

Degradation rates were calculated using the predicted remaining activity values given in Table 2.27.2a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 2.27.3a. Accelerated degradation test data for creatine kinase stored in the presence of 1mM EDTA. This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	1	12.0	67.78
37	1	69.0	80.63
26	1	79.0	91.35
37	2	36.0	65.00
37	4	17.0	42.26
26	4	66.0	69.63
37	13	6.0	6.08
26	13	41.0	30.83
26	22	36.0	13.65
26	34	12.0	4.6
4	34	24.0	65.82

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.3b. Predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	0.0134	7.3×10^{-3}	1.335
20	0.0558	1.5×10^{-3}	5.431
37	0.217	8.7×10^{-3}	19.466

Degradation rates were calculated using the predicted remaining activity values given in Table 3.27.3a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 3.27.4a. Accelerated degradation test data for creatine kinase stored in the presence of 10mM EDTA. This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	1	9.0	10.12
37	1	60.0	61.36
26	1	87.0	95.03
37	2	22.0	37.65
26	4	63.0	81.54
37	6	10.0	5.34
26	13	47.0	51.52
4	13	94.0	99.58
26	22	33.0	32.55
26	34	9.0	17.65
4	34	17.0	98.90

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.4b. Predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	3.3×10^{-3}	1×10^{-4}	0.033
20	0.0136	2×10^{-3}	1.377
37	0.4884	5×10^{-3}	38.638

Degradation rates were calculated using the predicted remaining activity values given in Table 3.27.4a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 3.27.5a. Accelerated degradation test data for creatine kinase stored in the presence of 5mM DTE. This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY OBSERVED	% ACTIVITY REMAINING (*) PREDICTED
45	1	2.00	72.34
37	1	32.00	83.16
26	1	49.00	92.24
37	2	10.00	69.15
37	4	3.00	47.82
26	4	37.00	72.40
26	13	28.00	35.01
4	13	24.00	85.61
26	22	20.00	16.93
26	34	6.00	6.43
4	34	7.00	66.61

(*)Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.5b. Predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	0.0132	2.5×10^{-3}	1.313
20	0.0512	2.7×10^{-3}	4.990
37	0.1857	0.014	16.949

Degradation rates were calculated using the predicted remaining activity values given in Table 3.27.5a using the Degtest programme as described by Kirkwood (1984) as described in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 3.27.6. Accelerated degradation test data for creatine kinase stored in the presence of BSA (80g/l). This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY (*) OBSERVED
45	1	28.00
37	1	71.00
37	2	57.00
45	2	4.00
26	4	92.00
37	6	42.00
37	13	5.00
4	13	98.00
26	22	85.00
26	34	19.00
4	34	20.00

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.7. Accelerated degradation test data for creatine kinase crosslinked with DMS and then stored in the presence of BSA (80g/l) and DTE (5mM). This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY (*) OBSERVED
37	1	86.00
37	4	34.00
26	4	54.00
37	6	26.00
26	13	44.00
4	13	68.00
26	18	37.00
26	34	9.00
4	34	12.00

(*) Relative to samples stored at -20°C.

Accelerated storage studies were performed as described in Section 3.14.

Table 3.27.8. Accelerated degradation test data for creatine stored in the presence of BSA (80g/l) and DTE (5mM). This was performed as described in Section 3.14.

TEMP. (°C)	TIME (days)	% ACTIVITY (*) OBSERVED
26	1	87.00
26	4	30.00
4	13	45.00
26	22	23.00
26	34	9.00
4	34	10.00

(*) Relative to samples stored at -20°.

Accelerated storage studies were performed as described in Section 3.14.

SUMMARY

Investigation of the irreversible thermoinactivation of creatine kinase suggests that inactivation results from conformational processes. Covalent processes such as peptide chain hydrolysis and deamidation of Asn and Gln residues, appeared not to be involved in the thermoinactivation of the enzyme. A number of approaches have given evidence to suggest that incorrect structure formation is the cause of irreversible inactivation. The cause of incorrect structure formation can be attributed to heat-induced oxidation of thiol groups, which allows disulfide formation to occur. The formation of these intra- and intermolecular disulfides seems to be the cause of the loss of activity of creatine kinase upon heating.

Accelerated storage studies performed on the enzyme in the presence of a number of different additives indicated that only BSA and DMS had any significant stabilizing effect on the enzyme.

DEVELOPMENT AND STANDARDIZATION OF A MICROASSAY FOR CREATINE KINASE

4.1 Introduction:

Work has recently been reported on the use of microplate readers to determine the activities of a number of enzymes (Cribb et al.,1989; Florini, 1989; Butler et al.,1988). This chapter describes the determination of creatine kinase activity using an assay which conforms to the recommendations of the Deutsche Gesellschaft fur Klinishe Chemie (Section 2.5.1) employing a programmable microplate reader. The advantages of such an approach include the speed of assay performance, economy in the use of reagents, high throughput of samples, the small sample volumes required, multiple analysis on each sample, automatic mixing of samples and good temperature control.

4.2 Materials:

Bovine heart creatine kinase and all of the reagents used to assay this enzyme were purchased from Sigma Chemical Co., Dorset, England. Creatine kinase was purified as described in Section 2.3. The Titertek Twinreader Plus type 381 was obtained from Flow Laboratories Ltd., Scotland. Nunc 96-well microtitre plates were purchased from Gibco, Ltd., Paisley, Scotland.

4.3 Methods:

The creatine kinase assay mixture contained 30mM creatine phosphate, 10mM Mg-acetate, 2mM ADP, 5mM AMP, 10μM diadenosine pentaphosphate, 2mM NADP, 20mM glucose, 20mM N-acetylcysteine, 2.5 U/ml hexokinase and 1.5 U/ml G6PDH in 0.1M imidazole-acetate buffer, pH 6.7. Aliquots of the assay mixture (250μl) were dispensed into the 96-well plates using the dispensing function of the microplate reader and the plates were incubated at 30°C for approximately 30min. The reaction was initiated by the addition of creatine kinase (10μl). This can be rapidly achieved using a multipipette or an automatic dispensing pipette. For blank values no creatine kinase was added. Accuracy in timing initiation of the reactions is not critical. The microplate reader reads one column at a time and each well is read at 60s intervals. The slope of the line is automatically calculated by the microplate reader which also subtracts the blank values. The automatic mix function on the microplate reader was used to shake the plate after the addition of creatine kinase to the wells. The plate was then incubated for an additional 60s which was necessary to eliminate any effects of the lag period which is often observed with this assay and which can probably be attributed to the time required for the accumulation of intermediates. Thereafter, the absorbance at 340nm was measured every 60s for 8min at 30°C. Serum samples were analysed by the method described and also the standard method

(Anon,1977). Undiluted and various dilutions of serum samples were tested (1:4 to 1:50). All samples were measured in quadruplicate. Creatine kinase activity measured on the microplate reader was determined using the following equation:

$$\text{Activity (U/l)} = \frac{\Delta A/\text{min} \times 10^6 \times 0.26 \times 0.74}{4{,}662 \times 0.01}$$

where $\Delta A/\text{min}$ = the absorbance change per minute at 340nm.

4,662 = the molar extinction coefficient of NADPH at 340nm and a pathlength of 0.74cm.

0.260 = total volume in the well (ml).

0.010 = volume of creatine kinase added to the well (ml).

0.74 = measured pathlength (cm).

The pathlength was determined for a volume of 260μl in the microtitre well. The manufacturers of the microtitre plate had specified that for a volume of 396μl, the height of the well was equal to 1.125cm. The microtitre plate reader reads the plates vertically, therefore, height = pathlength, and for a volume of 260μl, the pathlength was determined to be 0.74cm.

One unit of creatine kinase is defined as that amount of enzyme which catalyses the production of 1 μmole of NADPH per minute under the above conditions.

4.4 Results and Discussion:

It was necessary to obtain the optimum concentration range of creatine kinase for use with the microassay as the total well volume used was 260μl. Purified creatine kinase was used for these experiments. The rate of increase in absorbance of creatine kinase was linear over 8min during which it was determined for up to 186units/litre (Fig. 4.4.1) and the rate of increase in absorbance at each concentration of creatine kinase was also linear over this period (Fig. 4.4.2). The accurate range of measurement was found to be between 0.002 and 0.05 absorbance change per minute. At concentrations of creatine kinase which give a higher absorbance change per minute than this, the linear relationship was lost.

Measurement of serum creatine kinase activity is commonly employed as an adjunct for diagnosing myocardial infarction and a variety of myopathies (Konttinen and Somer,1973; Lee and Goldman, 1986; Leung et al.,1991). In order to determine the applicability of the miniaturised assay for measuring the activity in clinically relevant samples, serum containing normal (15-130 U/l) and elevated levels of creatine kinase was assayed and compared to the values obtained when the serum was assayed using the conventional procedure.

Initial experiments were performed to determine the range of linearity with the change in absorbance per minute of undiluted

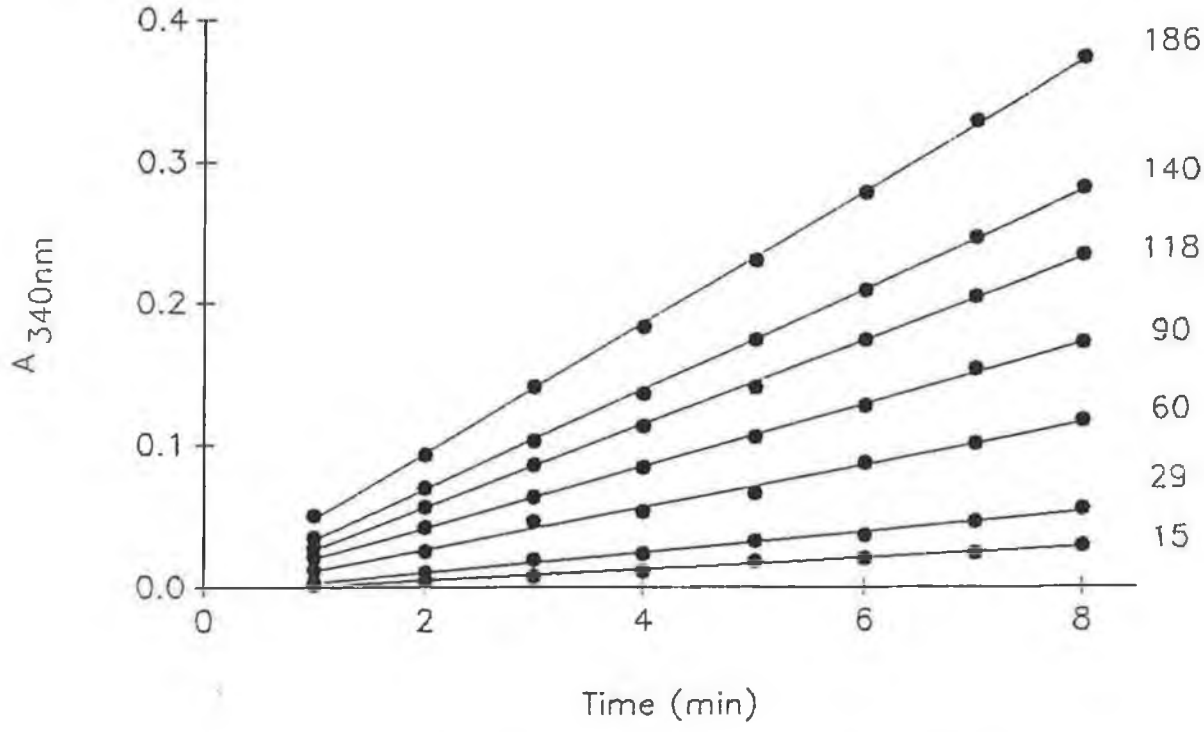


FIG.4.4.1. Time course of creatine kinase reaction in a microtitre plate. Incubation conditions were as specified earlier. The numbers near the lines on the right hand side are the units per litre of creatine kinase added per well. Each point represents the mean of four replicate samples.

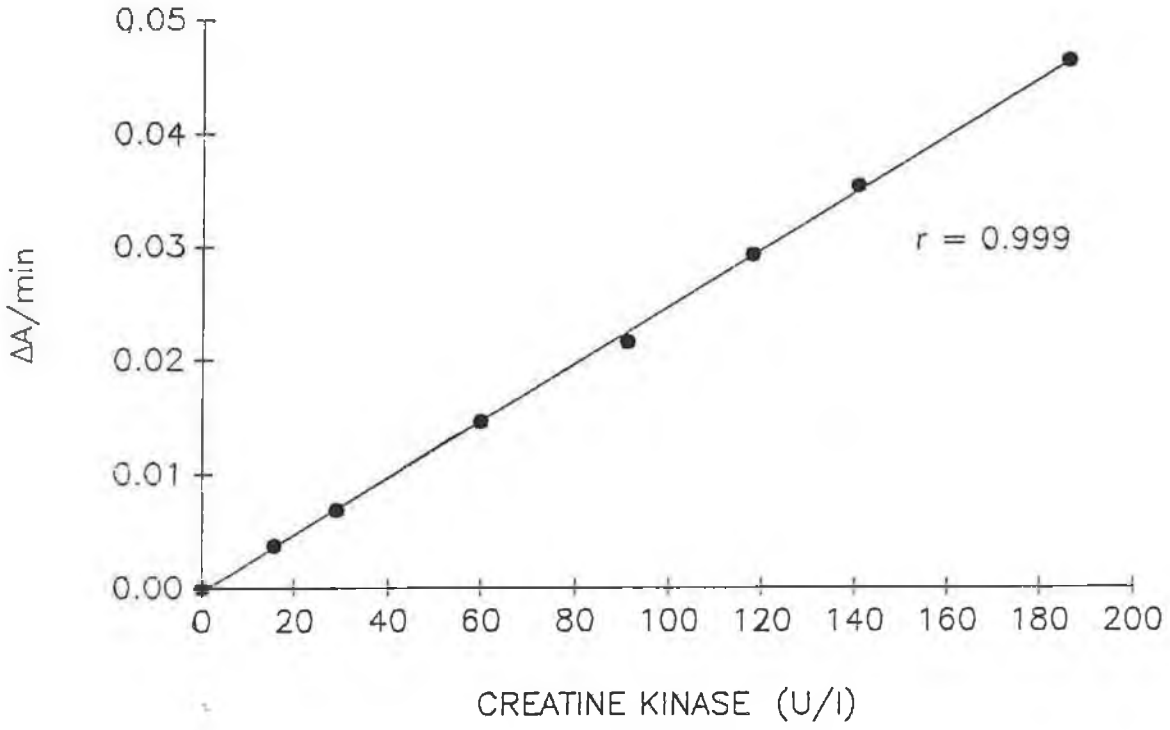


FIG.4.4.2. Linear relationship between rate of increase in absorbance per minute at 340nm and creatine kinase concentration. Creatine kinase activity is expressed as U/l of added sample. Absorbance was measured at 60s intervals over 8 min.

creatine kinase activity was up to an absorbance change per minute of 0.03. A standard curve was constructed from a serum sample containing elevated levels of creatine kinase (Fig. 4.4.4). Dilution of the serum increased the range of linearity up to $\Delta A/min$ of 0.07. The correlation coefficient for this line was 0.998. The rate of increase in absorbance at each concentration of creatine kinase was linear over the time period during which it was determined (Fig. 4.4.5). It is recommended that at least a 3-fold dilution of serum containing high levels of creatine kinase is necessary in order to obtain accurate values in the microassay.

Serum creatine kinase assayed using the miniaturised procedure was then compared to the values obtained when the enzyme was assayed using the conventional procedure (Section 2.5.1). The results are shown in Fig. 4.4.6. The correlation coefficient was 0.998, indicating that the two assay methods compared extremely well. The serum was also assayed using a Beckman kit and measured on a Centrifuchem Centrifugal Analyser (Fig. 4.4.7). Again, the microassay compared very well with these results. The above results indicate that the miniaturised procedure is suitable for determining the activity of creatine kinase.

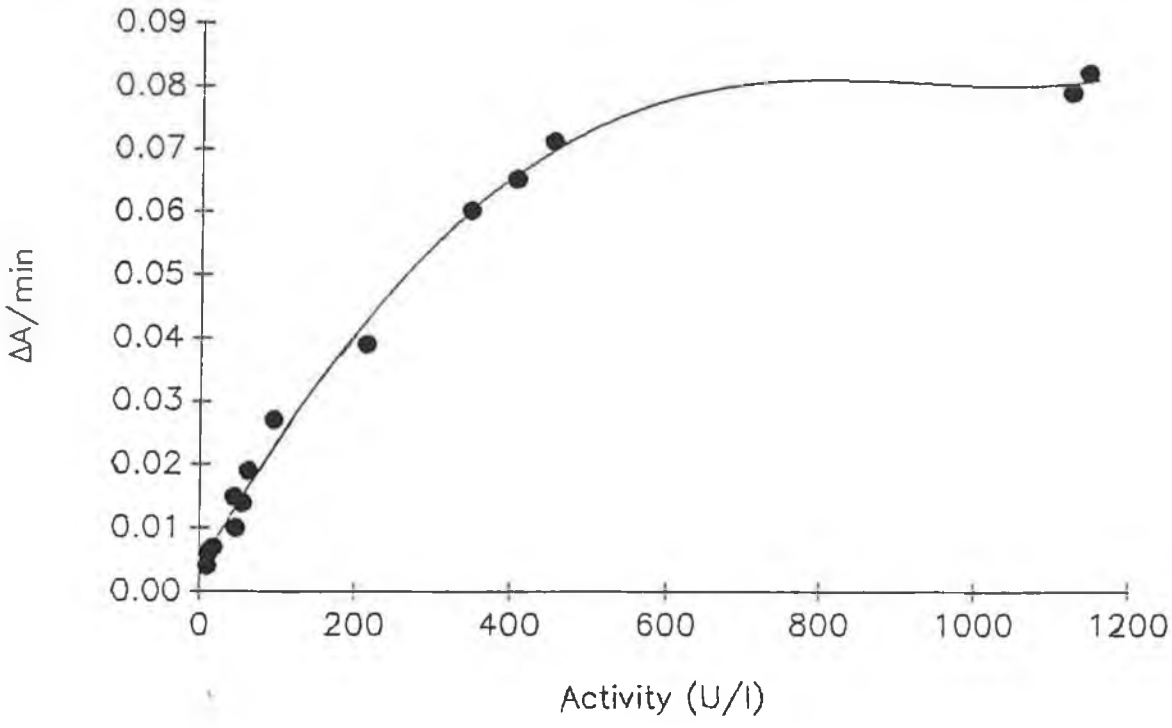


FIG.4.4.3. The rate of change of absorbance per minute (ΔA/min) as a function of undiluted serum creatine kinase reaction in a microtitre plate.

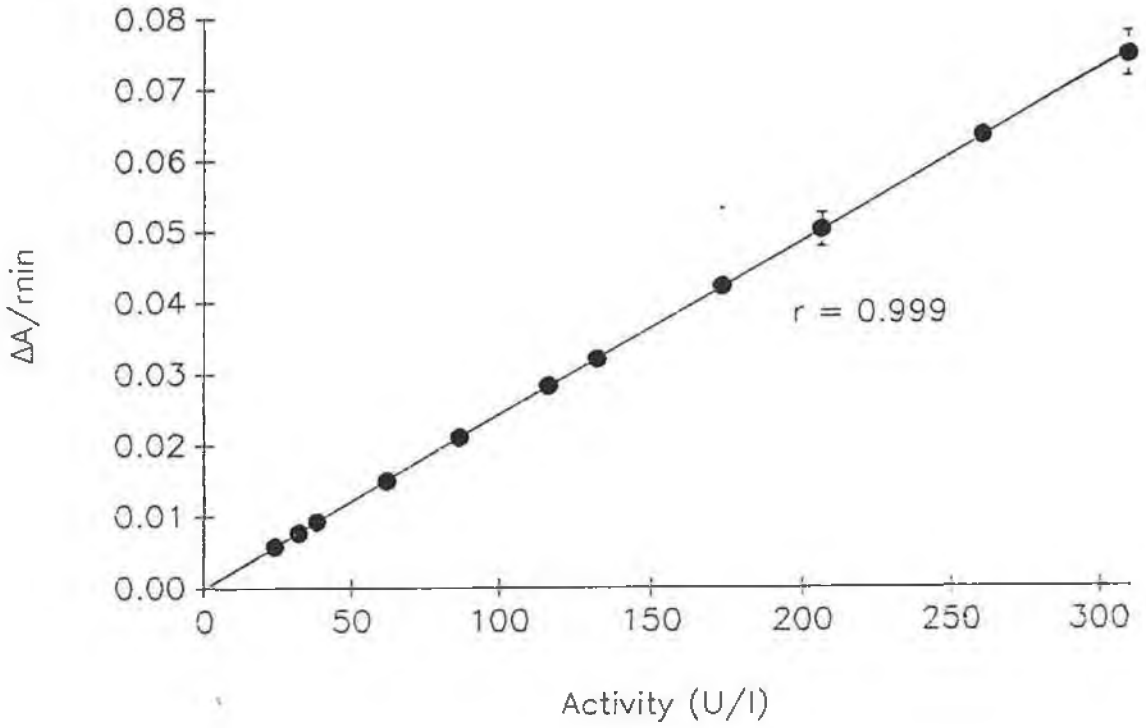


FIG..4.4.4. Linear relationship between rate of increase in absorbance per minute at 340nm and serum creatine kinase concentration. SEM bars are plotted, but in most cases are smaller than the points and thus are not visible.

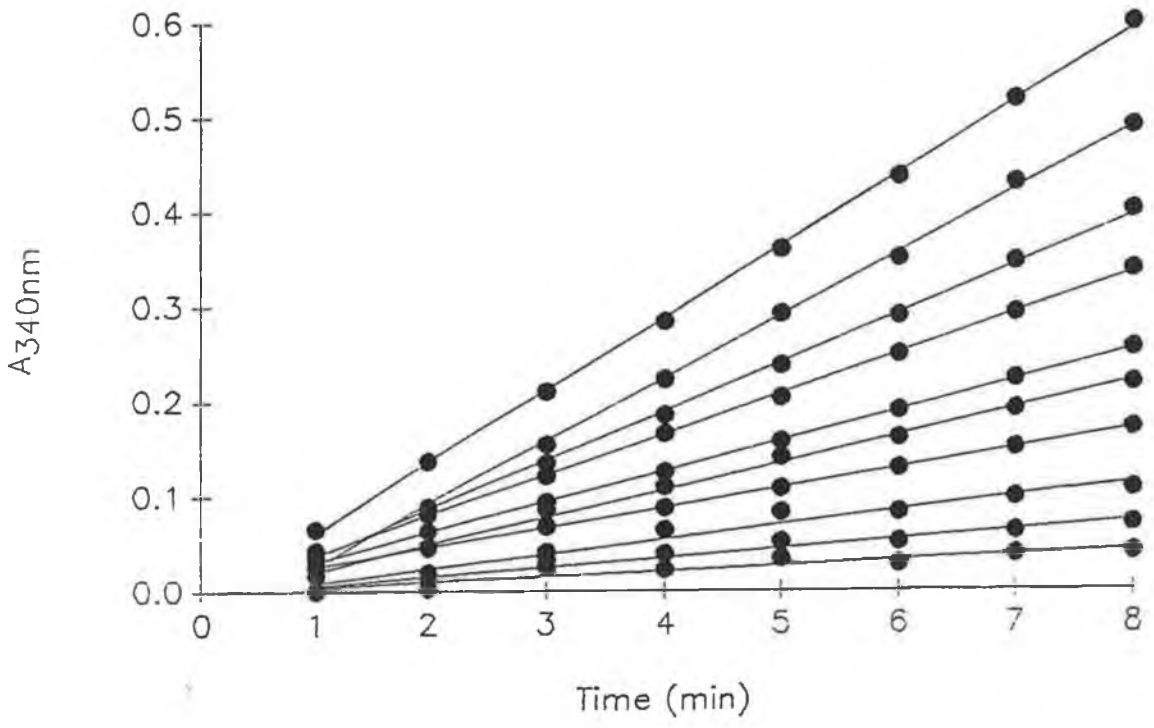


FIG.4.4.5. Time course of serum creatine kinase reaction in a microtitre plate showing the linearity observed with time at 340nm of the serum samples from Fig.4.4.4.

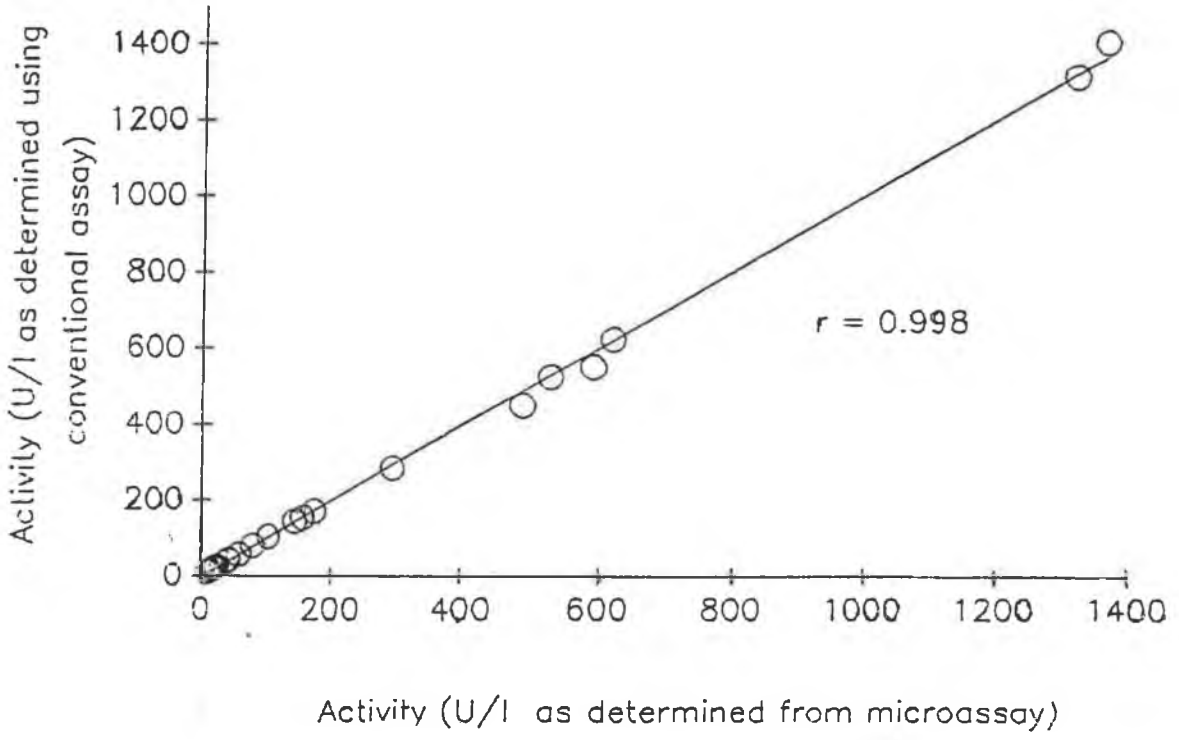


FIG.4.4.6. Correlation of creatine kinase levels obtained using the miniaturised assay procedure on serum samples with the activity levels determined on the same samples using the full scale conventional assay procedure.

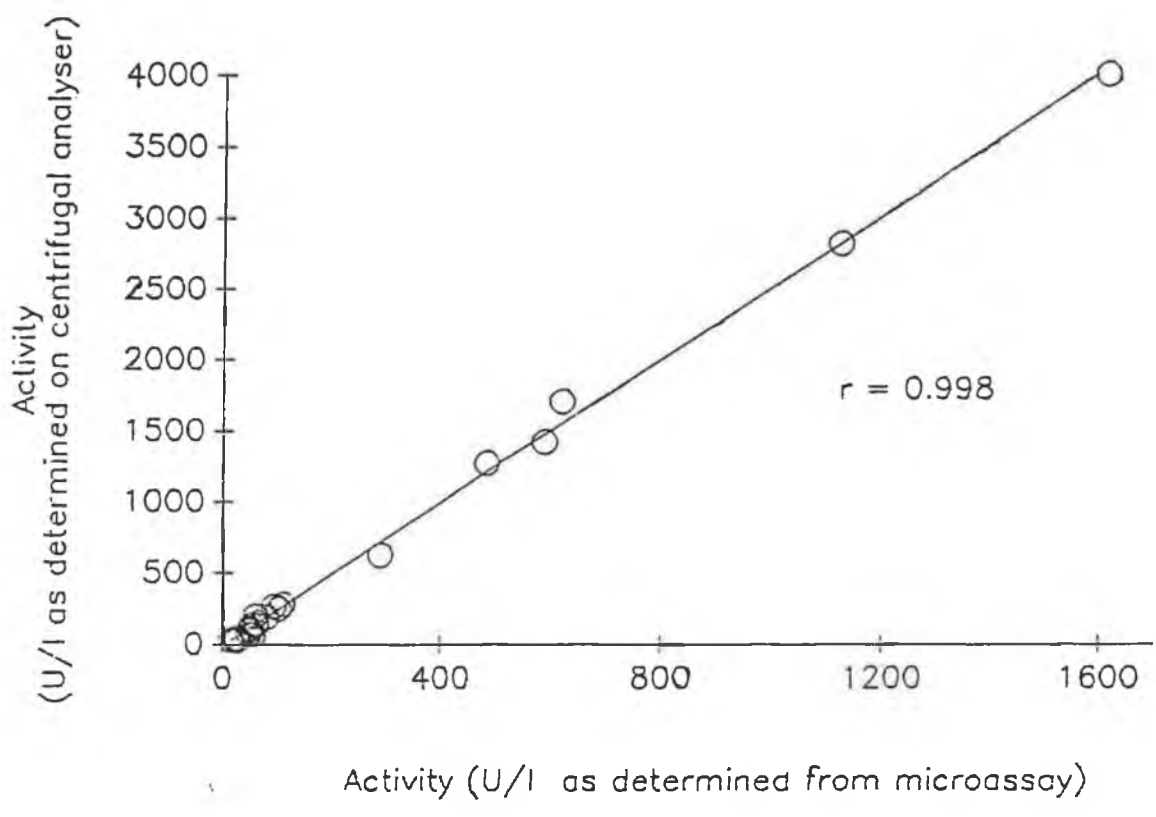


FIG.4.4.7. Correlation of creatine kinase activity levels obtained using the miniaturised assay procedure on serum samples with the activity levels on the same samples using a Beckman kit and a Centrifuchem analysis system.

SUMMARY:

This work has described the adaptation of an existing assay for measuring creatine kinase and its implementation for use with a programmable microplate reader and microtitre plates. It can be used to measure serum creatine kinase levels and has proved extremely useful in studies performed on the stabilization of purified creatine kinase (see Chapter 3). The method allows for a large number of samples to be assayed at one time, it is rapid and, in view of the expensive reagents required for the assay, very economical.

N-TERMINAL SEQUENCE ANALYSIS OF BOVINE HEART CREATINE KINASE

5.1 INTRODUCTION:

To date, little or no information has been available in the literature concerning the amino acid composition or the sequence data for bovine heart creatine kinase. This information is obviously necessary if studies such as improving the thermal stability of the enzyme using site-directed mutagenisis is to be performed. The aim of this work was to determine the N-terminal amino acid sequence of bovine heart creatine kinase. This in itself might prove difficult as most of the published kinase sequences have been obtained through translation of mRNA sequences (Hossle et al.,1988; Roman et al.,1985; Giraudat et al.,1984) as the protein has appeared to be difficult to purify and sequence. If an N-terminal sequence could be determined, this would enable an oligonucleotide primer to be made, which could be used to probe a cDNA bovine heart library in order to isolate the cloned creatine kinase gene. Having sequenced the gene, the open reading frame could then be translated into an amino acid sequence. This would allow studies on the site-directed mutagenisis of creatine kinase to be carried out with a view to improving the thermal stability of the enzyme.

5.1 Materials:

Polybrene and the C18 Microbore RP-column were purchased from Applied Biosystems. All other reagents used for reduction,

carboxymethylation and sequencing were Analytical or sequencing grade reagents.

Methods:

5.2 Reduction and carboxymethylation:

This was performed as described by Allen, (1989) using the following procedure.

The protein was dissolved at 1-20mg/ml in a solution containing 6M guanidium chloride, 0.1M Tris and 1mM EDTA, and adjusted to pH 8.3 with HCl. Dithiothreitol was added to a concentration of 2mM. Nitrogen was passed through the solution, which was then sealed under the inert atmosphere and incubated at 37°C for 1 h. A solution of 5mM iodoacetamide was added to give a 1.1-fold molar excess over total thiol groups in the solution. Nitrogen was then blown over the surface of the reaction mixture, the vessel was sealed, and alkylation was allowed to proceed in the dark at 37°C for 1 h. 2-Mercaptoethanol was then added to a concentration of 1%(v/v), and the solution was dialysed exhaustively against 50mM NH_4HCO_3/0.01% (v/v) thiodiglycol, and freeze-dried.

5.3 Amino acid sequence determination:

Protein samples were analysed using the Applied Biosystems pulsed liquid sequencer type 475A with the on-line PTH-analyzer type 120A. The material was applied onto a glass fiber filter disc which had already pre-run in the presence of 1mg Polybrene (1,5-dimethyl 1,5-diazaun-decamethylene,). The sequence run was then directly started using standard cycles. The temperature of the cartridge and of the conversion flask were 45°C and 55°C, respectively. The PTH derivatives were on-line analyzed using a C18 Microbore RP-column at 55°C. Separation was achieved using a gradient of 5% THF in water and acetonitrile (including 50nmol DMPT U/l). Detection was done at 270nm (0.02 AUFS).

RESULTS AND DISCUSSION:

5.4 Determination of the N-terminal sequence of creatine kinase from bovine heart:

Reduction and carboxy-amidoethylation were performed on purified creatine kinase as described in Section 5.2. Amino acid sequence determination of the modified protein was then carried out as described in Section 5.3. The following N-terminal sequence was found:

?-Phe-Gly?-Asn-Thr-His?-Asn-

This sequence was in fairly good agreement with the N-terminal sequences of the creatine kinase M-chain from rabbit in particular, and also from chicken which are as follows:

Rabbit Met-Pro-Phe-Gly-Asn-Thr-His-Asn-Lys-Tyr-Lys-Leu-Asn-Tyr-Lys-Ser-

Chicken Met-Pro-Phe-Ser-Ser-Thr-His-Asn-Lys-Tyr-Lys-Leu-Lys-Phe-Ser-Ala-

However, as some of the residues in the above sequence for bovine heart were not absolutely conclusive, it was decided to attempt another N-terminal analysis. In this instance, the crude protein sample (18mg) was reduced and carboxyamidoethylated as described before. The material was then purified by SDS-polyacrylamide electrophoresis and electroblotted onto Problott, a PVDF membrane especially designed for use in the Applied Biosystems Sequencer. The monomeric band of about 40kD was cut out and analysed (see Fig.5.4). The outcome of the analysis was the following:

Pro-Phe-Gly-Asn-Thr-His-Asn-Lys-Asp-Lys-Leu-Asn-Phe-Lys-Ala-Glu-

The above sequence of 16 amino acids was in full agreement with the earlier analysis and also gave additional information for those residues which had not been assigned before. Therefore, this N-terminal sequence of the M-chain of bovine heart creatine kinase, should provide the groundwork to facilitate the isolation of the creatine kinase gene from genomic libraries. Bovine heart cDNA libraries have been constructed (Gay and Walker, 1984; Breen et al.,1988) and would probably be available upon request.

In view of the results presented in Chapters 2 and 3, a basic knowledge of which residues are involved in maintaining stability and which residues contribute to instability is known. This could provide an indication of which amino acid residues could be changed in site-directed mutagenisis studies. This area is now proving to be very successful in improving the thermal stability of enzymes (Eijsink et al.,1991) and if pursued, could contribute significantly to improving the thermal stability of bovine heart creatine kinase. Stabilization work, on the results described, is now in progress in our laboratory.

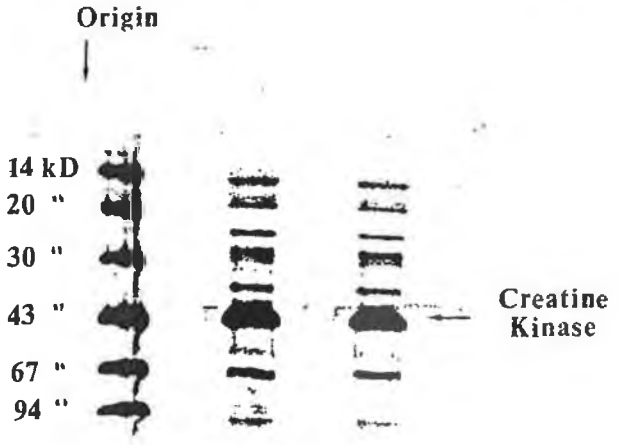


Fig. 5.4. Crude creatine kinase after reduction and carboxyamidoethylation was electrophoresed on an SDS-polyacrylamide gel before being purified by electroblotting onto Problott. The creatine kinase band of about 40kD (see arrow) was then cut out and analysed. Marker proteins and their molecular weights are indicated.

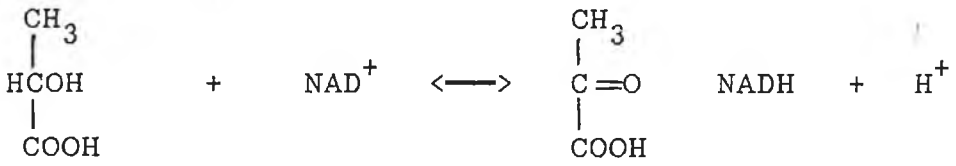
CHEMICAL MODIFICATION OF LACTATE DEHYDROGENASE

6.0 INTRODUCTION:

The enzymes, lactate dehydrogenase (EC 1.1.1.27) and aspartate aminotransferase (EC 2.6.1.1) are widely used as clinical diagnostic markers. Their levels in serum act as a key indicator of liver and other functions, in a variety of clinical states, particularly hepatitis and myocardial disease (Schwartz, 1971).

Commercial control sera containing known amounts of diagnostically important enzymes are used to validate the results obtained in clinical laboratories. However, the stability of enzyme activities in such controls is limited. Therefore, a brief study was undertaken, using some of the methodologies described in Chapter 2 for the modification of creatine kinase, in order to identify possible means of modification which might lead to the increased stabilities of these two clinically relevant enzymes.

LDH catalyses the following reaction:



Lactate Pyruvate

The enzyme is a tetrameric molecule (Markert and Moller, 1959). It contains two polypeptide chains, the distribution of which is dependant on whether the enzyme extract originated in aerobic

tissue such as heart (where the H_4 isozyme predominates) or in anaerobic tissue as in skeletal muscle (where the M_4 isozyme predominates) (Pfleiderer and Wachsmuth, 1961). The molecular weight of the tetramer is 140,000 daltons with a subunit molecular weight of 35,000 daltons (Pickles et al., 1964). The enzyme contains one essential cysteine residue per subunit. No disulfide bridges have been reported for LDH. The enzyme also contains an essential histidine residue.

The following chapter reports the results obtained when LDH from chicken heart was subjected to chemical modification and crosslinking with a view to improving the thermal stability of the enzyme.

6.1 Materials:

Chicken heart LDH was a kind gift from Dr. A. Posner, Baxter-Dade, Miami 33152-0672. Carboxymethyl- (CM-52) cellulose was obtained from Whatman, UK. All chemicals used in the assay of LDH and all chemicals used for LDH modification were purchased from Sigma Chemical Co., Dorset, England.

6.2 Purification of Lactate Dehydrogenase from Chicken Heart:

This was performed as follows: An ammonium sulfate suspension of LDH was dialysed overnight against 4 x 1l of 0.02M sodium

sulphate, pH 6.0. The enzyme was then applied to a CM-52 column (2.5cm x 10cm),which had been previously equilibrated with the above buffer. The active fractions were pooled and immediately lyophilized. Purity of the enzyme was established by SDS-polyacrylamide gel electrophoresis.

6.3.1 Assay of Lactate Dehydrogenase:

LDH activity was determined according to the method described by Bergmeyer (1974) which follows the rate of decrease in absorbance at 340nm as NADH is oxidised by pyruvate.

$$\text{Pyruvate} + \text{NADH} + H^+ \quad \langle\!=\!=\!=\!\rangle \quad \text{Lactate} + NAD^+$$

The following reagents were required for the assay.

Reagent A: Phosphate / pyruvate (50mM phosphate, pH 7.5; 0.63mM pyruvate).

Reagent B: Reduced nicotinamide-adenine dinucleotide (11.3mM β-NADH).

3 ml of reagent A was added to a cuvette followed by 50μl of reagent B. Finally, 100μl of LDH sample was added, the contents of the cuvette were mixed and the mean absorbance change per minute (ΔA/min) at 340nm was then determined and used to calculate LDH activity. All units are expressed as μmol/min at 25°C.

Calculation of activity: Units/litre (25°C) = 5,064 x A_{340nm}/min.

6.3.2 Protein measurement:

The protein concentration for pure enzyme was determined by measuring the absorbance at 280nm and then using the relationship $A^{1\%} = 13.8$ (Tuengler and Pfleiderer, 1977) and a molecular weight of 140,000 daltons (Pickles et al., 1964).

6.3.3 Total Activity of LDH:

The total activity was defined as the number of units in 1 ml of enzyme solution multiplied by the total volume of that solution.

6.3.4 Specific Activity of LDH:

The specific activity was defined as the number of units of activity per mg of pure protein.

6.4 Thermodeactivation of LDH:

Thermodeactivation of LDH (100μg/ml) was performed in 0.1M phosphate buffer, pH 7.5, at 75°C. Accelerated degradation studies were performed in the same buffer at temperatures of 45°C, 37°C and 26°C. The reference samples were stored at -20°C. All samples were filtered through low protein binding 0.22 μm membrane filters

prior to storage in order to sterilise the samples.

6.5 Guanidination with O-methylisourea:

This was performed as described by Minotani et al.,(1979) and Cupo et al., (1980). LDH was dissolved in 0.1M phosphate buffer, pH 10. Varying amounts of O-methylisourea were added to the solution and the reaction allowed to proceed at 4°C for 24h. The reaction was stopped by passage through a Sephadex G-25 column (1.5 x 7cm) using 0.1M phosphate, pH 7.5, as the eluting buffer.

6.6 Crosslinking with glutaraldehyde:

Crosslinking was performed as described by Klemes and Citri, (1979). 1, 2 and 5μl of a 25% (v/v) solution of glutaraldehyde were added to 500μl of a 1mg/ml solution of LDH in 0.05M sodium phosphate buffer, pH 7.8. The reaction was allowed to proceed for 20min, after which time it was terminated by the addition of sodium borohydride to a final concentration of 0.05M, and incubated for an additional 20min at 4°C. The enzyme derivative was separated from low molecular weight material by fractionation through a Sephadex G-25 column as described previously.

6.7 Reaction with Succinic Anhydride:

This was performed as follows: 1, 0.2 and 0.05mg of succinic anhydride were added to a 0.5mg/ml solution of LDH (650µl) in 0.1M phosphate, pH 8.0. The reaction time was 12hrs after which time it was stopped by gel filtration through a Sephadex G-25 column.

6.8 Crosslinking with Dimethyl Adipimidate:

Crosslinking was performed as described by Minotani et al., (1979). Reaction of the enzyme (1mg/ml) with dimethyl adipimidate was performed in 0.1M phosphate buffer, pH 10, for 1h at room temperature. The reaction was terminated by passing the solution through a Sephadex G-25 column.

RESULTS AND DISCUSSION:

6.9 Purification of lactate dehydrogenase from chicken heart:

The enzyme was purified as described in Section 6.2. Typical results from a purification are shown in Table 6.9 and a typical elution profile is shown in Fig. 6.9.1. A 55% recovery of LDH activity and a 96% recovery of protein was obtained. The high protein recovery was due to the fact that only very minor high molecular weight bands were present in the LDH preparation prior to purification. These proteins remained bound to the CM-52 column, whereas the LDH did not bind at all, but was eluted immediately. The reason for the low recovery of LDH activity was not clear. The purified protein had a specific activity of 287 U/mg. SDS-polyacrylamide gel electrophoresis of the lyophilized protein was performed as described in Section 2.4 and revealed only one protein band (Fig.6.9.2) corresponding to the molecular weight of the monomer of approximately 35,000 daltons.

Table 6.9. Purification of chicken heart lactate dehydrogenase:

Purification Step	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)
Crude LDH preparation	14.5	5,956	86,360	11.9	173	499
After purification on CM-52	44	1,084	47,680	3.8	166	287

Protein was measured by absorbance at 280nm as described in Section 6.3.2 and LDH was assayed as described in Section 6.3.1 .

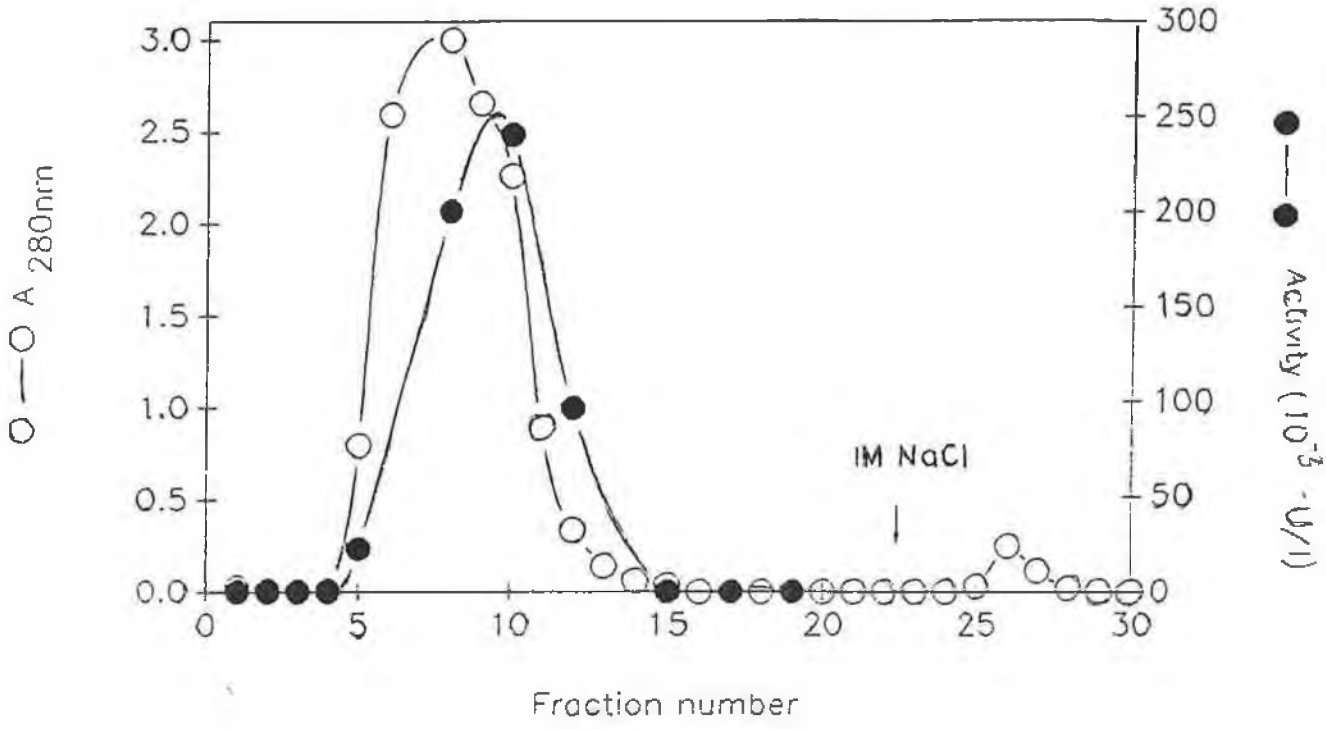


Fig. 6.9.1. The elution profile of LDH after chromatography on a CM52 ion exchange column. The enzyme was assayed as described in Section 6.3.1. and protein measurements were performed as described in Section 6.3.2.

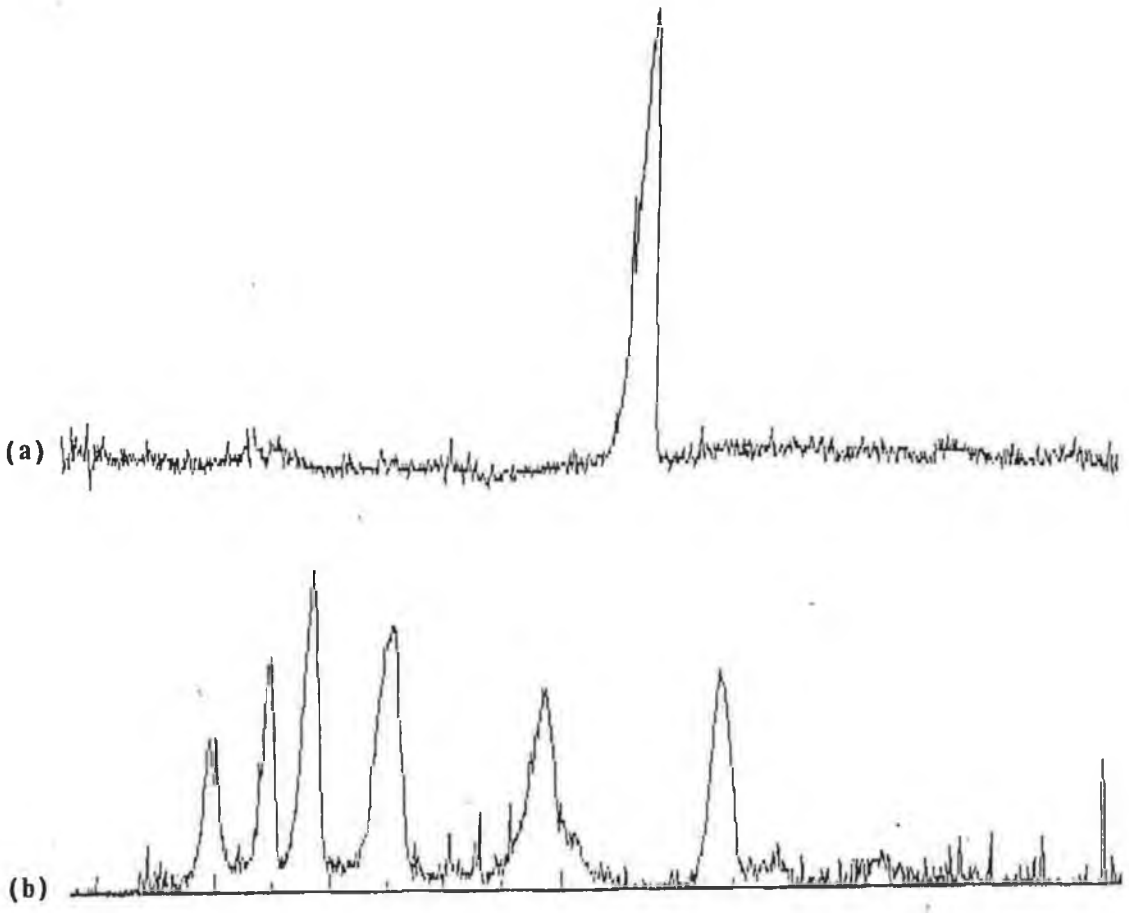


Fig. 6.9.2. The SDS-PAGE electrophoretic profiles of LDH after purification. SDS-PAGE was performed as described in Section 2.4. on a 10% gel. (a) shows pure LDH with an estimated molecular weight of 36,000 daltons. (b) shows the molecular weight markers used, which are from left to right as follows; myosin (205,000 Da); β-galactosidase (116,000 Da); phosphorylase B (97,400 Da); bovine albumin (66,000 Da); ovalbumin (45,000 Da); and carbonic anhydrase (29,000 Da).

6.10 Guanidination with O-methylisourea:

This was performed as described in Section 6.5. Varying amounts of O-methylisourea (2, 5, 10 and 20 mg), were added to a 0.64mg/ml solution of LDH to initially try and determine the optimum amount of O-methylisourea required. LDH treated with 10 and 20mg of O-methylisourea lost all of its original activity after the reaction. LDH treated with 2 and 5mg retained all of its activity. Thermodeactivation was performed on these samples and the results are presented in Fig. 6.10. As can be seen, there was some degree of stabilization, although not very significant. Since the LDH modified with 5mg O-methylisourea showed the best stability, it was decided to try to modify the enzyme with amounts of the reagent varying between 5 and 9mg. The results of thermodeactivation of those samples are shown in Table 6.10.1.

It was of interest to note that after the reaction with O-methylisourea, the enzyme lost very little of its original activity. At 8mg, LDH had lost only 8% activity. However, when reacted with 9mg, the enzyme lost 62% of its original activity and a precipitate was observed.

It appeared as if maximum stabilization was occurring when between 8 and 9mg of O-methylisourea were used and so amounts of the reagent varying between 8 and 9mg were used and thermodeactivation performed. For clarity, the results are presented in tabular form in Table 6.10.2. Once again, the 9mg

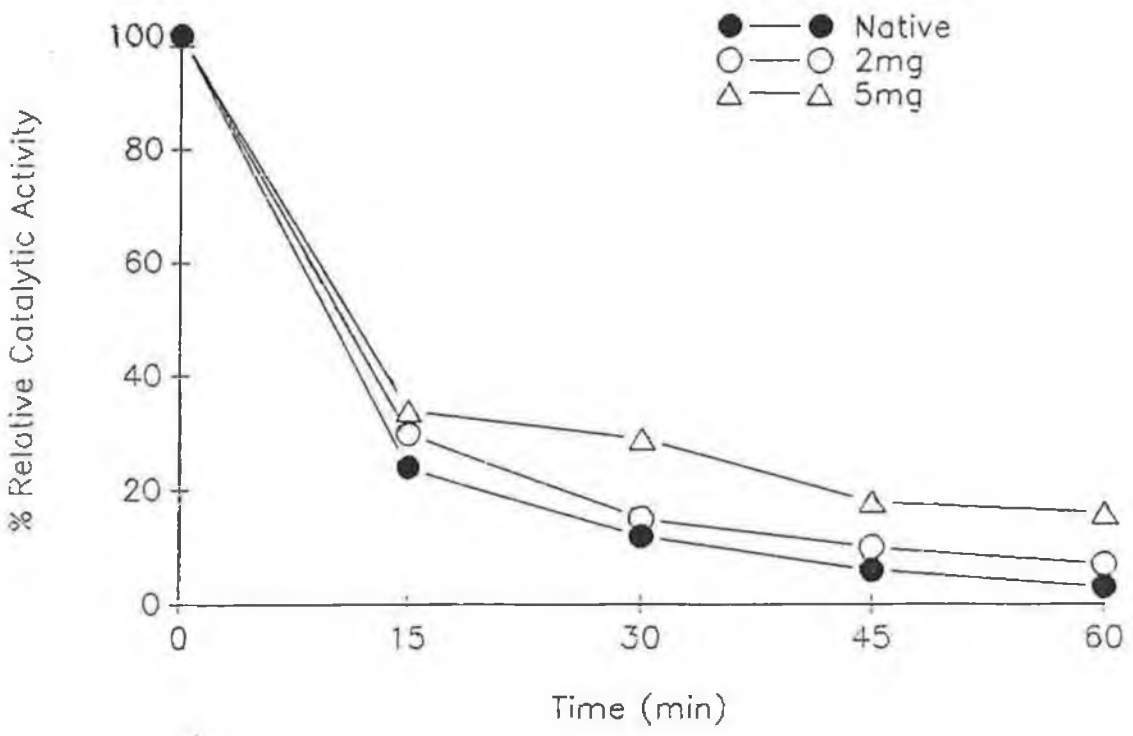


Fig. 6.10. Thermodeactivation at 75°C of native and O-methylisourea modified LDH. O-methylisourea (2 and 5mg) was added to a 0.64mg/ml solution of LDH in 0.1M phosphate, pH 10, as described in Section 6.5. Thermodeactivation was performed as described in Section 6.4.

Table 6.10.1. Thermodeactivation of native LDH and LDH modified with varying amounts of O-methylisourea.

SAMPLE	% RCA AT 0 TIME	% RCA AT 10 MIN	% RCA AT 20 MIN	% RCA AT 30 MIN
Native	100	34	22	4
5 mg	100	43	32	15
6 mg	100	45	30	16
7 mg	100	45	32	16
8 mg	100	46	33	18
9 mg	100	56	39	35

LDH was modified with O-methylisourea as described in Section 6.5 and thermodeactivation at 75 °C was performed as described in Section 6.4. % RCA refers to % relative catalytic activity.

Table 6.10.2. Thermodeactivation of native LDH and LDH modified with varying amounts of O-methylisourea.

SAMPLE	% RCA AT 0 TIME	% RCA AT 10 MIN	% RCA AT 20 MIN	% RCA AT 30 MIN
Native	100	29	18	7
8.0 mg	100	42	28	22
8.3 mg	100	45	31	22
8.5 mg	100	47	32	24
8.8 mg	100	46	34	25
9.0 mg	100	53	34	28

LDH was modified with O-methylisourea as described in Section 6.5 and thermodeactivation at 75 C was performed as described in Section 6.4. % RCA refers to % relative catalytic activity.

treated sample had the most stabilizing effect. Not much difference was observed between the other samples.

Accelerated storage studies were performed on some of the above samples, namely the 5, 7 and 9mg treated samples. The results of these are presented in Tables 6.10.3 to 6.10.6. In all cases, the observed % activity remaining, correlated extremely well with the predicted value. The 5mg-modified enzyme exhibited the greatest stability, which was an unexpected finding. The % loss per day at 4°C, 20°C and 37°C was less in each case than the native enzyme. The 7 and 9mg treated samples both had greater stability at 37°C than the native enzyme, but at 20°C and 4°C, they had approximately the same or an even greater rate of denaturation. Thus, it would appear that contrary to the results obtained from thermodeactivation studies, the less the number of modified lysine residues, the greater the long-term stabilization. However, as the stabilization effect was still not that significant, it was decided not to pursue the modification of LDH with O-methylisourea any further.

6.11 Crosslinking with Glutaraldehyde:

This was performed as described in Section 6.6. The results of thermodeactivation of these samples are shown in Fig. 6.11. As can be seen, the enzyme crosslinked with 1μl of the 25% (v/v) solution of glutaraldehyde proved to be the most thermostable. It lost only

8% of its original activity after modification. The 2 and 5μl modified enzymes lost 8 and 60%, respectively, of their activities. Both modified enzymes were more unstable at 75°C than the native enzyme.

Since the 1μl treated sample was the most thermostable, it was decided to proceed with accelerated storage studies. The results are shown in Tables 6.10.3 and 6.11.2. Rather surprisingly, the glutaraldehyde treated sample denatured even more rapidly than the native protein at most of the temperatures studied indicating that treatment with glutaraldehyde would not prolong the shelf-life of chicken heart LDH.

6.12 Reaction with Succinic Anhydride:

This was carried out as described in Section 6.7. After the reaction, the samples that had been treated with 1, 0.2 and 0.05mg of succinic anhydride lost 51, 30 and 4%, respectively, of their original activities. After 10min at 75°C, all had lost total activity.

Succinic anhydride converts amino groups to acidic groups (Hollecker and Creighton, 1982). The effect of reversing the charge on LDH would probably have been to decrease specific salt-bridges between oppositely charged groups, causing a rapid destabilization when the enzyme was heated since the absence of these salt-bridges would result in the enzyme unfolding at a much

faster rate.

6.13 Reaction with Dimethyl Adipimidate:

This was performed as described in Section 6.8. The modified enzyme retained all of its activity after the reaction. The results of thermodeactivation are shown in Fig. 6.13. As can be seen, a very significant stabilization was observed with the crosslinked enzyme. After 90min at 75°C, the native enzyme had lost all activity while the DMA-modified enzyme still retained 52% of its activity.

Accelerated storage studies were performed on the enzyme modified with 1.3mg DMA and the results are presented in Tables 6.10.3 and 6.13.2. On examination of the observed activity remaining, the modified enzyme appeared to be much more stable than the native enzyme at the temperatures studied. However, the crosslinked protein did not appear to obey a first order denaturation rate when the % RCA remaining was plotted against time. Thus, while the results of the accelerated degradation study were analysed using the Degtest programme, and are quite accurate in the case of the native enzyme, they should be interpreted with caution for the modified enzyme. In particular, the predicted degradation rates for the modified enzyme at 20°C and 4°C are probably overestimated.

However, the above results do suggest that a more thermostable

LDH can be brought about by modification with dimethyl adipimidate and possibly with the rest of the bis-imidates. This particular area appears promising and is worthy of further study.

SUMMARY:

Modification of LDH with O-methylisourea and glutaraldehyde at a number of different concentrations did not result in any significant stabilization of the enzyme. Succinic anhydride caused LDH to completely inactivate upon thermoinactivation, probably by decreasing specific salt bridges between oppositely charged groups. However, stabilization was observed when LDH was crosslinked with dimethyl adipimidate. This stabilization occurred within a very narrow concentration range and proved to be quite significant.

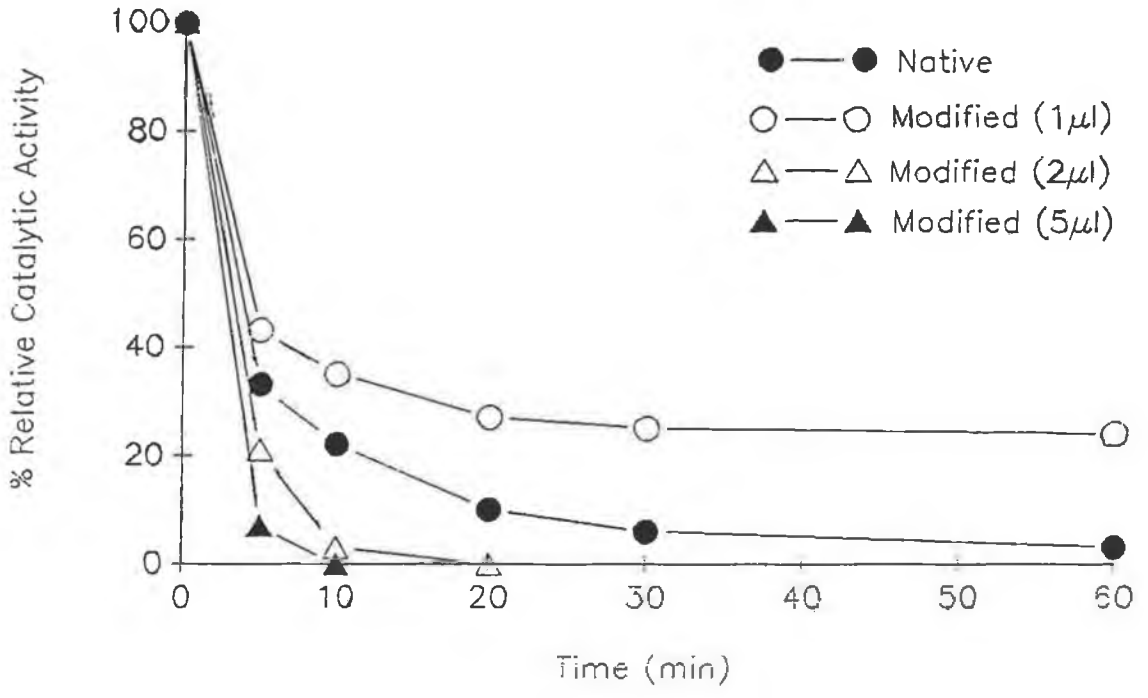


Fig. 6.11. Thermodeactivation of native and glutaraldehyde modified LDH. Modification was performed as described in Section 6.6 and thermodeactivation at 75° C was performed as described in Section 6.4.

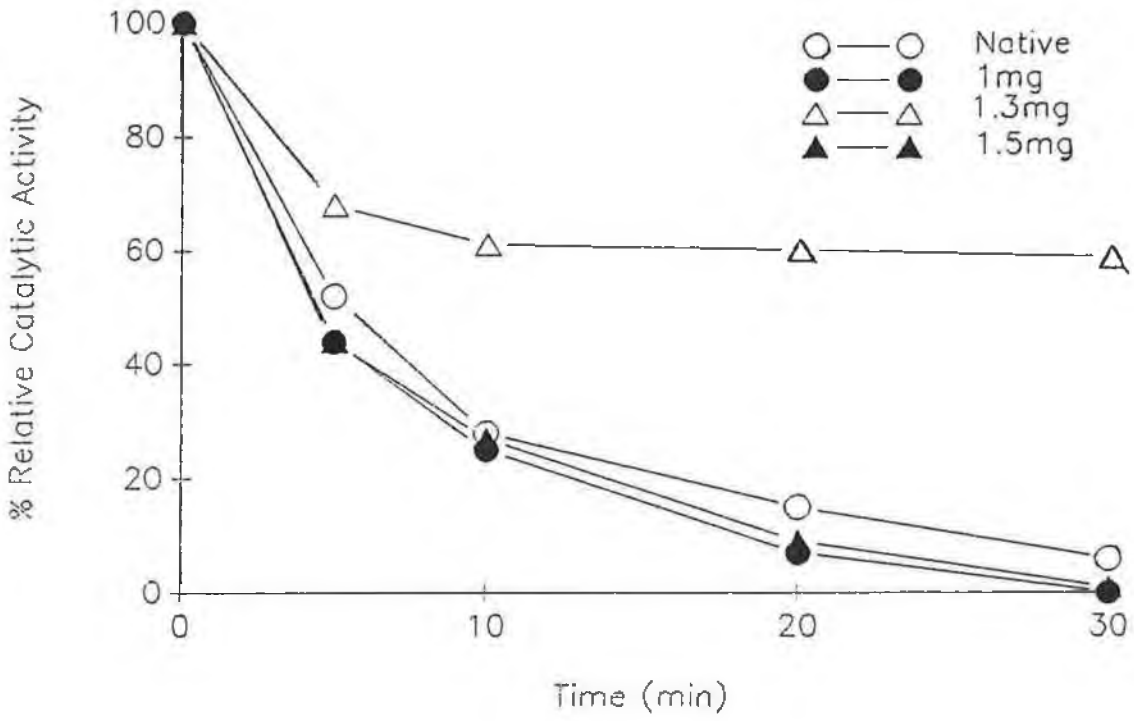


Fig. 6.13. Thermodeactivation of native LDH and LDH modified with varying amounts of dimethyl adipimidate. Crosslinking was performed as described in Section 6.8 and thermodeactivation was performed as described in Section 6.4.

Table 6.10.3a. Accelerated degradation test data for native LDH.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	5	4.50	3.38
37	5	36.00	27.57
37	7	21.00	16.47
37	8	10.70	12.73
26	8	47.00	60.57
37	11	5.00	5.88
26	11	44.00	50.19
26	15	34.00	39.06
26	18	26.00	32.37
26	26	23.00	19.61

(*) Relative to samples at -20° C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.10.3b. Predicted degradation rates for native LDH at 4° C, 20° C and 37° C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	2.65×10^{-3}	1×10^{-4}	0.265
20	2.77×10^{-2}	5.2×10^{-4}	2.736
37	0.257	8.5×10^{-4}	22.718

Degradation rates were calculated using the predicted remaining activity values from Table 6.10.3a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 6.10.4a. Accelerated degradation test data for LDH modified with 5mg of O-methylisourea.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	5	12.60	10.25
37	5	46.00	42.97
37	7	39.00	30.65
37	8	25.00	25.89
26	8	59.00	72.87
37	11	15.00	15.60
26	11	64.00	64.71
26	15	51.00	55.24
26	18	47.00	49.06
26	26	36.00	35.75

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.10.4b. The predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	1.54×10^{-3}	2×10^{-5}	0.154
20	1.7×10^{-2}	1.2×10^{-4}	1.699
37	0.169	5.4×10^{-4}	15.544

Degradation rates were calculated using the predicted remaining activity values from Table 6.10.4a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 6.10.5a. Accelerated degradation test data for LDH modified with 7mg of O-methylisourea.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	5	12.30	16.72
37	5	51.00	45.99
37	7	52.00	33.70
37	8	31.00	28.85
37	11	22.00	18.10
26	8	66.00	69.30
26	11	65.00	60.39
26	15	52.00	50.28
26	18	46.00	43.82
26	26	31.00	30.36

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.10.5b. The predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	$3.0. \times 10^{-3}$	9×10^{-5}	0.299
20	2.27×10^{-2}	3.4×10^{-4}	2.247
37	0.155	9.2×10^{-4}	14.398

Degradation rates were calculated using the predicted remaining activity values from Table 6.10.5a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 6.10.6a. Accelerated degradation test data for LDH modified with 9mg of O-methylisourea.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	5	19.00	25.05
37	5	50.00	49.56
37	7	52.00	37.43
37	8	32.00	32.52
26	8	63.00	66.02
37	11	23.00	21.34
26	11	66.00	56.50
26	15	49.00	45.91
26	18	38.00	39.29
26	26	25.00	25.94

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.10.6b. The predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	5.68×10^{-3}	1.8×10^{-4}	0.566
20	2.9×10^{-2}	3.7×10^{-4}	2.904
37	0.140	1.3×10^{-3}	13.127

Degradation rates were calculated using the predicted remaining activity values from Table 6.10.6a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 6.11.2a. Accelerated degradation test data for LDH crosslinked with glutaraldehyde.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	1	75.00	57.22
45	2	48.00	32.75
45	7	1.40	2.01
37	7	20.00	18.88
26	7	61.00	61.92
26	9	54.50	54.00

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.11.2b. The predicted degradation rates at 4°C, 20°C and 37°C.

TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	4.2×10^{-3}	4.9×10^{-4}	0.422
20	3.3×10^{-2}	2.0×10^{-3}	3.288
37	0.238	5.8×10^{-3}	21.201

Degradation rates were calculated using the predicted remaining activity values from Table 6.11.2a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

Table 6.13.2a. Accelerated degradation test data for LDH crosslinked with dimethyl adipimidate.

TEMP. (°C)	TIME (DAYS)	% ACTIVITY REMAINING (*) OBSERVED	PREDICTED
45	1	86.00	87.43
45	2	79.50	76.43
45	3	67.00	66.82
45	6	12.00	44.66
37	1	86.50	92.49
37	8	81.50	53.55
26	2	85.00	93.22
26	8	82.50	75.51
4	8	85.50	95.71
37	10	64.00	45.81
26	10	76.00	70.38
37	13	32.00	36.24
26	13	82.00	63.35
4	13	85.50	93.12

(*) Relative to samples at -20°C.

Accelerated degradation studies were performed as described in Section 6.4.

Table 6.13.2b. The predicted degradation rates at 4°C, 20°C and 37°C.

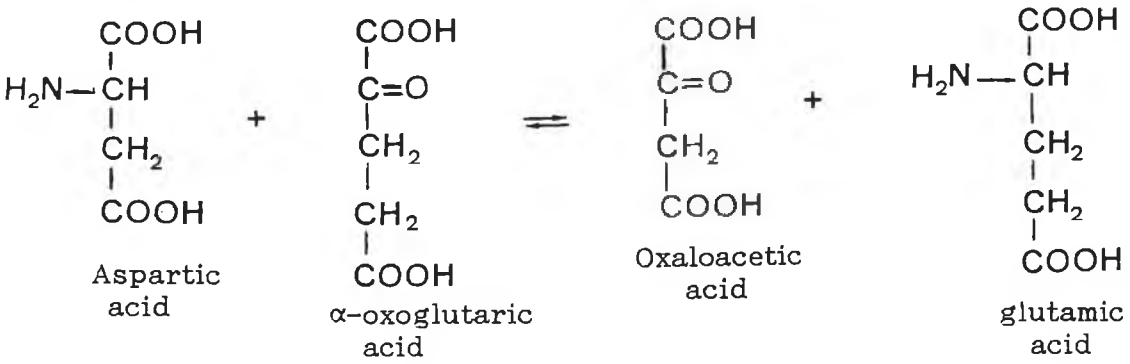
TEMP. (°C)	K	S.E.(K)	% LOSS PER DAY
4	6.1×10^{-3}	5.4×10^{-4}	0.609
20	2.2×10^{-2}	1.0×10^{-3}	2.242
37	7.8×10^{-2}	7.7×10^{-4}	7.568

Degradation rates were calculated using the predicted remaining activity values from Table 6.13.2a which were obtained using the Degtest programme as described by Kirkwood in Section 2.7. K refers to the degradation rate and S.E. refers to the standard error associated with the degradation rate.

CHEMICAL MODIFICATION OF ASPARTATE AMINOTRANSFERASE

7.0 Introduction:

The importance of aspartate aminotransferase as a clinical diagnostic marker and the need to improve the stability of the enzyme has been discussed in Section 7.0. The enzyme catalyses the following reaction:



AAT has a molecular weight of 92,000 daltons (Doonan et al., 1981). The enzyme consists of two identical subunits, each of which has a molecular weight of 46,000 daltons. The enzyme contains no disulfide groups, and has essential lysine, histidine and tyrosine residues (Braunstein, 1973).

The following chapter describes the results obtained when the protein was modified in an attempt to increase the stability of the enzyme.

7.1 Materials:

Porcine heart aspartate aminotransferase was purchased from Sigma Chemical Co., Dorset, U.K. DEAE-cellulose was obtained from

Whatman, U.K. All reagents used in the assay of AAT and all reagents used to modify and crosslink the enzyme were purchased from Sigma.

7.2 Purification of Aspartate Aminotransferase (AAT) from Porcine Heart:

The procedure used was based on that described by Rej et al., (1972). The enzyme was first desalted by (a) passage through a column of Sephadex G-25 using 5mM sodium phosphate, pH 6.8, as eluting buffer or (b) by dialysis against the same buffer (4 x 1l) at 4°C.

The enzyme solution was then applied to a DE52 column (2.5cm x 10cm) which had been previously equilibrated with 5mM sodium phosphate, pH 6.8. The column was washed with 2-3 column volumes of the buffer. The buffer was then changed and 0.02M sodium acetate, pH 4.73, was applied to the column which resulted in the elution of a peak of AAT activity. Fractions containing ASAT activity were pooled and immediately lyophilized. Purity of the protein was established by analysis of the lyophilized sample using SDS-polyacrylamide gel electrophoresis as described in Section 2.4.

7.3.1 Assay of Aspartate Aminotransferase:

AAT activity was determined according to the method recommended by the International Federation of Clinical Chemistry (Bergmeyer, 1985). The assay is based on a method that couples AAT with the reaction catalysed by malate dehydrogenase and follows the decrease in absorbance at 340nm as the NADH is oxidised.

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightleftharpoons{AAT} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} + H^+ \xrightleftharpoons{MDH} \text{L-malate} + NAD^+$$

The following reagents were required for the assay.

Reagent A: 264mM L-aspartate/88mM Tris, pH 7.8.

Reagent B: 0.198mM NADH.

Reagent C: 13.2mM α-oxoglutarate, pH 7.8.

Reagent D: Malate dehydrogenase ≥ 0.46 U/ml; Lactate dehydrogenase ≥ 0.66 U/ml in 50% glycerol.

2.0 ml of reagent A was pipetted into the cuvette, followed by 20μl each of reagents B, C and D. Finally, 200μl of AAT sample was pipetted into the cuvette. The contents were mixed and incubated for 1min at 30°C, after which time the mean absorbance per min (ΔA/min) at 340nm was determined and used to calculate AAT activity as follows:

Calculation of AAT activity:

Units/litre (30°C) = 1,746 x ΔA_{340nm}/min.

All units are expressed as μmol/min at 30°C.

7.3.2 Protein Measurement:

The protein concentration for pure enzyme was determined by measuring the absorbance at 280nm and then using the relationship $A^{1\%} = 14$ (Barra et al., 1976) and a molecular weight of 92,000 daltons for the protein (Doonan et al.,1981).

7.3.3 Total Activity of Aspartate Aminotransferase:

The total activity was defined as the number of units in 1ml of enzyme solution multiplied by the total volume of that solution.

7.3.4 Specific Activity of Aspartate Aminotransferase:

The specific activity was defined as the number of units of activity per mg of pure enzyme.

7.4 Thermodeactivation of Aspartate Aminotransferase:

Solutions of native or modified enzyme, in 0.05M sodium phosphate, pH 7.8, were incubated at 65°C. Samples were taken at

appropriate time intervals and catalytic activity measurements were carried out after preliminary cooling on ice.

7.5 Accelerated storage studies:

Accelerated storage studies were carried out as described in Section 2.7 except that the buffer for storage was 0.05M sodium phosphate, pH 7.8.

7.6 Crosslinking with Diacids:

This was performed as described by Torchilin et al., (1982) and is fully described in Section 2.8.

7.7 Crosslinking with Diamines:

This was performed as described by Torchilin et al., (1978) and is fully described in Section 2.9.

7.8 Reductive Alkylation with Glyoxylic Acid:

Reductive alkylation was performed as described by Melik-Nubarov et al., (1987) and is described in Section 2.10.

7.9 Guanidination with O-methylisourea:

Guanidination was performed as described by Minotani et al., (1979) and Cupo et al., (1980) and is described in Section 2.11.

7.10 Crosslinking with Dimethyl Suberimidate:

Crosslinking was performed according to Renobales and Welch (1980). DMS (1mg) was added as a dry powder to a 1mg/ml solution of AAT in 0.1M Tris-HCl, 0.1M 2-mercaptoethanol, 0.05M K_2SO_4, pH 8.0. The reaction was allowed to proceed for 2hr at room temperature, after which time, crosslinking was stopped by dialysis against 0.1M Tris-HCl, pH 8.0.

All reactions were performed in the presence of 100mM L-aspartate and 40μM pyridoxal phosphate.

RESULTS AND DISCUSSION:

7.11 Purification of AAT from Porcine Heart:

Initial attempts to purify AAT by ion-exchange chromatography on DE52 and CM52 cellulose using linear salt gradients proved unsuccessful. However, a procedure was finally developed to purify the enzyme utilising the principle of stepwise elution i.e. a change in the pH of the eluting buffer which is described in Section 7.2. A typical elution profile is shown in Fig. 7.11.1. As can be seen, a peak of AAT activity emerged as the pH was changed from 6.8 to 4.73. The vast majority of the contaminants remained bound to the column and were only eluted when the column was washed with 1M NaCl. For the purification procedure reported here, 2,100 units of activity (equivalent to 15mg of protein; specific activity = 140 U/mg) were recovered from 30mg of protein applied to the column. Purity of the active fractions from the DE52 column was judged by SDS-polyacrylamide gel electrophoresis where the presence of one protein band corresponding to the subunit molecular weight of 46,000 daltons was observed (see Fig. 7.11.2).

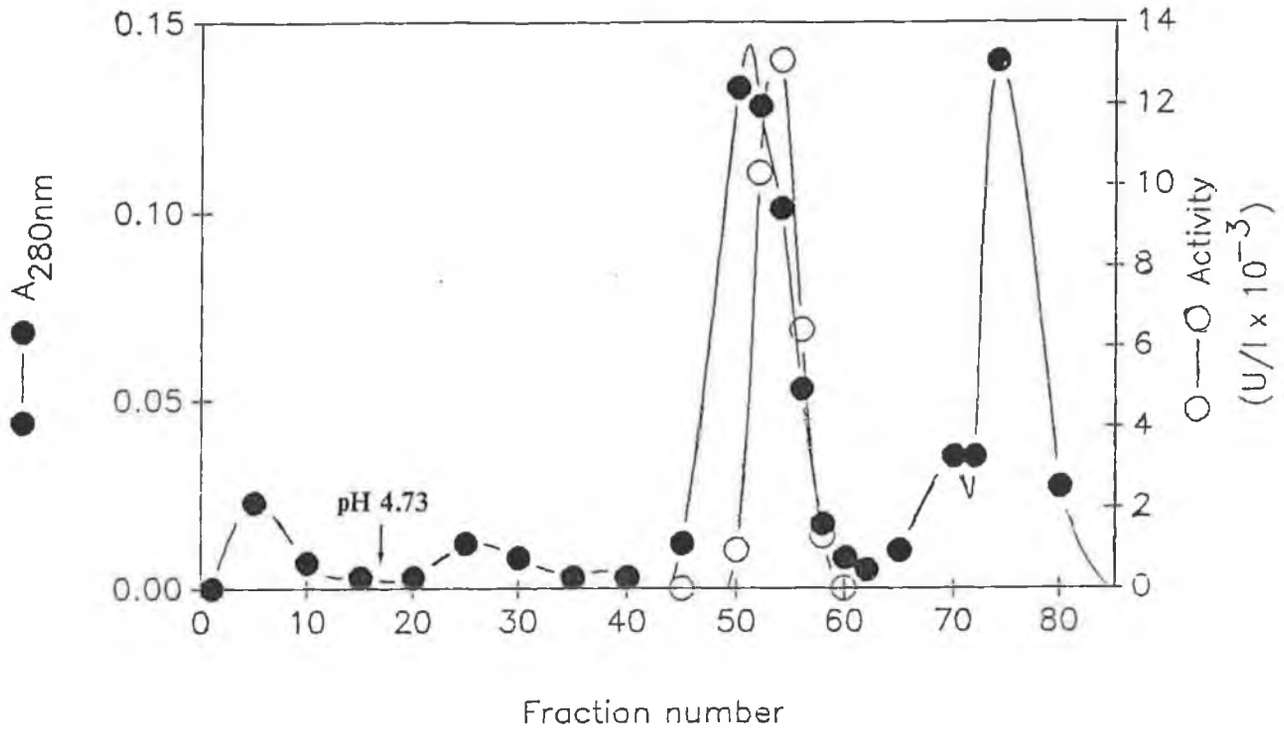


Fig. 7.11.1. Elution profile of aspartate aminotransferase purified on a DE52 column by stepwise elution as described in Section 7.2. The enzyme was assayed as described in Section 7.3.1 and protein measurements were performed as described in Section 7.3.2.

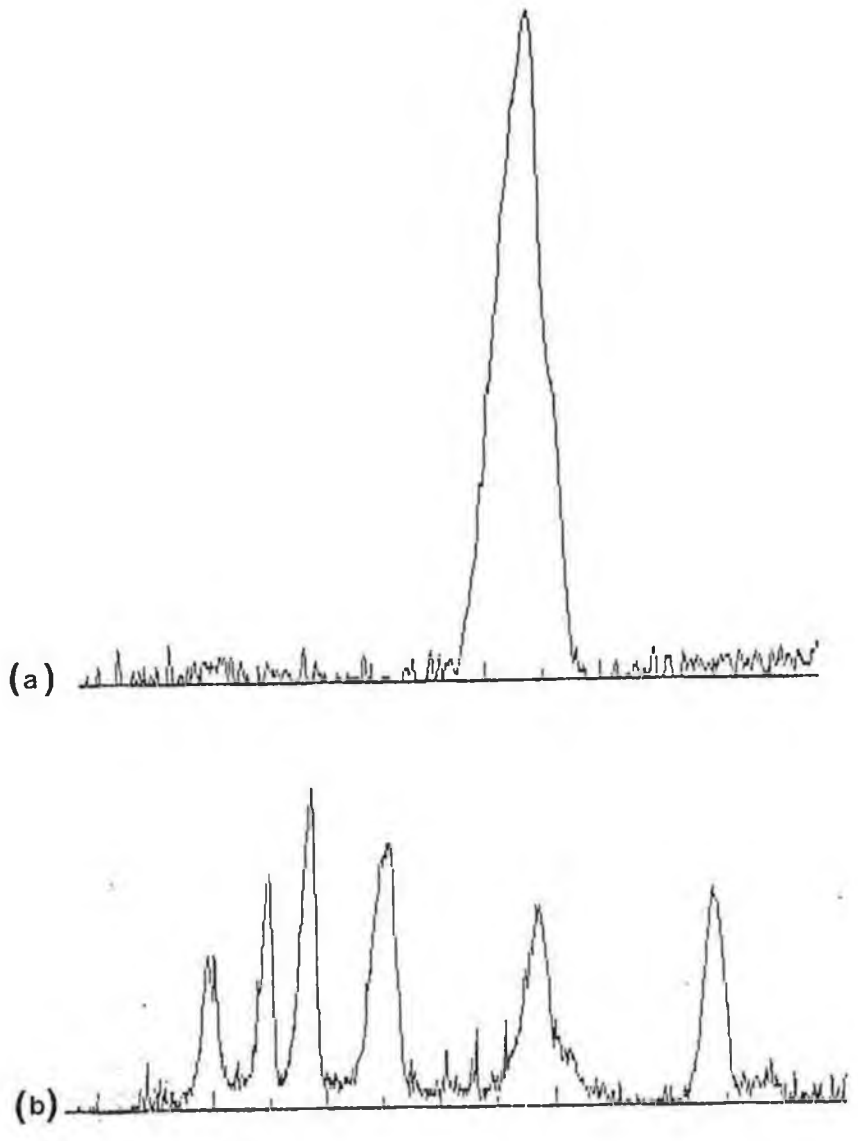


Fig. 7.11.2. The SDS-PAGE electrophoretic profiles of aspartate aminotransferase after purification on a DE52 column. SDS-PAGE was performed as described in Section 2.4. (a) shows the densitometer trace of pure aspartate aminotransferase with an estimated molecular weight of approximately 46,000 daltons (b) shows the molecular weight markers used which are as follows: myosin (205,000 Da); β-galactosidase (116,000 Da); phosphorylase B (97,000 Da); Bovine albumin (66,000 Da); ovalbumin (45,000 Da) and carbonic anhydrase (29,000 Da).

7.12 Crosslinking with Diacids:

Crosslinking was carried out as described in Section 2.8 and thermodeactivation was performed as described in Section 7.4. The results of thermodeactivation are shown in Fig. 7.12. At 65°C, the monofunctional control, acetic acid, had the most stabilizing effect on AAT. At t = 60min, the acetic acid-treated enzyme retained 26% of its original activity. The native enzyme retained 4% of its activity, while the other diacid-treated samples retained less than 6% of their activities. These results would seem to indicate that a one-point modification of the enzyme was occurring rather than crosslinking of the protein. It is of interest to note that modification of the enzyme does not result in substantial loss of activity. The pimelic acid-treated sample lost most activity after modification - losing 13% of its original activity.

Accelerated storage studies were performed as described in Section 7.5. Samples were stored at 45°C, 37°C and 4°C. The results of accelerated storage at 45°C are shown in Table 7.12.1 and support the results obtained in the thermodeactivation experiment at 65°C. After 25 days at 45°C, the acetic acid-treated enzyme retained 18% activity, while the native and other diacid-treated samples retained less than 5% activity.

However, after 64 days at 37°C (see Table 7.12.2), the acetic

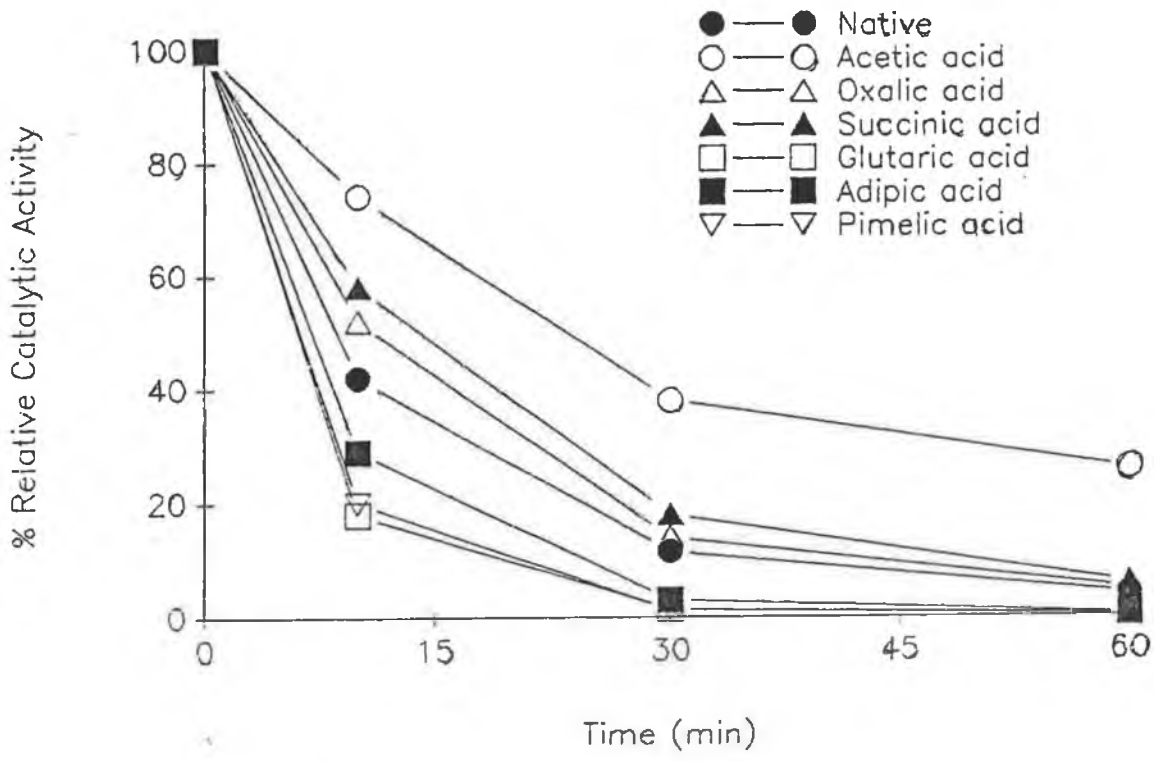


Fig. 7.12. Thermodeactivation of native and diacid-treated aspartate aminotransferase samples. Modification with diacids was performed as described in Section 2.8 and thermodeactivation at 65 °C was carried out as described in Section 7.4.

Table 7.12.1. The % relative catalytic activity remaining after accelerated storage studies at 45°C of native and diacid-modified aspartate aminotransferase.

Diacid	0 Time	1 day	2 day	3 day	7 day	16 day	25 day	64 day
Native enzyme	100	73	62	61	50	29	3	0
Acetic acid	100	93	92	89	85	64	18	9
Oxalic acid	100	83	70	64	61	46	5	0
Succinic acid	100	85	80	78	71	46	2	0
Glutaric acid	100	52	44	42	41	34	4	0
Adipic acid	100	52	38	31	21	9	0	0
Pimelic acid	100	47	34	29	21	13	0	0

Aspartate aminotransferase was modified with diacids as described in Section 2.8 and then accelerated storage studies were performed as described in Section 7.5.

Table 7.12.2. The % relative catalytic activity remaining after accelerated storage studies at 37°C of native and diacid-modified aspartate aminotransferase.

Diacid	0 Time	7 day	25 day	64 day
Native enzyme	100	46	20	9
Acetic acid	100	32	2	2
Oxalic acid	100	67	50	42
Succinic acid	100	55	50	35
Glutaric acid	100	54	29	16
Adipic acid	100	58	44	32
Pimelic acid	100	64	51	36

Aspartate aminotransferase was modified with diacids as described in Section 2.8 and then accelerated storage studies were performed as described in Section 7.5.

acid-treated enzyme retained only 2% activity, the native enzyme 9%, and the other diacid-treated samples retained activity ranging from 16% to 42%, with the oxalic acid having the most stabilizing effect on the enzyme.

A different picture emerged again at 4°C. As can be seen in Table 7.12.3, the acetic acid-treated enzyme rapidly loses activity. However, after only 25 days, no activity remained in any of the other samples, with the exception of the pimelic acid-treated sample, which retained 17% of its activity.

The above results were unexpected. Not only were different stabilization effects being seen at different temperatures, but degradation appeared to be most rapid at 4°C, a temperature where most enzymes would undergo a slow rate of degradation. The change in stabilization effect seemed to occur at a temperature between 45°C and 37°C.

An example of this type of effect has been reported by Urabe et al., (1973). It was found that acetylation of α-amylase increased its resistance to thermal denaturation for temperatures greater than 70°C, but reduced resistance for temperatures below 67°C. This was termed the "compensation effect" and the temperature of compensation, T_c, was approximately 68°C. The effect seemed to be due to the conformational change of the enzyme caused by acetylation. Urabe and coworkers (1973) recommended care in the interpretation of the effect of experimental conditions or modification of a protein on its stability, because no effect will

Table 7.12.3. The % relative catalytic activity remaining after accelerated storage studies at 4°C of native and diacid-modified aspartate aminotransferase.

Diacid	0 Time	7 day	25 day
Native enzyme	100	71	0
Acetic acid	100	8	0
Oxalic acid	100	81	0
Succinic acid	100	87	0
Glutaric acid	100	83	0
Adipic acid	100	85	0
Pimelic acid	100	90	17

Aspartate aminotransferase was modified with diacids as described in Section 2.8 and then accelerated storage studies were performed as described in Section 7.5.

be seen at T_c, and opposite effects will be observed at both sides of T_c. It appears as if this "compensation effect" might also be evident in the heat inactivation of AAT, with the temperature of compensation being somewhere in the region between 45°C and 37°C, with opposite effects of the diacids being observed on either side of this temperature.

The reason for the rapid degradation of native AAT at 4°C was unclear. However, it was obvious from the above results that the accelerated degradation test could not be used to predict the stability of AAT. The use of the Arrhenius equation in this test assumes that denaturation occurs with first-order kinetics, therefore, in this case, it would not have been appropiate to proceed with fitting the Arrhenius equation.

7.13 Crosslinking with Diamines:

Crosslinking with diamines was performed as described in Section 2.9 and thermodeactivation was carried out as described in Section 7.4. The results of thermodeactivation are shown in Table 7.13.1. In this experiment, the monofunctional control, ethylamine caused nearly complete loss of activity in the enzyme, indicating that a one-point modification of the enzyme was not occurring. The shortest chain length diamine, hydrazine, also resulted in a very large loss of activity. Ethylenediamine had the most stabilizing effect on the enzyme. At t = 180min, the enzyme retained 59% of

its activity, with the native and other diamine-treated samples retaining between 33% and 42% activity. After modification, the ethylenediamine-treated sample had lost 33% of its original activity.

The results of accelerated storage studies at 45°C are presented in Table 7.13.2. At 21 days, the native enzyme retained 4% activity. All the other diamine-treated samples (with the exception of ethylamine and hydrazine) retained a higher amount of activity, with diaminopropane having the most stabilizing effect, retaining 27% of its original activity.

At 37°C, ethylenediamine had the most stabilizing effect with 13% of its activity left after 21 days. The native enzyme and the other modified samples all had 2% activity left after this time (see Table 7.13.3).

At 4°C, the native enzyme had lost practically all activity after 9 days. The ethylenediamine and diaminopropane-treated samples had retained 66% and 50%, respectively, of their original activities (see Table 7.13.4). After 21 days, none of the samples had any remaining activity. Rapid degradation at 4°C was therefore again observed. Unlike the situation with the diacids, however, different stabilization effects at different temperatures were not seen with the diamines. Ethylenediamine consistently showed a greater stabilizing effect on the enzyme with the exception of the accelerated degradation test at 45°C.

Once again, it was not possible to predict stability using the

Arrhenius equation, due to the atypical response of AAT to storage at different temperatures, particularly at 4°C.

Table 7.13.1. The % relative catalytic activity remaining after thermodeactivation of native and diamine-treated aspartate aminotransferase at 65°C.

Diamine	0 time	10 min	30 min	60 min	120 min	160 min
Native	100	80	66	60	47	42
Ethylamine	100	0	0	0	0	0
Hydrazine	100	6	0	0	0	0
Ethylenediamine	100	87	79	74	66	59
Diaminopropane	100	67	56	50	46	42
Tetramethylenediamine	100	86	65	59	44	40
Pentamethylenediamine	100	68	52	44	37	33
Hexamethylenediamine	100	78	65	54	43	40

Modification of aspartate aminotransferase with diamines was performed as described in Section 2.9 and thermodeactivation of the native and modified enzyme was performed as described in Section 7.5.

Table 7.13.2. The % relative catalytic activity remaining after accelerated storage studies of native and diamine-treated aspartate aminotransferase at 45°C.

Diamine	0 days	9 days	21 days
Native	100	15	4
Ethylamine	100	0	0
Hydrazine	100	0	0
Ethylenediamine	100	35	11
Diaminopropane	100	51	27
Tetramethylenediamine	100	45	16
Pentamethylenediamine	100	24	6
Hexamethylenediamine	100	39	18

Modification of aspartate aminotransferase with diamines was performed as described in Section 2.9 and accelerated storage studies of the native and modified enzyme were performed as described in Section 7.5.

Table 7.13.3. The % relative catalytic activity remaining after accelerated storage studies of native and diamine-treated aspartate aminotransferase at 37°C.

Diamine	0 days	9 days	21 days
Native	100	5	2
Ethylamine	100	0	0
Hydrazine	100	0	0
Ethylenediamine	100	30	13
Diaminopropane	100	8	2
Tetramethylenediamine	100	3	2
Pentamethylenediamine	100	4	2
Hexamethylenediamine	100	3	2

Modification of aspartate aminotransferase with diamines was performed as described in Section 2.9 and accelerated storage studies of the native and modified enzyme were performed as described in Section 7.5.

Table 7.13.4. The % relative catalytic activity remaining after accelerated storage studies of native and diamine-treated aspartate aminotransferase at 4°C.

Diamine	0 days	9 days	21 days
Native	100	1	0
Ethylamine	100	0	0
Hydrazine	100	0	0
Ethylenediamine	100	66	0
Diaminopropane	100	50	0
Tetramethylenediamine	100	15	0
Pentamethylenediamine	100	32	0
Hexamethylenediamine	100	3	0

Modification of aspartate aminotransferase with diamines was performed as described in Section 2.9 and accelerated storage studies of the native and modified enzyme were performed as described in Section 7.5.

7.14 Reductive Alkylation with Glyoxylic Acid:

Reductive alkylation was performed as described in Section 2.10. This procedure carboxymethylates the amino groups of the enzyme resulting in a more hydrophilic protein. However, this procedure resulted in complete loss of enzyme activity. The experiment was performed in both the presence and absence of substrates, but the results were identical in both cases. Reductive alkylation, therefore, caused a total destabilization of the enzyme. This would imply that the lysines residues must have a role in stabilizing the enzyme in the native state, perhaps via electrostatic interactions if carboxymethylation produced such a dramatic loss of enzyme activity. Alternatively, the presence of substrates may not have protected the active site, and an essential lysine residue may have been modified causing the loss of catalytic activity.

7.15 Guanidination with O-methylisourea:

Guanidination was performed as described in Section 2.11. The results of thermodeactivation are shown in Fig. 7.15. As can be seen, the modified enzyme exhibited only a very slight increase in stability. In this reaction the positive charge of the original amino group is retained. The fact that the enzyme was not destabilized once again points to the role of the amino groups in

electrostatic interactions.

7.16 Crosslinking with Dimethyl Suberimidate:

This was carried out as described in Section 7.10. Following crosslinking with DMS, the enzyme lost 30% of its activity. Thermodeactivation of the native and modified enzyme revealed very similar rates of degradation (see Fig. 7.16) implying that DMS was not conferring any additional stability to AAT.

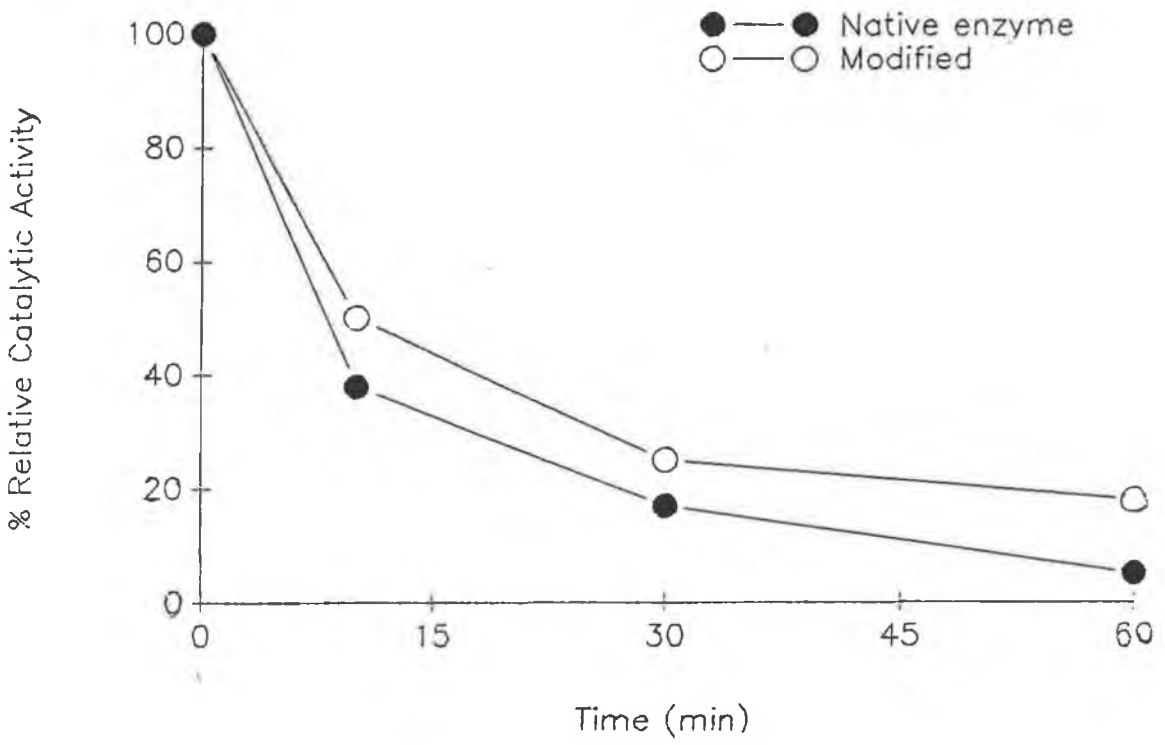


Fig. 7.15. Thermodeactivation of native and O-methylisourea modified aspartate aminotransferase. Guanidination with O-methylisourea was performed as described in Section 2.11 and thermodeactivation was carried out as described in Section 7.4.

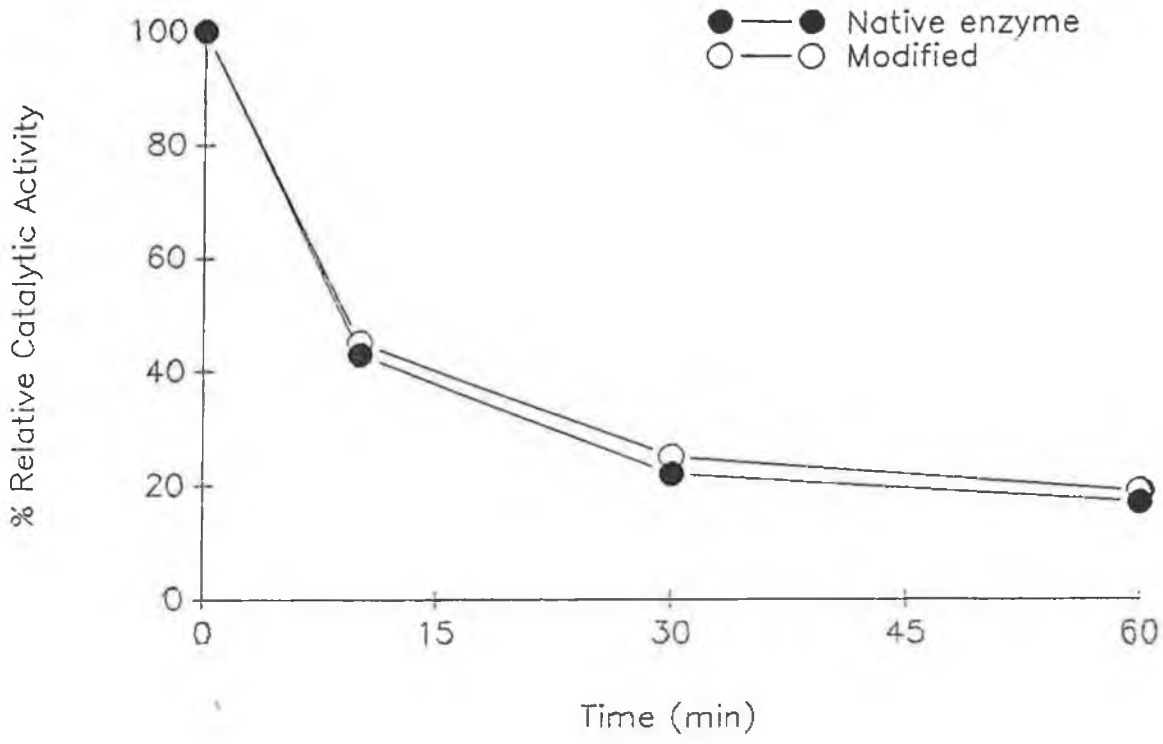


Fig. 7.16. Thermodeactivation of native and crosslinked aspartate aminotransferase. Crosslinking with dimethyl suberimidate was performed as described in Section 7.16 and thermodeactivation was performed as described in Section 7.4.

SUMMARY:

The crosslinking of aspartate aminotransferase with various diacids produced rather unusual results with stabilization occurring above 45°C when the monofunctional control, acetic acid was used. Below this temperature, pimelic acid appeared to have the most stabilizing effect on the enzyme. Also, the enzyme appeared to degrade very rapidly at 4°C in comparison to its storage at higher temperatures. This effect was also observed when the enzyme was modified with diamines. In this instance, ethylenediamine seemed to have the most stabilizing effect on the enzyme. Carboxymethylation of the enzyme resulted in a drastic destabilization of the enzyme and modification of AAT by guanidination or crosslinking with dimethyl suberimidate appeared to have no real stabilizing effect.

CONCLUSION

CONCLUSION:

Bovine heart creatine kinase was purified to homogeneity. Following purification, the enzyme was then chemically modified with a number of different modifying reagents in an effort to improve the thermal stability of the protein. It was observed that modification reagents which disturbed the charge of amino or carboxyl groups, usually resulted in destabilization of the enzyme. This seemed to infer that electrostatic interactions such as salt bridges were involved in stabilizing the native protein. When this was investigated further, it was found that salt bridges were required for stabilization of the native enzyme, and that stabilization was achieved by rigidifying the structure of the enzyme, thereby hindering its unfolding.

One of the modifying reagents used, dimethyl suberimidate, did not alter the charge of groups involved in electrostatic interactions. This reagent was found to significantly increase the thermal stability of creatine kinase at a number of different concentrations. Having optimized the procedure, the effects of crosslinking on the enzyme were examined. As judged from the electrophoretic profiles of native and modified enzyme, intermolecular crosslinks were introduced in creatine kinase, giving rise to a protein with increased thermal stability. This enhanced stability is presumably due to the prevention of the unfolding step in denaturation and the dissociation of the protein

into subunits. Tetramers are present in maximal quantities which is not surprising in view of the high concentrations of DMS used. This may also mean, however, that the stabilization effect can be further increased if some other intramolecular and intersubunit crosslinking agent is used.

Modification of the enzyme resulted in a decrease in specific activity. It is known that there are two lysine groups/molecule which are essential for catalytic activity. However, inclusion of substrates to protect the active site of the enzyme did not prevent any loss of activity. The Michaelis constants of the modified protein were not significantly altered, which again seemed to indicate that the active site of the enzyme had not been affected by crosslinking. It would appear, therefore, that the decreased specific activity might be due to the formation of catalytically inactive oligomers. Optimum stabilization occurred when 43% of all the available amino groups were crosslinked. This same degree of stabilization was observed when up to 90% of the amino groups were blocked, but a progressive decrease in specific activity also accompanied this.

Crosslinking also conferred a resistance to the action of denaturing agents. Strong denaturants, such as guanidine-HCl disrupt noncovalent interactions in proteins leading to their unfolding. The covalent bridges formed by DMS with the amino groups of creatine kinase should be resistant to this action, maintaining the enzyme structure when the noncovalent bonds have

been broken and, thus, stabilizing the protein against denaturation.

Accelerated storage studies indicated that the modified enzyme would be more stable than the native enzyme at lower temperatures. From the results obtained in this study, it is evident that bovine heart creatine kinase is a rather labile enzyme and so the above result was of special interest, as it meant that the crosslinked water-soluble enzyme preparation was significantly more stable than the native enzyme at room temperature and also at 4 °C.

A mechanistic investigation as to the cause of irreversible thermoinactivation of creatine kinase was undertaken. It appeared that inactivation of the enzyme followed first-order kinetics, although the course of inactivation did not seem to be independent of the initial enzyme concentration. Although aggregation occurred in parallel with irreversible inactivation at the higher enzyme concentrations, the former was not the cause of the latter, but rather coincidental. The enzyme was "irreversibly" thermoinactivated in the presence of guanidine-HCl. Reactivation of the enzyme by dilution occurred at the three pH values studied, indicating that conformational, as opposed to covalent processes were playing a major role in the inactivation of creatine kinase. The formation of incorrect structures was again demonstrated to play a part in inactivation when the enzyme was reactivated after thermal inactivation in which approximately 90% of the enzymes original activity had been lost. At the three pH values studied,

the enzyme was able to regain between 58% and 90% of "recoverable" activity. Since refolding of the thermoinactivated enzyme could not occur if thermoinactivation was due solely to covalent processes, this result demonstrated conclusively, that "irreversible" inactivation was caused by the formation of incorrect structures. Further evidence to support this result was obtained when the tryptophan residues of the enzyme were titrated before and after heating. The number of accessible tryptophans increased from 3 to 4, reflecting a more unfolded conformation of the enzyme. The difference spectra of creatine kinase after thermoinactivation at pH 6.7 was also examined. Differences in the spectra were observed between 220 and 200nm, which is characteristic of changes in chain conformation. Polyclonal antibodies were raised against creatine kinase with a view to measuring the extent of unfolding of the enzyme. However, while these antibodies could not adequately distinguish changes in protein conformation, they may prove useful in any ongoing work with creatine kinase, as is described later.

While accepting that conformational processes play a large role in the thermoinactivation of creatine kinase, the possible involvement of covalent processes in this was also investigated. It was found that peptide bond hydrolysis did not occur at either pH 4, 6.7 or 8. Similarly, deamidation of Asn and/or Gln residues, a process which has been identified as contributing to the thermodeactivation of several enzymes, did not cause irreversible

inactivation of creatine kinase at 55°C at the three pH values studied. Deamidation did occur at 100°C at pH 6.7 and 8, but the process was too slow to account for the inactivation of the enzyme at that temperature.

Since the destruction of free sulfhydryl groups has been found to be involved in the covalent inactivation of several enzymes, this process was also investigated in relation to the thermodeactivation of creatine kinase. During this investigation, it was determined that contrary to previously published reports, intra- and interchain disulfide bonding was occurring in the enzyme. The addition of the reducing agent, dithioerythritol, was found to be able to increase the activity of the enzyme by 100% implying that the presence of disulfide bridges were associated with a loss of activity in creatine kinase.

Further investigation of this phenomenon revealed that at pH 6.7, the presence of Cu^{2+} accelerated the thermoinactivation of the enzyme, probably by causing O_2 oxidation of cysteine residues. Electrophoresis (under non-reducing conditions) of the native thermoinactivated enzyme was then performed, the results of which clearly showed differing degrees of disulfide bonding occurring as the enzyme was progressively heated. The banding pattern of the enzyme which had been heated for 10min, was very similar to the banding pattern obtained when Cu^{2+} had been added to the enzyme. These results would seem to indicate that heat-induced oxidation of thiol groups in creatine kinase caused disulfide formation.

These intra- and intermolecular disulfides are the incorrect structures of creatine kinase which are responsible for the "irreversible" loss of activity of the enzyme upon heating.

Oxidation is a major inactivation force in nature. In vitro, enzymes are rather vulnerable to oxidation, even under normal atmospheric conditions. However, it has been recently shown (Shami, 1991), that in the presence of its corresponding polyclonal antibodies, the enzyme, subtilisin, showed a greatly enhanced resistance to both high temperature and oxidation. In view of the role oxidation plays in the thermal inactivation of creatine kinase, this methodology might also be applied to this enzyme in any future work. The physical shielding by the antibodies of vulnerable sites on the surface of the enzyme, may possibly prevent oxidation and hence, inactivation.

Accelerated storage studies were performed on creatine kinase in the presence of a number of additives which were thought might prevent this inactivation. However, the presence of EDTA and DTE did not improve the stability of the enzyme. BSA and DMS, which were also included in these studies, did bring about a significant degree of stabilization.

The existing assay for measuring creatine kinase activity was adapted for use with a programmable microplate reader and microtitre plates. This assay system was able to accurately measure purified and serum creatine kinase activity , and compared extremely well with other assay procedures. The main advantage of

the microassay was the speed of assay performance. A large number of samples could be assayed within an 8min time period. This assay proved to be extremely useful during the course of this work and should prove invaluable to workers involved in assaying creatine kinase without the benefit of expensive automated equipment. This system has subsequently been successfully applied to study the stability of chemically modified creatine kinase in our laboratory.

The N-terminal amino acid sequence of bovine heart creatine kinase was determined for the first 16 residues. This information should provide the groundwork necessary to facilitate the isolation of the creatine kinase gene from genomic libraries, with a view to sequencing the entire gene. Studies on the site-directed mutagenisis of creatine kinase might then be performed, allowing the thermal stability of this enzyme to be investigated further.

Limited studies were performed on two other clinically important enzymes, lactate dehydrogenase and aspartate aminotransferase. The enzymes were chemically modified in an attempt to improve their thermal stabilities. In the case of lactate dehydrogenase, the only significant stabilization observed was when the enzyme was crosslinked with dimethyl adipimidate. Aspartate aminotransferase was stabilized to some extent by modification with diacids and diamines. However, the enzyme exhibited an atypical response to degradation at 4°C, with inactivation occurring rapidly at this temperature, compared to

the inactivation observed at the higher temperatures. Having established which reagents stabilize and destabilize lactate dehydrogenase and aspartate aminotransferase, the information obtained regarding the chemical modification of these two enzymes, may provide a basis to any further studies in this laboratory which may be carried out in the future, with a view to enhancing the thermal stabilities of these enzymes.

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