STUDIES ON THE SUBUNITS OF CREATINE KINASE

BY

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DEDICATION

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To my wife Emily and my daughter Pamela for their patience and constant support, particularly during the many evenings in which they had to forgo the pleasure of my company.

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I would like to thank my supervisor Dr N C Price for his invaluable guidance and encouragement during the course of this work.

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Gordon Bickerstaff.

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ABBREVIATIONS

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ADP	:	adenosine 5'-diphosphate
ATP	:	adenosine 5'-triphosphate
Ci	:	curie
c.d.	:	circular dichroism
CNBr	:	cyanogen bromide
DTT	:	dithiothreitol
EDTA	:	ethylenediaminetetraacetate
GdnHCl	:	guanidine hydrochloride
h	:	hour
IAM	:	iodoacetamide
Km	:	Michaelis constant
Ks	:	dissociation constant for substrate-enzyme complex
NADH	:	nicotinamide-adenine dinucleotide
Nbs_2	:	5,5'-dithiobis-(2-nitrobenzoic acid)
PEP	:	phospho(enol)pyruvate
Phosphate buffer	:	10mM-sodium phosphate buffer at pH 8.0 containing 1.0mM-EDTA
SDS	:	sodium dodecyl sulphate
Tris	:	2-amino-2-hydroxymethyl-propane-1,2-diol
U or unit	:	1 unit of enzyme activity refers to the consumption of 1.0 µmol of substrate/minute

SYNOPSIS

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1. A detailed study of the denaturation and renaturation of native soluble creatine kinase (EC 2.7.3.2) has revealed that denaturation by guanidine hydrochloride is essentially completely reversible with 95% regain of enzyme activity after 1.0 h of renaturation at 20°C. In addition the product of renaturation, the fully renatured enzyme, was found to be identical to the native soluble enzyme in terms of active site structure, homogeneity and overall conformation.

2. The dimeric enzyme was immobilised via a single subunit to CNBr-activated Sepharose 4B to yield a matrix-bound enzyme derivative containing 400 μg of protein/ml of packed gel, with substantial retention of enzyme activity. The matrix-bound enzyme was subsequently treated with guanidine hydrochloride to facilitate dissociation of the subunits, followed by renaturation to yield a catalytically active matrix-bound subunit derivative containing 200 μg of protein/ml of packed gel.

3. The effects of pH on the enzyme activities of the soluble enzyme and the matrix-bound derivatives were studied. In all cases there was a broad peak in the pH-activity profile with maximum activity being observed at pH 9.0. Kinetic parameters for the matrix-bound derivatives were generally similar to those for the soluble enzyme although some variations in the values of the Km for MgATP were observed with those for the matrix-bound derivatives being raised approximately 2.5-fold compared with the soluble enzyme.

4. In common with the findings of studies on glucose 6-phosphate dehydrogenase and tryptophanase by other workers (see Chapter 6), it was found that immobilisation of creatine kinase led to an enhancement of stability towards either thermal inactivation or unfolding by guanidine hydrochloride. In both types of study the order of stabilities observed was matrix-bound dimeric enzyme > matrix-bound subunit enzyme > soluble enzyme.

5. The numbers of reactive thiol groups in the soluble enzyme and the matrix-bound derivatives were determined by two different methods: (i) spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid); (ii) reaction with radioactive iodoacetamide. The results of these two studies indicated that both the soluble enzyme and the matrix-bound dimeric enzyme possess one reactive thiol group per subunit, whereas the matrix-bound subunit enzyme was found to possess two reactive thiol groups per subunit. Further studies on the reactivity of the thiol groups of the soluble enzyme and the matrix-bound derivatives were obtained by measuring the rate of inactivation of the enzyme by iodoacetamide. The data obtained showed that the reactivity of the thiol groups in the presence of various combinations of substrates and ligands of the soluble enzyme and the matrix-bound derivatives were largely similar.

6. The remarkable similarity in the properties of the native and immobilised creatine kinase attests to the lack of significant conformational restraints imposed by the matrix.

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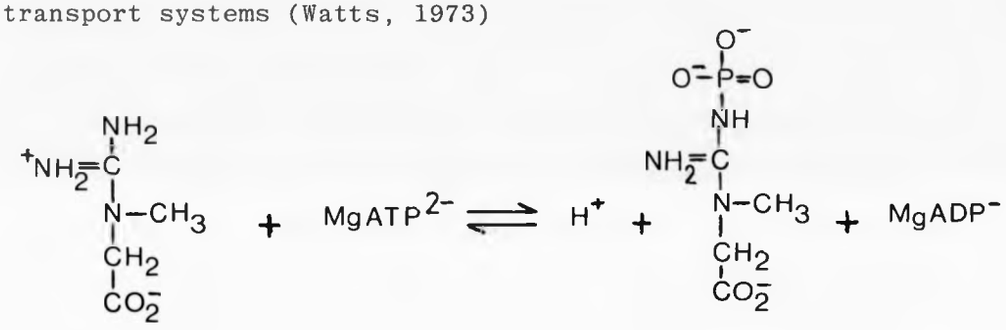
CHAPTER 1

INTRODUCTION

CREATINE KINASE

Introduction

The aim of this section is to describe some recent work on the enzyme creatine kinase (adenosine 5'-triphosphate-creatine N-phosphotransferase, EC 2.7.3.2) which has led to a greater understanding of the enzyme mechanism and subunit behaviour. The primary function of the enzyme, which catalyses the reaction shown below, seems to be associated with regeneration of ATP in conjunction with contractile or transport systems (Watts, 1973)



Creatine Phosphocreatine

There has been considerable interest in clinical assays of creatine kinase activity (Szasz et al., 1976) as it has been proposed as a measure of myocardial infarct size (Sobel, 1976; Sobel et al, 1977) and as an indication of muscular dystrophy and other disorders (Sherwin et al., 1969; Roy, 1974). Other recent articles have dealt with the various physiological aspects of the enzyme, such as its rôle in muscle (Berson, 1976; Seraydarian and Abbott, 1976) and adipose tissue (Berlet et al., 1976). The work reviewed in this section will however refer to the enzyme isolated

from rabbit skeletal muscle as it was the form studied in the work described in this Thesis.

Structural features of the enzyme

Creatine kinase consists of two very similar, if not identical subunits of molecular weight 41000 daltons (Watts, 1973). The enzyme possesses one reactive thiol group per subunit, modification of which by a variety of reagents (e.g. iodoacetate, iodoacetamide and 1-fluoro-2,4-dinitrobenzene) leads to inactivation of the enzyme (Watts, 1973). Dissociation of the dimer can be brought about by agents such as guanidinium hydrochloride and urea (Yue et al., 1967) and subsequent re-association of subunits (upon removal of the denaturing agent) has been observed by several workers (Dawson et al., 1967; Yue et al., 1967). Only limited sequence information is available, accounting for less than 15% of the total amino acid content of the molecule (Watts, 1973). Particular attention has been focused on the sequence of a peptide containing the reactive thiol group, and there appears to be considerable sequence homology between this peptide and the corresponding peptide from other isoenzyme forms of creatine kinase (Watts, 1973) and from arginine kinase isolated from lobster muscle (Morrison, 1973) (see Table 1.1).

Some preliminary X-ray diffraction measurements on the enzyme have been made (McPherson, 1973). Of the 3 crystal forms studied, two appeared to possess an asymmetric unit consisting of two molecules (i.e. 160000 daltons), whereas in the third (orthorhombic) form, the asymmetric unit

Table 1.1 Comparative amino acid sequences around the reactive thiol residues of creatine kinases and arginine kinase (adapted from Roy, 1974)

Creatine kinase					Arginine kinase
Rabbit muscle	Human muscle	Chicken muscle	Ox brain	Ox muscle	Lobster muscle
Val	Val	Ile	Ile	Val	-
Leu	Leu	Leu	Leu	Leu	Glu
Thr	Thr	Thr	Thr	Thr	Thr
Cys	Cys	Cys	Cys	Cys	Cys
Pro	Pro	Pro	Pro	Pro	Pro
Ser	Ser	Ser	Ser	Ser	Thr
Asn	Asn	Asn	Asn	Asn	Ser
Leu	Leu	Leu	Leu	Leu	Asn
Gly	Gly	Gly	Gly	Gly	Leu
Thr	Thr	Thr	Thr	Thr	Gly
Gly	Gly	Gly	Gly	Gly	Thr
Leu	Leu	Leu	Leu	Leu	Val
Arg	Arg	Arg	Arg	Arg	Arg

consisted of a single protein subunit. From the unit cell dimensions, it was calculated that the thickness of a creatine kinase subunit was of the order of 5 nm, and considerations of the space group suggested that the enzyme molecule possesses a two-fold axis relating two identical subunits.

Mechanism of action

The reaction catalysed is a transfer of a phosphoryl group from $MgATP^{2-}$ to creatine. There is no evidence for any phosphorylated enzyme intermediate (Watts, 1973). Detailed kinetic studies of the reaction, including the use of product inhibition have shown that the mechanism is of the rapid-equilibrium, random order type with synergism in substrate binding i.e. the binding of the metal-nucleotide to the enzyme facilitates the subsequent binding of creatine and vice versa (Morrison and James, 1965). An important finding was that certain small, planar, anions (notably nitrate and formate) led to a remarkable decrease in the reactivity of the thiol groups when added to a mixture of $MgADP^{-}$ and creatine (Milner-White and Watts, 1971). On the basis of this work and other kinetic and magnetic resonance studies (Reed and Cohn, 1972; James and Cohn, 1974; McLaughlin et al., 1976) it has been proposed that the anions can occupy the site normally occupied by the γ-phosphoryl group of $MgATP^{2-}$, so that a complex is formed which resembles the "transition state" for the enzyme catalysed reaction (Fig. 1.1).

In a recent paper Milner-White and Kelly have used the

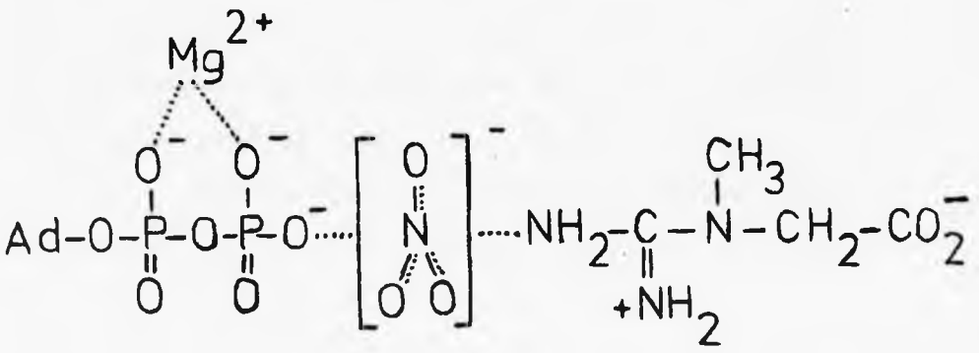


Fig 1.1 The "transition state analogue" complex for the reaction catalysed by creatine kinase. Various other anions can replace nitrate in this complex, in which the thiol groups have only a very low reactivity towards iodoacetamide. It is postulated that in the transition state of the enzyme catalysed reaction, the planar metaphosphate ion (PO_3^-) would replace nitrate.

reactivity of the reactive thiol group, on each subunit, towards iodoacetamide as a measure of the conformational state of the "working enzyme" i.e. while it is catalysing the reaction between $MgATP^{2-}$ and creatine (Milner-White and Kelly, 1976). From their results it appears that about 80% of the enzyme molecules are complexed in the $MgADP^{-} - PO_3^{-}$ - creatine form (in which the thiol groups are assumed to be virtually unreactive by analogy with their behaviour in the "transition state analogue" complex), with the remainder present as a Michaelis complex (in which the thiol groups are fully reactive). This finding lends support to the idea that the transition state analogue complex is relevant as an important state in the enzyme catalysed reaction.

Structural studies on the arrangement of substrates at the active site of the enzyme have relied heavily on magnetic resonance methods, as X-ray diffraction work is still at a preliminary stage. The magnetic resonance work (McLaughlin et al., 1976) has shown that the transferable phosphoryl group on one substrate is in apposition to the acceptor moiety on the second substrate. In addition the divalent metal ion is probably liganded to the α- and β-phosphates of the nucleotide substrate and not directly liganded to the guanidino substrate.

The actual mechanism of phosphoryl transfer in the catalytically active complex is still unresolved. Transfer could proceed via an "Sn2-type" mechanism in which the guanidino nitrogen would attack the phosphorus of the γ-phosphoryl group of ATP, simultaneously weakening the P-O

bond. This would involve the γ-phosphorus assuming a pentacoordinate trigonal bipyramid geometry in the transition state of the reaction (Milner-White and Watts, 1971). The alternative "Sn1-type" mechanism of transfer would involve the participation of the planar, highly reactive metaphosphate ion (PO_3^-). The effect of nitrate and other planar anions in forming a "transition state analogue" complex would be consistent with either type of mechanism. From detailed studies of the mechanism of phosphocreatine hydrolysis, it has been concluded that the reaction does involve production of metaphosphate which then reacts with water to produce phosphate (Allen and Haake, 1973, 1976). This is depicted in Fig 1.2, which also shows the proposed alternative fate of metaphosphate in the presence of $MgADP^-$ and creatine kinase i.e. to yield $MgATP^{2-}$.

This mechanism has several important implications for the enzyme catalysed reaction among which are the following: (1) The phosphocreatine must be protonated before it can break down to yield PO_3^- and presumably an amino acid side chain which can function as an acid catalyst must be close to the phosphocreatine site on the enzyme. (2) The metaphosphate produced must be in very close association with the $MgADP^-$ on the enzyme so that reaction occurs to form only $MgATP^{2-}$ and not phosphate (HPO_4^{2-}). The active site of the enzyme must be such as to exclude water, since no enzyme catalysed breakdown of phosphocreatine occurs in the absence of $MgADP^-$. (3) Since water is to be excluded from the active site, it is proposed that there must be specific binding sites for the phosphate dianion of phosphocreatine, for the guanidinium

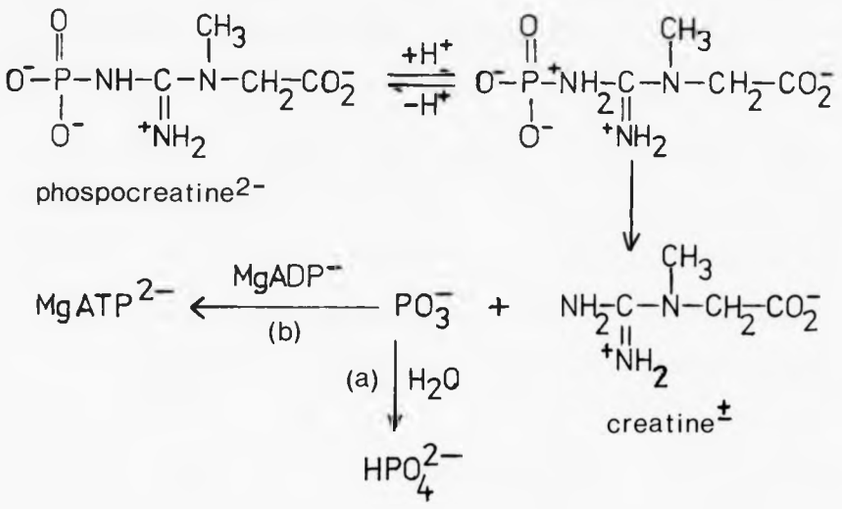


Fig 1.2 Proposed mechanism for hydrolysis of phosphocreatine (a) and its relevance to the enzyme catalysed transphosphorylation reaction (b) (Allen & Haake, 1976).

cation and for the carboxylate anion (Bickerstaff and Price, 1978). As yet none of these binding sites has been identified with certainty.

A good deal of effort has been expended in attempts to identify particular groups on the enzyme which are involved in the catalytic process. Most of the work has involved the technique of chemical modification of amino acid side chains and the most important results are outlined in the following:

(a) Thiol groups. It has long been known that creatine kinase possesses one reactive thiol group per subunit, the modification of which by a variety of thiol-blocking reagents, leads to complete or very nearly complete inactivation of the enzyme (Okabe et al., 1970). It has now been shown that modification of one thiol group per subunit does not always lead to inactivation of the enzyme (Smith et al., 1975). The importance of this thiol group in the mechanism of action of the enzyme has been the subject of considerable debate and will be examined in detail in Chapter 7.

(b) Lysine groups. Previous work has indicated that a lysine group was present at or near the active site of the enzyme, on the basis of modification by reaction with dansyl chloride or ρ-nitrophenyl acetate (Watts, 1973). Further work using magnetic resonance methods (James and Cohn, 1974) has led to the conclusion that the ε-CH_2 group of the lysine is in close proximity to the proton of the formate anion in the "transition state analogue" complex (enzyme-$MgADP^-$-formate-creatine) and hence by implication to the transferred phosphoryl group in the catalytically active complex.

(c) Arginine groups. Work with arginine-specific reagents butanedione and phenylglyoxal has shown that one arginine group per subunit can be modified with complete loss of activity (Borders and Riordan, 1975). The modified enzyme is incapable of binding nucleotides and inclusion of $MgATP^{2-}$ of $MgADP^{-}$ afforded protection against modification. On the basis of these results it was proposed that an arginine group is involved in the binding of nucleotides, presumably by an electrostatic interaction with the negatively charged oligophosphate moiety. It is reasonable to suppose that the arginine (and/or lysine) groups referred to may fulfill certain of the binding rôles suggested for the model studies on phosphocreatine breakdown mentioned earlier.

(d) Other groups. Chemical modification work has been performed on histidine (Pradel and Kassab, 1968) and tyrosine (Fattoum et al., 1975), and although modification leads to inactivation of the enzyme, these experiments have not been interpreted in as much detail as those described earlier. It is possible that a histidine group could act as the proton donating group towards phosphocreatine postulated from the model studies of Allen and Haake (1976).

Subunit behaviour

Creatine kinase consists of two freely dissociable subunits, each containing a single polypeptide chain and possessing no disulphide bridges (Bayley and Thomson, 1967). Sedimentation studies revealed that the calculated frictional ratio (f/f_o) was only 1.21 and the axial ratio 4.4 (for an assumed anhydrous prolate ellipsoid), indicating a compact

rather cigar-shaped molecule (Yue et al., 1967). A useful finding was that sodium dodecyl sulphate (SDS) causes dissociation of the subunits without apparent loss of structural organisation and the dissociated subunits were found to have the same frictional ratio and the same axial ratio as the native dimer (Yue et al., 1967). Hence the molecule may be envisaged as consisting of two cigar-shaped subunits lying side by side rather than as two egg-shaped subunits arranged end to end.

Creatine kinase occurs in three distinct forms readily distinguishable by their electrophoretic behaviour (Dawson et al., 1965) and these isoenzymes are termed MM (muscle type), MB (hybrid) and BB (brain type), in order of increasing mobility toward the anode at pH values above neutral, to indicate the major tissue of origin. Preparation of the hybrid (MB) can readily be achieved from purified MM and BB enzymes by using 4M-guanidine hydrochloride as the subunit dissociating agent. After recombination of the subunits by dilution or dialysis to remove the denaturant, the three isoenzymes can be separated by eletrophoresis (Dawson et al., 1967). Of particular interest has been the formation of hybrids between the BB forms of rabbit or ox with the dimeric arginine kinase from the muscles of the sea cucumber (Holothuria forskali)(Watts et al., 1972). These findings demonstrate that among the phosphagen kinases from invertebrates to vertebrates the essential features of the intersubunit region has been concerned through evolution .

Until recently, there has been very little evidence for any subunit interactions in rabbit muscle creatine kinase, although some unusual features of the reaction of the enzyme, from

other sources, with thiol-modifying agents has been interpreted in terms of subunit interactions (Watts, 1973). However, using a fluorescent probe technique McLaughlin (1974) reported that the binding of ADP to the rabbit muscle enzyme in the presence of creatine and nitrate (with or without Mg^{2+} displayed features similar to negative cooperativity.

A further indication of negative cooperativity has come from a study on the kinetics of modification of the reactive thiol group on each subunit with a variety of reagents (7-chloro-4-nitrobenzofurazan, iodoacetate and Nbs_2) (Price and Hunter, 1976). With each of these reagents the thiol groups on the two subunits reacted at the same rate as each other, both in the absence of ligands and in the presence of combinations of Mg^{2+}, creatine and ADP. However, in the presence of $MgADP^-$, creatine and nitrate the reactions deviated markedly from normal second order kinetics, indicating that the thiol groups were no longer reacting at the same rate as each other. A study of ADP binding by the enzyme using the equilibrium dialysis method also showed that in the presence of the transition state analogue complex, the binding of ADP shows features characteristic of either negative cooperativity or non-identical binding sites (Price and Hunter, 1976).

The non-identical behaviour could arise either from an inherent asymmetry of the enzyme in the "transition state analogue" complex or from an asymmetry induced by dissociation of nucleotide from one subunit or by modification of the thiol group on one subunit. These possibilities might be distinguished if data on the symmetry of the enzyme were available e.g. from X-ray diffraction studies. The

significance of the non-identical behaviour in this complex (which is thought to resemble the structure of the transition state of the enzyme catalysed reaction) is open to question. A rapid reaction study of the enzyme catalysed reaction has indicated that there is no large transient or lag phase in either direction i.e. that the actual chemical reaction is not rate limiting and that isomerisation of the enzyme must be rapid (Engelborghs et al., 1975). This would argue against a "flip-flop" mechanism of the type described by Lazdunski (1972) for the dimeric alkaline phosphatase of E.coli (in which the events at one active site are linked to those at the other via conformational changes in the enzyme) being of importance in the case of creatine kinase.

IMMOBILISED ENZYMES

Introduction

It is now becoming increasingly apparent that relatively few intracellular enzymes actually exist *in vivo* as free protein molecules in an aqueous environment. They are instead either membrane-bound, involved in solid-state assemblies like those found in mitochondria or are present in gel-like surroundings (Mosbach, 1976). Ideally one would wish to study intracellular enzymes in their natural environment by recombining isolated enzymes with the gel-like or matrix surroundings with which they are normally associated inside the cell, but progress in this direction is beset with many difficulties. An alternative and much more practical approach is to attach isolated enzymes to mechanically stable artificial supports such as hydrophilic polymers. Such systems can not only provide valuable models of how enzymes behave in their natural milieu but also can serve as efficient biological catalysts with many practical (including medical) applications.

At a meeting on "Enzyme Engineering" in 1973 under the auspices of the Engineering Foundation Conference, several recommendations regarding immobilised enzymes were made (Sundaram and Pye, 1974). It was agreed to use the terms "immobilised" and/or "matrix-bound" to differentiate between the native soluble enzyme and the insoluble enzyme derivative. In addition four major categories of immobilised enzymes were recognised: (a) Enzymes immobilised by chemical or physical adsorption; (b) Enzymes covalently bound to insoluble supports; (c) Enzymic species arising from crosslinking of the protein

molecules; (d) Enzymes entrapped into gels, membranes or within microcapsules. The spectrum of immobilisation possibilities encompassed by these four groups is very large and therefore we shall confine our attentions to group (b) which is the group with the greatest significance for the work presented in this Thesis.

Selection and activation of a matrix

When selecting a support or matrix for the covalent attachment of proteins, a number of factors are taken into consideration to optimise protein binding and immobilised enzyme activity. The most important factors are:

(a) Mechanical properties of the matrix, e.g. rigidity and durability.

(b) Physical form (granules, beads, sheets, etc.)

(c) Resistance to chemical and microbial attack.

(d) Hydrophilicity (ability to incorporate water into its structure.

(e) Permeability to high molecular weight proteins.

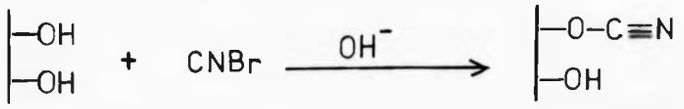
(f) Ease of matrix activation and protein retention factors.

(g) Price and availability.

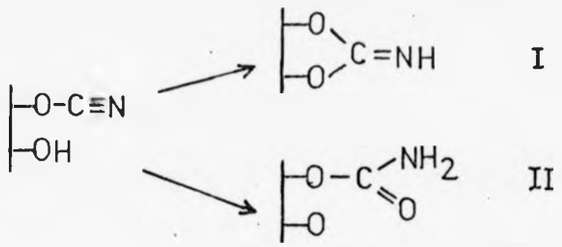
From experience (Porath and Axen, 1976) it has been found that hydrophilicity is a crucial factor for the preservation of enzymes in a highly active state after their immobilisation to the solid support. This fact places hydrophilic gels in a favourable position among the matrices used as supports for immobilised enzymes. The most important polysaccharide gel supports are (1) starch, (2) cellulose, (3) dextran and (4) agar. Starch and cellulose have certain disadvantages which make them less suitable as matrices, the most serious of

which are a non-uniform macroporus structure and a strong susceptibility to microbial disintegration (Porath and Axen, 1976). Dextran in a cross-linked form (Sephadex) is considerably more suitable and Sephadex-bound enzymes are in general more active than cellulose-bound enzymes (Axen et al., 1967). Agar and agarose (bead form) approach, in many aspects, the ideal solid matrix for binding proteins and an extensive analysis of the properties of agarose can be found elsewhere (Porath and Axen, 1976).

The coupling procedure most widely used at present to bind proteins to agarose is based on the reaction of cyanogen halides with the hydroxyl groups of the agarose matrix. The reaction is carried out in alkaline solution either in water or in water-miscible organic solvents (March et al., 1974; Axen et al., 1967; Nishikawa and Bailon, 1975). Hydrolysis of the reagent, usually cyanogen bromide, is an undesirable side reaction that cannot be avoided but can be suppressed by optimising the conditions for imidocarbonate formation. At and below room temperature the optimum pH for the desired reaction lies in the interval 11 - 12.5. The most important reactions which occur during the activation are:

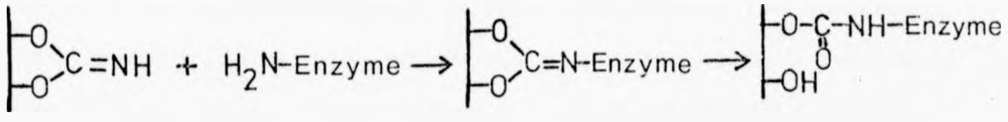


Cyanates are very labile and the reaction will proceed to give the reactive imidocarbonate (I) and the unreactive carbonate (II)



A part of the imidocarbonate is undoubtedly in the form of cross-links (III) since the gel becomes insoluble in boiling water.

The presence of species I, II and III has been indicated by infrared (IR) analysis (Axen and Ernback, 1971) and it has been found that hydroxide ions are consumed in most reactions with concomitant decrease in pH. Early activation procedures monitored the overall reaction in a pH-stat under continuous addition of alkali (Axen et al., 1967), however more recent methods perform the reaction in the presence of strong carbonate buffer (March et al., 1974). The coupling of enzyme occurs entirely or at least predominantly through the free amino groups of the ligand protein. As revealed by IR spectra and studies on low molecular weight model compounds (Axen and Ernback, 1971), the following reactions have been found to occur:



The amine should be unprotonated, which means that the reaction will occur at an optimum rate when the coupling pH is greater than the pKa of the amino groups and generally a coupling pH within the range pH 8 - 10 is used.

Applications of matrix-bound enzymes

It is outwith the scope of this Thesis to describe the very large number of potential applications and current uses of immobilised enzymes as this subject is dealt with very comprehensively in several excellent books (Mosbach, 1976; Katchalski et al., 1971; Dunlap, 1974). Rather we shall confine our attention to the use of immobilised enzymes as tools for studies of fundamental problems in biochemistry, and in particular those problems concerning intermolecular interactions of enzyme subunits.

Immobilisation allows the study of enzymes under conditions in which they would normally aggregate. For example, when ligand binding is accompanied by association or dissociation of an oligomeric enzyme, immobilisation offers a means for uncoupling the interaction so that those changes associated with ligand binding can be assessed independently from those resulting from protein association. Glutamate dehydrogenase is an enzyme of molecular weight 336000 and the oligomeric unit is composed of 6 identical polypeptide chains; the oligomeric units aggregate in the presence of ADP to form linear aggregates with a molecular weight exceeding 2 million (Horton et al., 1974). To determine whether such aggregate formation is a prerequisite for the increased activity observed, or whether ADP acts as an allosteric modulator directly on the monomeric subunit with aggregation of oligomeric units being a secondary phenomenon, bovine liver enzyme was covalently bound in its oligomeric form to porous glass beads (Horton et al., 1974). It was found that the immobilised oligomer was subject to activation by ADP and since under the conditions employed no

free enzyme was available to aggregate, the observed activation was shown to be independent of association of the active oligomeric units.

Refolding of some disulphide-containing proteins such as trypsinogen from a denatured state leads to aggregate formation and incorrect folding. However by immobilising trypsinogen to Sepharose 4B it has been found that this problem is largely eliminated (Sinha and Light, 1976). Yields of 60 - 70% regenerated trypsinogen were obtained after 24 h and the regenerated trypsinogen displayed similar properties to the native molecule.

Immobilised subunits

Immobilisation can be used to prevent the spontaneous association between subunits of an oligomeric protein (Chan, 1976). With this approach it is possible to determine whether the subunit form of an enzyme is catalytically active or not. The technique was first used by Chan and co-workers to study the properties of isolated subunits of aldolase (Chan, 1970) and transaldolase (Chan et al., 1973a). Subsequently the method has been applied to various enzymes and some of the results are collected in Table 1.2. From the Table it can be seen that the enzymes aldolase, arginase, transaldolase and triose-phosphate isomerase do not require the intact oligomeric structure for the expression of catalytic activity. However phosphorylase, lactate dehydrogenase and phosphoglucose isomerase do require subunit interactions for enzyme activity. In the case of phosphorylase it was neatly demonstrated that the inactivity of the matrix-bound subunit was not an artifact produced by the

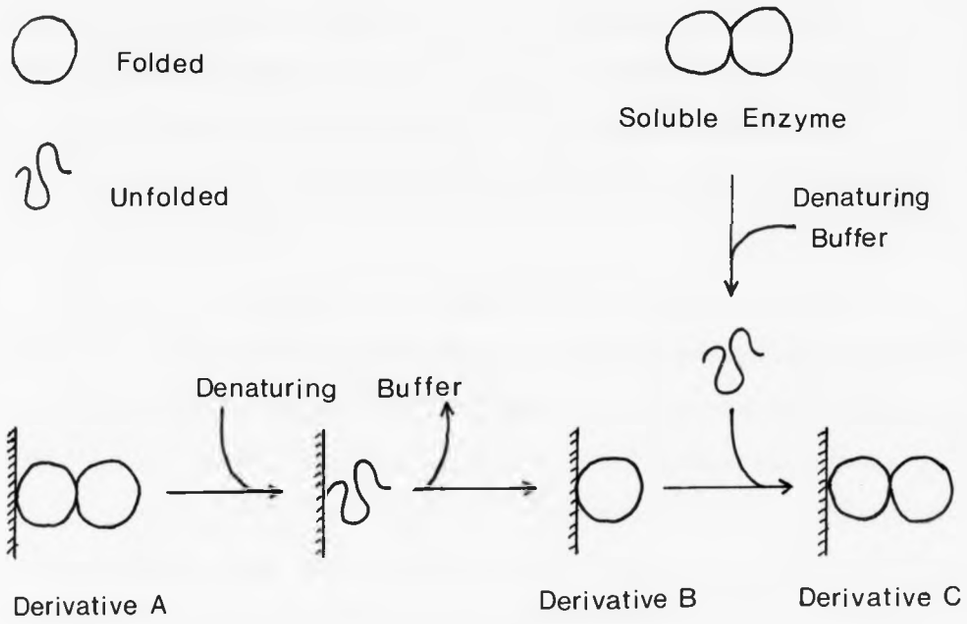
Table 1.2 Some proteins studied by the immobilised subunit approach

Protein	No. of Subunits	Properties of Subunit Form	Reference
Aldolase	4	Active	Chan (1970)
Arginase	4	Active	Carjaval et.al.(1977)
Phosphorylase b	2	Inactive	Feldman et.al.(1972)
Transaldolase	2	Active	Chan et. al. (1973a)
Fructose diphosphatase	4	Dimers Active	Grazi et.al. (1973)
Lactate dehydrogenase	4	Inactive	Chan & Mosbach (1976)
Phosphoglucose isomerase	2	Inactive	Bruch et. al. (1976)
Triose-phosphate isomerase	2	Active	Fell & White (1975)

matrix (Feldman et al., 1973). The matrix-bound subunit containing bound cofactor pyridoxal phosphate was re-associated with soluble subunits of native enzyme which had bound an inactive analogue of pyridoxal phosphate and therefore were enzymically inactive. The reconstituted dimer exhibited the activity of only one subunit as the inactive subunit containing the analogue generated activity in the matrix-bound subunit by providing the essential subunit interactions.

If the immobilised subunit is active then comparison of its enzymic properties with those of the corresponding matrix-bound oligomer can yield valuable information regarding the effects of subunit interactions on enzyme function. An important prerequisite for the detailed interpretation of experiments involving the matrix-bound subunit forms of enzymes is that renaturation with good recovery of enzyme activity can be achieved after subunit dissociation (Chan, 1976).

The principles of the method for preparing matrix-bound subunits are outlined in Scheme 1.1. One of the basic features of this approach is that the oligomeric form of the enzyme is coupled to the matrix via only one of its subunits to yield derivative A (Scheme 1.1). The subunit form is then generated by treating derivative A with a denaturing agent which produces dissociation of the subunits and subsequent removal of the non-covalently bound subunits. On removal of the denaturing agent, the matrix-bound subunit (derivative B) can refold but is prevented from re-associating with other matrix-bound subunits by the rigidity of the matrix (Chan, 1976). A useful test to demonstrate the presence of matrix-bound subunits is to prepare the re-associated matrix-bound oligomer



Scheme 1.1 Scheme depicting the relationships between the matrix-bound derivatives of a dimeric enzyme

The soluble enzyme is coupled to CNBr-activated Sepharose 4B to yield the matrix-bound enzyme (derivative A). Successive treatment of derivative A with a denaturing buffer then a renaturing buffer produces the matrix-bound subunit (derivative B). The matrix-bound re-associated enzyme (derivative C) is prepared from derivative B by re-association with added subunits of denatured soluble enzyme.

(derivative C) by re-associating with added subunits of soluble enzyme (Scheme 1.1). The interconversions between the various derivatives can be conveniently monitored by following the changes in protein content of the derivatives (Chan et al., 1973a).

In order to prevent the coupling of oligomers via more than one subunit to the matrix, or the interaction between the immobilised subunits after preparation, it is necessary to limit the density of activated points on the matrix (Chan, 1970). In practice, an activation level of 1-5 mg of CNBr/ml of packed Sepharose gel, has generally given preparations with acceptably low contamination of immobilised oligomers (Chan, 1970; Chan et al., 1973a; Chan and Mosbach, 1976).

IMMOBILISED CREATINE KINASE

Introduction

In their native form, many enzymes are found to contain subunits (Klotz et al., 1970) and it has been often assumed that the subunit structure has some rôle in the function of the enzyme. Certainly in the case of many regulatory enzymes, the allosteric properties have been explained successfully in terms of co-operative interaction between subunits (Koshland, 1970). There remain, however, a large number of oligomeric enzymes which show no signs of co-operative or other effects which can be attributed to their quaternary structure. Creatine kinase belongs to this latter category and it was decided, therefore, to investigate in more detail the role of the dimeric structure in the function of the enzyme. The main part of this work therefore is concerned with the preparation and study of the subunits of creatine kinase.

There have been two principal approaches to the study of the activity of subunits of oligomeric enzymes. The first approach involves an analysis of the kinetics of regain of activity during re-association of the dissociated enzyme (Chan et al., 1973b). The second approach involves the preparation of matrix-bound subunits as described earlier; a comparison of the properties of the matrix-bound oligomer with those of the matrix-bound subunit should provide valuable information regarding the importance of subunit interactions in the properties considered. In this present work the second procedure was employed in order to investigate the effects of subunit interactions on creatine kinase function.

An important prerequisite for the detailed interpretation of experiments involving matrix-bound subunit forms of enzymes is that substantial regain of enzyme activity of the native soluble enzyme can be achieved upon renaturation from a denatured state. A study was undertaken, therefore, to evaluate and where possible improve the renaturation of soluble creatine kinase after denaturation. Creatine kinase was then immobilised on Sepharose 4B and several properties of the matrix-bound enzyme were studied in comparison with the soluble enzyme in order to establish whether or not immobilisation adversely affected the activity of the enzyme. In this respect the properties of the immobilised enzyme proved largely similar to those of the native soluble enzyme.

The immobilised subunit form of the enzyme was prepared from the immobilised dimer creatine kinase and the subunit form retained substantial enzyme activity, clearly demonstrating that subunit interactions are not essential for the expression of catalytic activity in creatine kinase. Studies on the properties of the matrix-bound subunit of the enzyme also revealed that the dimeric structure is not important with regard to enzyme kinetics, iodoacetamide inactivation in the presence of various combinations of substrates and ligands, and pH optimum.

Although studies with matrix-bound subunit forms of enzymes provides much useful information, the presence of the matrix precludes the use of a number of physicochemical techniques (e.g. fluorescence spectroscopy and ultracentrifugation). It was therefore of interest to study the process of re-association of subunits with the matrix-bound subunit

derivative and added soluble subunits with a view to preparing, if possible, soluble non-associating enzyme subunits. The results of studies on the matrix-bound subunit derivative of creatine kinase demonstrated that it should be possible to design suitable procedures for the preparation of isolated soluble subunits, and some progress in this direction was achieved in this present work.

CHAPTER 2

MATERIALS & METHODS

MATERIALS

Enzymes

Creatine kinase was isolated from rabbit skeletal muscle as described in the Methods Section. Pyruvate kinase (200 U/mg) and lactate dehydrogenase (550 U/mg) were obtained as ammonium sulphate suspensions from Boehringer, Lewes, Sussex, U.K. Pyruvate kinase was also isolated from rabbit skeletal muscle as described in the Methods Section and stored in an ammonium sulphate suspension. Prior to use the suspensions were centrifuged at 2000g for 5 minutes and the resulting pellet dissolved in 0.1 M-glycine/NaOH solution at pH 9.0. Trypsin (1100 BAEE units/mg) was obtained from Sigma Chemical Co., Eastbourne, Sussex, U.K.

Protein standards

Cytochrome c (horse heart), myoglobin (horse heart), ovalbumin (hen egg) and serum albumin (bovine) were obtained from Sigma Chemical Co., Eastbourne, Sussex, U.K.

Cyanogen bromide-acetonitrile

Both cyanogen bromide (CNBr) and acetonitrile were purchased from Koch-Light Ltd., Colnbrook, U.K. The acetonitrile was twice re-distilled (80^{o}C) and dried with anhydrous magnesium sulphate. A stock solution of CNBr was prepared by adding 100 ml of the acetonitrile to 20 g of solid CNBr. This solution was stable for several months at room temperature.

Radioactive compounds

Iodo[1-^{14}C]acetamide (58 Ci/mol) and [8-^{14}C]adenosine 5'-diphosphate (50 Ci/mol) were both obtained from the Radiochemical Centre, Amersham, Bucks., U.K. A stock solution of radioactive iodoacetamide (IAM) was prepared by adding 50 μCi of radioactive IAM to 3 ml of 10mM-iodoacetamide in 0.1 M-glycine/NaOH at pH 9.0. A stock solution of radioactive ADP was prepared by adding 0.03 ml (approx. 1.5 μCi) to 1.0 ml of 0.5 mM-ADP in 50mM-Tricine/NaOH at pH 8.0. In both cases the stock solution was divided into ten equal aliquots, and then stored frozen at -15°C. Aliquots were then thawed and used as required.

Guanidine hydrochloride

Guanidine hydrochloride (Gdn HCl) was obtained from BDH Chemicals, Poole, Dorset, U.K., and was re-crystallised from ethanol (98%, v/v) at least three times or until the absorbance at 280 nm of an aqueous 6 M solution was less than 0.1.

Buffer solutions

All buffer solutions were prepared by dissolving the appropriate amount of solid in double distilled water and adjusting the solution to the required pH with dilute solutions of acid or alkali as appropriate. Double distilled water was used for all solutions and was obtained from a Fisons all glass Bi-distillation unit.

Other reagents

Phospho(enol)pyruvate was prepared by chemical synthesis

as described in the Methods Section. ATP, ADP, NADH, dithiothreitol, 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) and 3-bromopyruvic acid were obtained from Sigma Chemical Co., Eastbourne, Sussex, U.K. Creatine, glycine, Tricine, Tris, trimethyl phosphite, cyclohexylamine and iodoacetamide were obtained from BDH Chemicals, Poole, Dorset, U.K. The iodoacetamide was twice re-crystallised from aqueous 98% (v/v) ethanol. Sepharose 4B, Sephadex G-100 and Blue Dextran were obtained from Pharmacia (G.B.) Ltd., London, U.K. All other reagents were of the highest grade available and were used without further purification.

METHODS

Isolation of creatine kinase from rabbit skeletal muscle

(Ref. Kuby et al., 1954)

Preparation of homogenate

In the cold room the rabbit muscle was cut into small segments, minced into a beaker and homogenised with 10 mM-Tris/HCl at pH 9.5 containing 10 mM-KCl and 1.0 mM-EDTA (2 litres/kg of muscle). The resulting homogenate was then stirred at $0^{o}C$ for not longer than 15 minutes before the extract was centrifuged at 3000 g for 20 minutes at $0^{o}C$.

Precipitation of the homogenate

In the cold room the volume (S_1) of the supernatant was measured and then solid ammonium chloride was added with stirring to a final concentration of 0.1 M. The pH of the solution was adjusted to 9.0 with 5 M-ammonia solution and the mixture then stirred at $0^{o}C$ for 30 minutes. A volume ($1.5 \times S_1$) of ethanol (98%, v/v) was then added to the mixture, slowly to avoid the temperature rising above $0^{o}C$. The mixture was then stirred at room temperature for 2 h before the precipitate was removed by centrifugation at 3000 g for 20 minutes at $10^{o}C$.

Extraction of magnesium proteinates

The volume (S_2) of the supernatant was measured and then a volume (V) of 0.1 M-Tris/HCl at pH 9.0 containing 2M-$MgSO_4$ was added with stirring to a final concentration of 30 mM (with

respect to $MgSO_4$). A volume (1.5 x V) of ethanol (98%, v/v) was then added to the solution and the mixture stirred for 30 minutes at room temperature before the precipitate was collected by centrifugation at 3000 g for 20 minutes at 0^oC. In the cold room the precipitate was then twice thoroughly re-suspended and extracted at 0^oC with 70 mM-magnesium acetate solution at pH 9.0, in volumes equal to 6% and 4% of the volume of the first supernatant (S_1). Each time the insoluble portion was separated by centrifugation at 25000 g for 20 minutes at 0^oC. The exact volumes of magnesium acetate used for extraction and the volume of the combined extracts were noted for the purpose of calculating the amount of alcohol in the extract (see Appendix 1).

Alcohol fractionation

To the combined extracts was added cold ethanol (98%, v/v), slowly to prevent the temperature rising above 0^oC, to a final concentration of 36%. The mixture was then stirred at 0^oC for 30 minutes before the precipitate was collected by centrifugation at 25000 g for 20 minutes at 0^oC. To the clear supernatant was added further cold ethanol to a final concentration of 50% and then a further period of 30 minutes stirring at 0^oC before the precipitate was collected by centrifugation at 25000 g for 20 minutes at 0^oC. The precipitate was then dissolved in a minimum volume of 50 mM-ammonium citrate solution at pH 9.0.

Final purification and storage

The solution was then dialysed overnight at 4^oC

successively against 1.0 mM-EDTA solution at pH 9.5 (4 litres), 0.01 mM-EDTA solution at pH 9.5 (4 litres) and finally 10 mM-Tricine/NaOH solution at pH 9.0 (2 litres). The solution was then filtered through a Millipore filter (pore size 0.22 μm) before adding dithiothreitol to a concentration of 2 mM. Finally an equal volume of glycerol was added to the solution to give final concentrations of 50% glycerol and 1.0 mM-dithiothreitol. The solution was stored at -18°C. The glycerol-enzyme solution was dialysed against the appropriate buffer solution for 24 h at 4°C before use.

Isolation of pyruvate kinase from rabbit skeletal muscle

(Ref. Tietz and Ochoa, 1958)

Extraction

In the cold room the muscle was cut into small pieces and minced into a beaker. The tissue was then mixed thoroughly with 1.0 mM-EDTA solution at pH 7.0 (in the proportion 1.0 litre/kg muscle) and the extraction continued for a further 30 minutes with occasional stirring. The mixture was then filtered through two layers of cheesecloth before the solid residue was extracted once more as above. The two extracts were combined for the next step.

Ammonium sulphate fractionation

Solid ammonium sulphate was added to the extract, with stirring, in the proportion of 320 g/litre (67% saturation). The precipitate was collected by centrifugation at 3000 g for 20 minutes at $0^{o}C$ and then dissolved in a volume (20 - 30 ml) of 20 mM-imidazole/HCl solution at pH 7.0, containing 1.0 mM-EDTA. The solution was then dialysed overnight against 10 mM-Tris/HCl buffer at pH 7.3 containing 1.0 mM-EDTA (2 litres), and any resulting precipitate removed by centrifugation at 25000 g for 20 minutes at $0^{o}C$.

Ethanol fractionation

The protein concentration of the solution was estimated (assuming OD_{280} = 1.0 for 1 mg/ml) and adjusted to approximately 20 mg/ml with 20 mM-imidazole/HCl buffer at pH 7.0 containing 1.0 mM-EDTA. The solution was then cooled to $2^{o}C$ in a bath of

acetone containing dry ice and then chilled ethanol (98%, v/v) was added with stirring to a final concentration of 12% by volume. The temperature of the solution was then lowered to -5°C and the stirring continued for a further 5 minutes. The precipitate was then removed by centrifugation at 25000 g for 15 minutes at -10°C. Further chilled ethanol was added to the supernatant to bring the ethanol concentration to 30% by volume while the temperature was maintained at -5°C in the acetone/dry ice bath. After 10 minutes stirring the precipitate was collected by centrifugation at 25000 g for 15 minutes at -10°C. The precipitate was dissolved in 20 mM-imidazole/HCl buffer at pH 7.0 containing 1.0 mM-EDTA as before and dialysed against 10 mM-Tris/HCl at pH 7.3 containing 1.0 mM-EDTA (2 litres).

Heat treatment

The protein concentration of the solution was estimated (as before) and adjusted to 20 mg/ml with 1.0 mM-EDTA solution at pH 7.0. A volume of 1.0 M-imidazole/HCl buffer at pH 7.0 containing 1.0 mM-EDTA was added to the solution to give a final concentration of 50 mM and the pH was then adjusted to 7.0 with 5 M-acetic acid solution. The enzyme solution was then rapidly brought to 60°C in a heated water bath and held at this temperature for 5 minutes with continuous stirring to avoid coagulation. The mixture was then cooled rapidly in a water bath containing crushed ice and the denatured protein removed by centrifugation at 25000 g for 15 minutes at 0°C. To the clear supernatant was added saturated ammonium sulphate solution to give a final concentration of

20% (v/v). The pH was then adjusted to 7.0 before the solution was rapidly brought to 65°C in a heated water bath and held at this temperature for 5 minutes. The solution was then rapidly cooled as before and any precipitate removed by centrifugation at 25000 g for 15 minutes at 0°C.

Ammonium sulphate precipitation

To the enzyme solution was added solid ammonium sulphate (in the proportion 30 g/100 ml) and the mixture stirred for 10 minutes. The precipitate was collected by centrifugation at 25000 g for 15 minutes at 0°C and dissolved in 20 mM-imidazole/HCl buffer at pH 7.0 containing 1.0 mM-EDTA. The protein concentration was estimated as before and adjusted to 20 mg/ml with further imidazole buffer. Saturated ammonium sulphate solution was added to the enzyme solution to give 50% saturation and the mixture left overnight to stand at 4°C. The resulting crystals were collected by centrifugation at 25000 g for 15 minutes at 0°C and dissolved in a small volume of 20 mM-imidazole/HCl buffer at pH 7.0 containing 1.0 mM-EDTA. The protein concentration of the enzyme solution was estimated (assuming OD_{280} = 0.54 for 1.0 mg/ml) and then to the solution was added an equal volume of saturated ammonium sulphate solution to yield an ammonium sulphate suspension of the enzyme which was stored at 4°C.

Chemical synthesis of phospho(enol)pyruvate

(Ref. J R Sargent, Personal communication)

Preparation of the cyclohexylammonium salt

To 100 ml of dry ether was added 4.3 g (35 m moles) of trimethyl phosphite and the mixture well stirred. To the mixture was then added 5.6 g (34 m moles) of 3-bromopyruvic acid and the solution allowed to stand at room temperature for 5 minutes before the ether was removed by rotary evaporation at room temperature. Further rotary evaporation facilitated the removal of unreacted trimethyl phosphite. To the colourless oil was added 43 ml of distilled water and 3.36 g (34 m moles) of cyclohexylamine and the solution well mixed. The mixture was allowed to stand at room temperature for 3 days before the water was removed by rotary evaporation under reduced pressure and using a water bath at $35^{o}C$.

The resulting solid was freeze-dried (6 h) and dissolved in methanol (45 ml) followed by an equal volume of ether. The mixture was allowed to stand at $4^{o}C$ overnight before collecting the crystals which were dried over phosphorus pentoxide.

Preparation of matrix-bound enzyme derivatives

Matrix-bound enzyme

Sepharose 4B was washed extensively with distilled water on a sintered glass funnel to remove any preservatives present. To the washed Sepharose (10 ml packed volume) was added 10 ml of distilled water and 20 ml of 2M-sodium carbonate solution. The mixture was then stirred gently before adding 50 mg of CNBr dissolved in 0.5 ml of acetonitrile (March et al., 1973) then the stirring was increased to ensure rapid mixing. After 2 minutes rapid stirring the gel was filtered and washed on the sintered glass funnel successively with 100 ml of cold 1.0 M-sodium bicarbonate solution at pH 9.0, 100 ml of distilled water and finally 100 ml of phosphate buffer (10 mM-sodium phosphate solution at pH 8.0 containing 1.0 mM-EDTA). To 10 ml of activated gel was added 10 mg of creatine kinase in 8 ml of phosphate buffer and the mixture stirred gently at $4^{o}C$ overnight.

Excess soluble enzyme was then removed from the gel by washing the gel alternately with phosphate buffer containing 1.0 M-NaCl and phosphate buffer containing no NaCl until no protein could be detected in the washings. A volume of 0.1 M-glycine/NaOH at pH 8.0 equal to the volume of packed gel was then added to the matrix-bound enzyme and the mixture allowed to stand at room temperature for 2 h. The gel was then washed and re-suspended in phosphate buffer. This matrix-bound derivative corresponds to derivative A in Scheme 1.1 (Chapter 1).

Packed gel volumes were determined after centrifugation at 200 g for 2 minutes in graduated centrifuge tubes. Stock suspensions of the matrix-bound derivatives were prepared by mixing

equal volumes of gel and phosphate buffer to form a 1:1 suspension. Accurate samples could then be removed from stirred suspensions by using an automatic plunger-type pipette with the plastic tips cut so as to increase the aperture.

Matrix-bound subunit derivative

A volume (4 ml packed gel) of matrix-bound enzyme from the previous step was mixed with 10 ml of denaturing buffer (0.1 M-Tris/HCl at pH 7.5 containing 5 mM-dithiothreitol and 6 M-GdnHCl) for 1.0 h at room temperature. The gel was then transferred to a sintered glass tube (1 cm x 10 cm) and washed with 40 ml of the denaturing buffer. The denaturing buffer was then washed out of the tube and subsequently replaced by renaturing buffer (0.1 M-Tris/HCl at pH 7.5 containing 5 mM-dithiothreitol). Renaturation was allowed to proceed for 3 h at 20°C followed by 18 h at 4°C. The gel was then washed extensively with phosphate buffer to displace the renaturing buffer and the gel was finally re-suspended in phosphate buffer. This matrix-bound derivative corresponds to derivative B in Scheme 1.1 (Chapter 1).

Matrix-bound re-associated enzyme

Small aliquots (0.05 ml) of a solution of denatured soluble enzyme (previously incubated with denaturing buffer) were added to a gently stirred suspension of matrix-bound subunit derivative from the previous step in renaturing buffer (gel/buffer, 1:5 v/v). After addition of a four fold excess of the dissociated soluble protein (expressed relative to the protein content of the matrix-bound subunit) the

mixture was allowed to renature for 3 h at 20^{o}C followed by 18 h at 4^{o}C. The gel was then transferred to a sintered glass tube (1 cm x 10 cm) and washed extensively with phosphate buffer and then re-suspended in phosphate buffer. This matrix-bound derivative corresponds to derivative C in Scheme 1.1 (Chapter 1).

Determination of molecular weight and homogeneity

Protein standards

The following protein standards were used in the three methods employed to determine molecular weight and homogeneity of the soluble enzyme.

Protein	Source	Molecular Weight	Reference
Cytochrome c	Horse heart	13400	Margoliash et al. (1962)
Myoglobin	Horse heart	17800	Edmunson and Hirs (1962)
Ovalbumin	Hen egg	45000	Andrews (1964)
Serum albumin	Bovine	67000	Andrews (1964)
Creatine kinase	Rabbit muscle	82000	Watts (1973)
Pyruvate kinase	Rabbit muscle	230000	Andrews (1964)

Column gel filtration

The column was packed in a vertical glass tube across which was fused a sintered glass disc to support the gel. Sephadex gel filtration media G-100 was suspended in phosphate buffer and allowed to swell for 2 - 3 days before the smallest particles were removed by decantation. The gel was then poured into the glass tube and even packing of the gel was checked by watching the passage through it of a coloured protein (e.g. cytochrome c). Columns were eluted continuously with phosphate buffer when not in use, and all experiments were conducted at $4^{o}C$. Proteins were dissolved in or dialysed against phosphate buffer and applied to the top of the column by layering under the buffer already present. The density of the protein solutions was increased slightly by the addition of a few crystals of sucrose.

Collection of column effluent in fractions of 1.0 ml was accomplished using a Gilson fraction collector, and was begun when half the protein solution was judged to have entered the column. Proteins were estimated spectrophotometrically by using the whole of each fraction in a semi-micro (1.0 ml capacity) quartz cuvette. The wavelength monitored was 280 nm except in the case of cytochrome c where it was 408 nm. Blue dextran was monitored at 625 nm. The elution volume (V_e) was determined from the elution profile as the effluent volume corresponding to the half height of the leading edge of the peak. A calibration graph for standard proteins was constructed and is presented in Fig. 2.1.

SDS polyacrylamide gel electrophoresis

Electrophoresis in 7.5% polyacrylamide gels in the presence of SDS was performed as described by Weber and Osborne (1969). Details of the preparation of buffers and gels are given in Appendix 2. To the protein solution (1.0 ml) was added sample buffer (9 ml) and 0.01 ml of mercaptoethanol in a pyrex boiling tube. The tube was covered with a glass marble and placed in a heated water bath at 100°C for 2 - 3 minutes. The solution was then cooled to room temperature before a sample (0.05 ml) was removed and mixed with 0.005 ml of mercaptoethanol, 0.005 ml of bromophenol blue and a few crystals of sucrose. After the components had been well mixed the solution was loaded on to the top of the gel which was then covered with reservoir buffer.

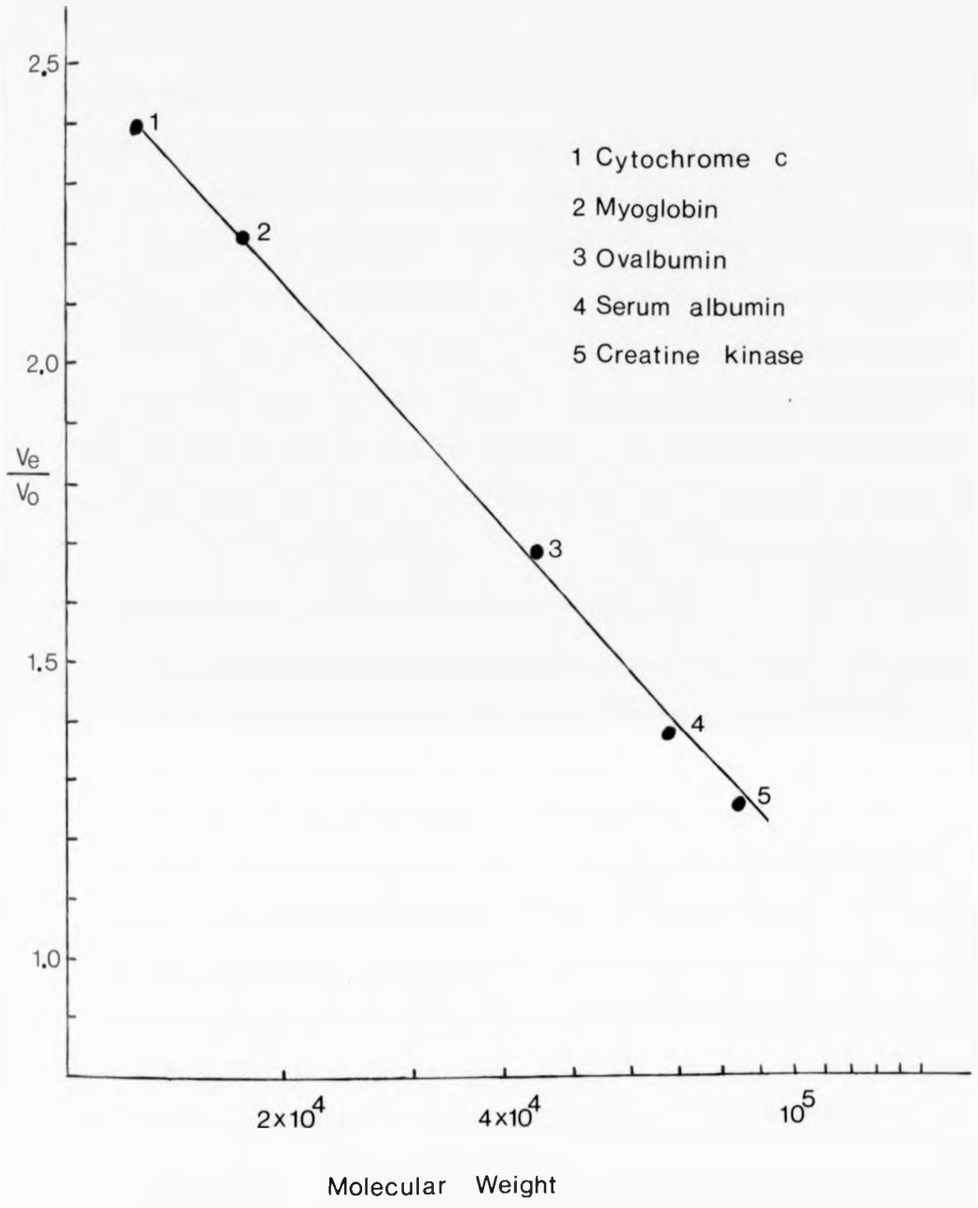


Fig 2.1 Plot of elution volume (Ve) relative to the elution volume of blue dextran (Vo), against log (molecular weight) for proteins on Sephadex G-100.

The apparatus was then connected to the power pack which was set to produce a constant current of 8 mA for each tube. Electrophoresis was stopped when the tracking dye neared the bottom of the tube in about 3 - 4 h. Gels were stained in Coomassie Brilliant Blue G250 (Appendix 2) for 2 h at room temperature and destained in methanol/acetic acid/water (1:1:8 by volume) overnight at room temperature. Gels were scanned at 545 nm in a Unicam SP 1800 spectrophotometer fitted with a gel scanning device. A calibration graph for standard proteins was constructed and is presented in Fig. 2.2.

Polyacrylamide disc gel electrophoresis

Electrophoresis in 7.5% polyacrylamide gels at pH 8.9 was performed as described in detail by Brewer et al. (1974). Details of the preparation of buffers and gels are given in Appendix 3. A Tris/glycine electrode buffer system was used with a running pH of 8.9. All samples well dialysed against the electrode buffer before a sample (0.05 ml) was removed and mixed with 0.005 ml of bromophenol blue and a few crystals of sucrose. After the components had been well mixed the solution was loaded onto the top of the gel which was then covered with electrode buffer. The apparatus was then connected to the power pack which was adjusted to produce a constant current of 2 mA per tube.

Electrophoresis was stopped when the tracking dye neared the bottom of the tubes in about 2 - 3 h. Gels were placed in stain solution (Appendix 3) for 1.0 h and then transferred to destain solution (Appendix 3) and allowed to destain overnight. Gels were scanned at 545 nm in a Unicam

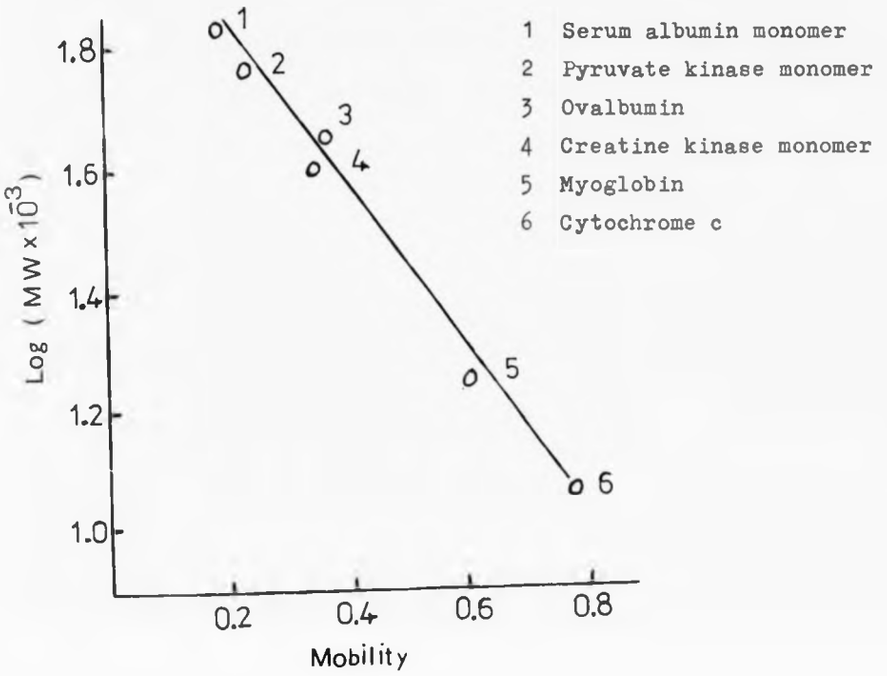


Fig 2.2 Plot of log (molecular weight x 10^{-3}) against mobility of proteins separated by polyacrylamide-SDS gel electrophoresis.

SP 1800 spectrophotometer fitted with a standard gel scanning device.

Assay of enzyme activity

Creatine kinase activity was determined in the forward direction (phosphocreatine synthesis) using a coupled assay system involving pyruvate kinase and lactate dehydrogenase as coupling enzymes. All assays were performed at 30°C in 0.1 M-glycine/NaOH at pH 9.0 and the standard assay system consisted of 40 mM-creatine, 4 mM-ATP, 5 mM-magnesium acetate, 0.1 M-sodium acetate and 24 units each of pyruvate kinase and lactate dehydrogenase in a final volume of 3 ml. Enzyme activity of matrix-bound derivatives was measured using an arrangement for stirring the contents of the assay cuvette (Chan et al. 1973a).

A standard 3 ml capacity cuvette was placed in a thermostated cell holder situated on a magnetic stirrer. The reaction was initiated by the addition of a small aliquot (0.05 — 0.10 ml) of suitably diluted suspension to the assay mixture. A micro-magnet (4 mm in length) was placed in the cuvette and the reaction mixture maintained at 30°C and continuously stirred. At suitable intervals (2 minutes) the cuvette was placed in an adjacent spectrophotometer to record the absorbance at 340 nm for a few seconds then the cuvette was returned to the cell holder for further temperature control and stirring. By allowing a chart recorder to run continuously, a series of points could be obtained which when joined together gave an estimate of the rate of change in optical density (see Scheme 4.1).

The suspensions employed in the cuvette were sufficiently stable for up to 5 seconds without stirring which was adequate for obtaining a recorder tracing. In this present work the

activity of the matrix-bound derivatives was sufficiently high that dilutions of the order 1:2400 (gel:buffer) were used. The contribution of any soluble enzyme activity was checked by filtration of the reaction mixture, and found to be less than 2% of the observed activity in all cases. All enzyme assays were performed on a Cecil CE 202 spectrophotometer in combination with a Bryans 2800 Recorder.

Estimation of radioactivity

Radioactivity was measured using a mixture (2:1) of scintillator solution and scintillation grade Triton X-100, and counting samples in a Phillips Liquid Scintillation Analyser. The scintillator solution consisted of 500 ml of toluene containing 2 g of PPO and 0.5 g of POPOP. Radioactivity of both soluble and matrix-bound derivatives was determined after hydrolysing an aliquot (0.1 ml) in 0.5 ml of 12 M-HCl, diluting it with 0.5 ml of distilled water and then adding 15 ml of scintillation mixture. The resulting white precipitate was completely dissolved after 5 minutes of vortex mixing.

ESTIMATION OF PROTEIN

The protein concentration of the soluble enzyme was determined from the absorbance at 280 nm assuming an absorbance of 0.9 for a 0.1% solution and a molecular weight of 82000 (Noda et al., 1960). Protein contents of matrix-bound derivatives were determined using a slightly modified version of the Lowry method (Lowry et al., 1951). The standard suspension (1:1) of gel in phosphate buffer (Bickerstaff and Price, 1976a) was used and samples (0.05 ml) were taken from a stirred suspension and made up to 0.6 ml with distilled water. To this was added 2.5 ml of a solution containing Na_2CO_3 (2%, w/v), NaOH (0.4%, w/v), $CuSO_4$ (0.1%, w/v) and sodium potassium tartrate (0.02%, w/v), and the mixture stirred for 10 minutes prior to the addition of 0.3 ml of Folin-Ciocalteu reagent (which had been diluted 1:2 with distilled water). The mixture was then stirred for a further 30 minutes before the reaction mixture was filtered through Whatman No 1 filter paper and the absorbance at 625 nm of the filtrate measured. A calibration graph was prepared using a standard protein solution of bovine serum albumin (Fraction V), and used to estimate the protein contents of the matrix-bound derivatives (Fig. 2.3).

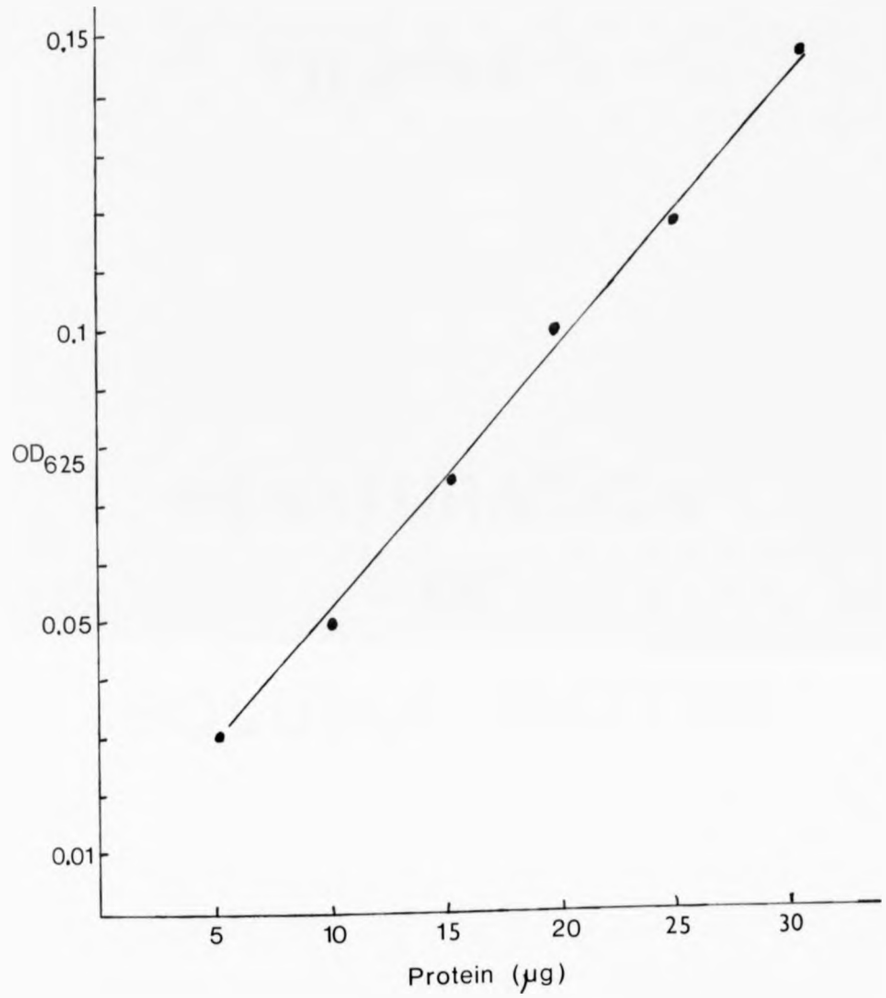


Fig 2.3 Plot of optical density at 625nm against protein. The modified Lowry procedure was used and the protein was bovine serum albumin (fraction V) (Chapter 2).

CHAPTER 3

RENATURATION OF SOLUBLE ENZYME

INTRODUCTION

Perhaps the most fundamental obstacle to studying the subunit form of an enzyme is that in most cases the native oligomer does not dissociate spontaneously. Artificial dissociation is usually accomplished by the addition of denaturants which normally result in substantial disruption of enzyme structure (Engelhard et al., 1976). It is therefore of prime importance that studies on single subunits involving denaturation-renaturation methods be preceded by a detailed investigation of the renaturation process. In particular the degree to which renaturation is possible and an examination of the renatured product with reference to the native enzyme would be the most appropriate points for consideration. Such an investigation is an important pre-requisite for the detailed interpretation of experiments involving matrix-bound subunit derivatives of enzymes (Chan, 1976).

There is well documented experimental evidence that oligomeric proteins, after denaturation, will spontaneously reassume an active conformation after removing the denaturant (Sund and Weber, 1966). These experiments provide convincing evidence that the information needed to specify the three-dimensional structure of a protein resides within its primary amino acid sequence. However, an investigation of the renaturation of a large number of enzymes revealed that the per cent regain of biological activity varied dramatically for different enzymes when they were renatured under identical conditions (Cook and Koshland,

1969; Neal et al., 1963). These observations clearly demonstrated that the regain of biological activity could (in many cases) be affected by the conditions under which the protein is renatured and that considerable improvements on the levels of renaturation could be made through simple alterations to the renaturation conditions.

Previous renaturation studies with rabbit muscle creatine kinase have demonstrated that the enzyme is readily dissociated in denaturing media e.g. GdnHCl (Yue et al., 1967), and that in common with many other proteins it can spontaneously renature from a denatured state upon removal of the denaturing agent (Dawson et al., 1965). The work of Dawson et al. (1965) showed that the level of creatine kinase renaturation as indicated by the regain of enzyme activity was rather low and subject to wide variations in similar renaturation protein concentrations. The apparent lack of any consistent pattern in the renaturation studies of Dawson et al. (1965) may be a result of the poorly defined renaturation conditions e.g. residual denaturant concentration too high, low quality denaturant (i.e. only partially purified) etc.

This present work was initiated with two main objectives: (1) to re-evaluate the work of Dawson et al. (1965) and make suitable improvements to the renaturation conditions that might increase the level of renaturation; (2) to prepare and fully characterise the product of renaturation. The studies included determining the influence of the residual concentration of denaturant in the renaturing medium, the effect of temperature and the effect

of protein concentration on the level of renaturation. A detailed analysis of the renaturation product was made, with reference to the native enzyme, in terms of active site structure, homogeneity and overall conformation as revealed by enzyme kinetic parameters, disc gel electrophoresis and circular dichroism studies respectively. A preliminary report of the results has been published (Bickerstaff and Price, 1977).

EXPERIMENTAL

Renaturation profiles

Creatine kinase was isolated from rabbit skeletal muscle as described in Chapter 2 and enzyme activity was assayed in the forward direction (phosphocreatine synthesis) by using the standard assay system described in Chapter 2. Denaturation of the enzyme was achieved by incubating the native enzyme (final concentration 5 mg/ml) with denaturing buffer consisting of 0.1 M-Tris/HCl at pH 7.5 containing 5 mM-DTT and 6 M-GdnHCl for 1 h at 20°C. Renaturation was accomplished by diluting (1:30) a sample of the denatured enzyme into renaturing buffer consisting of 0.1 M-Tris/HCl at pH 7.5 containing 5 mM-DTT. To determine the renaturation profile, renaturation was initiated then successive aliquots were removed at set times for assay of enzyme activity at that time. The renaturation profile was determined over several protein concentrations, at several temperatures and at several residual GdnHCl concentrations. In the last case the concentration of protein in the denaturation mixture was varied to ensure that the protein concentration in the renaturation mixture was similar to that in the other experiments (i.e. 0.167 mg/ml). Control experiments received the same treatment and dilutions except that the Gdn HCl was omitted.

Characterisation of the renaturation product

Kinetic parameters for substrates ATP and creatine were obtained by using the standard assay system (Chapter 2).

The studies were made by monitoring the effects on the initial velocity of the enzyme reaction as described in detail in Chapter 5. Circular dichroism (c.d.) studies in the 210 - 250 nm spectral range were obtained by using a Cary model 60 spectropolarimeter with model 6003 c.d. attachment. The cell compartment was maintained at 27°C and the instrument calibrated with D-10-camphorsulphonic acid. The slit width was programmed to yield constant energy over the wavelength range used and a scan speed of 5 nm/min was used. Using a 1.0 mm path length and a full scale deflection of 0.04 degrees, the spectra for both native and fully renatured enzyme were determined in the renaturation buffer at a protein concentration (for both) of 0.167 mg/ml. Molar ellipticity $[\theta]_\lambda$ values were calculated from the following relationship:

$$[\theta]_\lambda = \frac{\theta_\lambda . M}{10.d.C.}$$

where θ_λ is observed ellipticity in degrees at wavelength λ

M is the mean residue weight (112.5 based on a molecular weight of 82000 and 728 amino acid residues)

d is path length in cm

C is protein concentration in g/l.

The α-helix content for the native and fully renatured enzyme was calculated using the reference value, $[\theta]_{224.5}$ for molar ellipticity of a 100% α-helix at 224.5 nm of $-28,700$ deg.cm^2.d mole^{-1} (Chen et al., 1974).

A test was made on the homogeneity of the native and fully renatured enzyme using polyacrylamide disc gel electrophoresis. Electrophoresis was performed on 7.5%

polyacrylamide disc gels prepared in Tris/HCl at pH 8.9 with Tris/glycine buffer at pH 8.3 as the electrode buffer (Chapter 2). Before loading of the sample, the gels were pre-run at room temperature at a current of 2mA per tube for 30 - 60 minutes to remove excess persulphate. Gels were stained for protein and destained to reduce the background stain as described in Chapter 2. Gels were scanned at 545 nm using an SP 1800 spectrophotometer fitted with a conventional gel scanner. The molecular weight of the native and fully renatured enzyme was estimated using SDS polyacrylamide gel electrophoresis as described by Weber and Osborne (1969) and outlined in detail in Chapter 2.

A separate study was made on a routine preparation of rabbit skeletal muscle creatine kinase to establish the homogeneity, enzyme activity and molecular weight of the preparation. The procedures used in this study were identical to the corresponding methods outlined above.

RESULTS

Homogeneity of enzyme preparation

The results of this study to characterise the enzyme preparation from rabbit skeletal muscle are collected in Table 3.1. The molecular weight was estimated by SDS polyacrylamide gel electrophoresis and the homogeneity by polyacrylamide disc gel electrophoresis as described in Chapter 2. The kinetic parameters were determined by the procedure outlined in Chapter 5. The results are in excellent agreement with corresponding values published in the literature (Watts, 1973).

Renaturation profiles

The results obtained in the characterisation of the renaturation profile are collected in Fig. 3.1, and the regain of enzyme activity is taken as an indication of the extent of renaturation. In Fig. 3.1a it can be seen that the extent of renaturation is influenced by the residual concentration of GdnHCl and the graph demonstrates that optimum renaturation is possible when the concentration of GdnHCL is $\leq$ 0.1 M. In Fig. 3.1b the effect of temperature on the renaturation profile is shown to be less important at the higher temperatures of 20 - 35^{o}C. However the graph does show that at the lower temperatures of 0 - 5^{o}C, the renaturation process is considerably slower and the final level of renaturation after 24 h is lower than that obtained at 20^{o}C, although 100% regain of enzyme activity was obtained after 48 h at 4^{o}C. In Fig. 3.1c the effect of protein concentration on the renaturation profile is shown

Table 3.1 Characterisation of the creatine kinase isolated from rabbit skeletal muscle in this present work

Creatine Kinase Source	Molecular Weight	Specific Activity (μmol/min/mg)	pH Optimum	α-helix Content (%)
This Work	82000	135	9.0	31
Literature Value	82000	131	9.0	31

The literature values for molecular weight and α-helix content were obtained from Watts (1973). The literature value for pH optimum was obtained from Noda et. al. (1960) and the literature value for specific activity was obtained from Milner-White & Watts (1971). The molecular weight was determined by using polyacrylamide-SDS gel electrophoresis as described in Chapter 2, and the pH optimum was determined by the method described in Chapter 5. Other values were determined as described in Chapter 3.

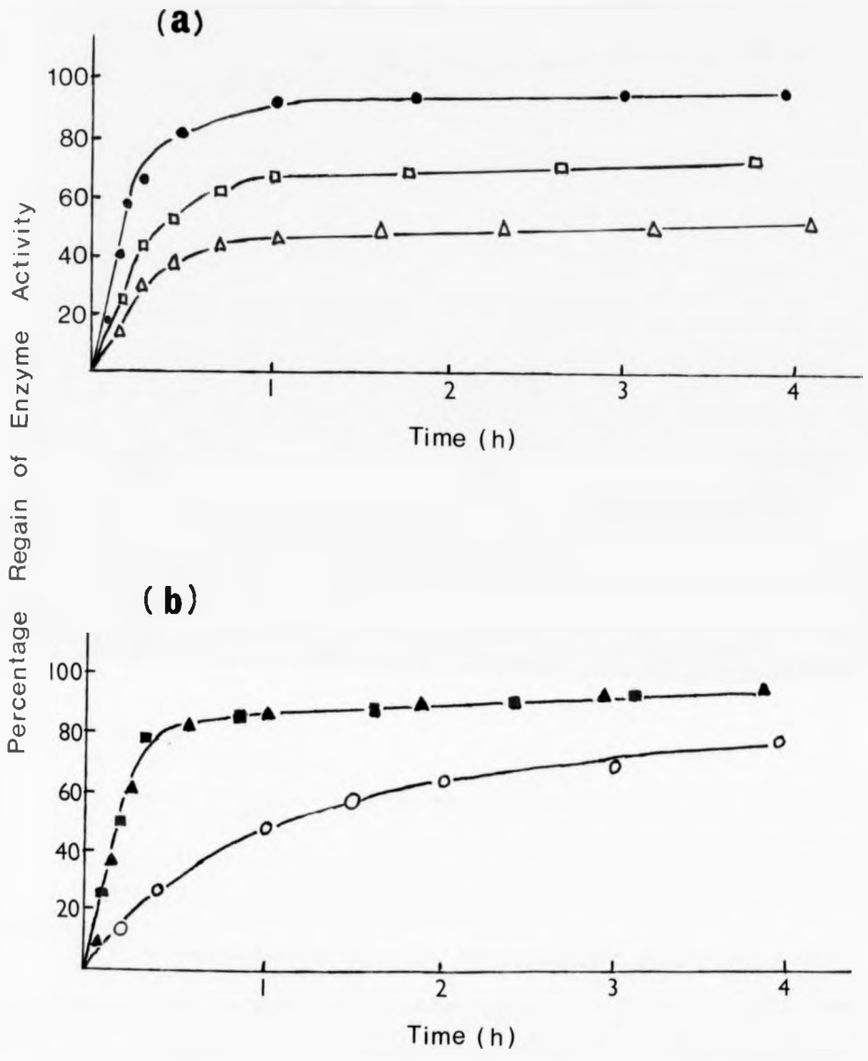


Fig 3.1 (a) Renaturation profile comparing the effect of residual [Gdn HCl] on the renaturation process. The residual concentrations of Gdn HCl were : ●, 0.1M; □, 0.15M; Δ, 0.3M. (b) Renaturation profile comparing the effect of temperature on the renatuation process. The temperatures were : ▲, 20°C; ■, 35°C; ○, 2°C.

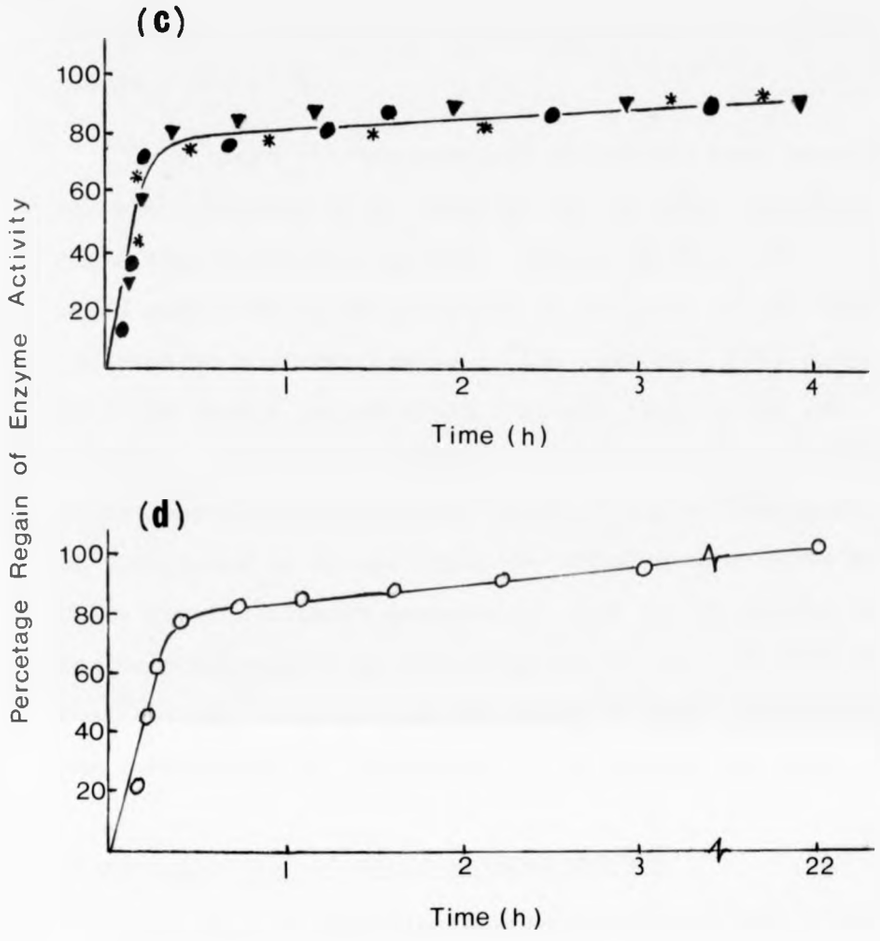


Fig 3.1 (c) Renaturation profile comparing the effect of protein concentration on the renaturation process. The protein concentrations were : *, 50µg/ml; ●, 100µg/ml; ▼, 200µg/ml. (d) Renaturation profile with the residual [Gdn HCl] 0.1M and protein concentration 0.167mg/ml. The temperature of renaturation was 25°C for the first 3h, followed by 19h at 4°C. In all cases, samples were removed at the times indicated and assayed for enzyme activity as described in Chapter 3.

to be minimal over the protein range considered which was 50 - 200 μg/ml.

From these results standard conditions were devised for the renaturation of creatine kinase from a denatured state (Experimental details). Under these conditions a rapid regain of enzyme activity to 95% over 3 h at 20°C, followed by a slower regain of the remaining (5%) enzyme activity over a period of 19 h at 4°C (Fig. 3.1d) was observed. The fully renatured enzyme was routinely obtained (Experimental details) after a 3 h period of renaturation at 20°C followed by an overnight period of renaturation at 4°C. These results clearly demonstrate that the denatured enzyme can be completely renatured readily in solution provided that (a) the residual concentration of GdnHCl is $\leq$ 0.1 M and (b) the temperature of renaturation lies within the range 20 - 35°C.

Characterisation of the renatured product

In order to determine whether or not the fully renatured enzyme was in any way altered from that of the native enzyme, several important properties were examined. The properties were carefully selected to reflect any changes in active site structure, molecular weight or overall protein conformation. The c.d. spectra for the native, fully renatured and denatured enzyme are presented in graphical form in Fig. 3.2. The overall shape of the spectra for both native and fully renatured enzyme was found to be similar over the whole range studied. The features of both spectra are consistent with an α-helical structure exhibiting an ellipticity minima at 222 nm which defines the n-π* peptide transitions. The

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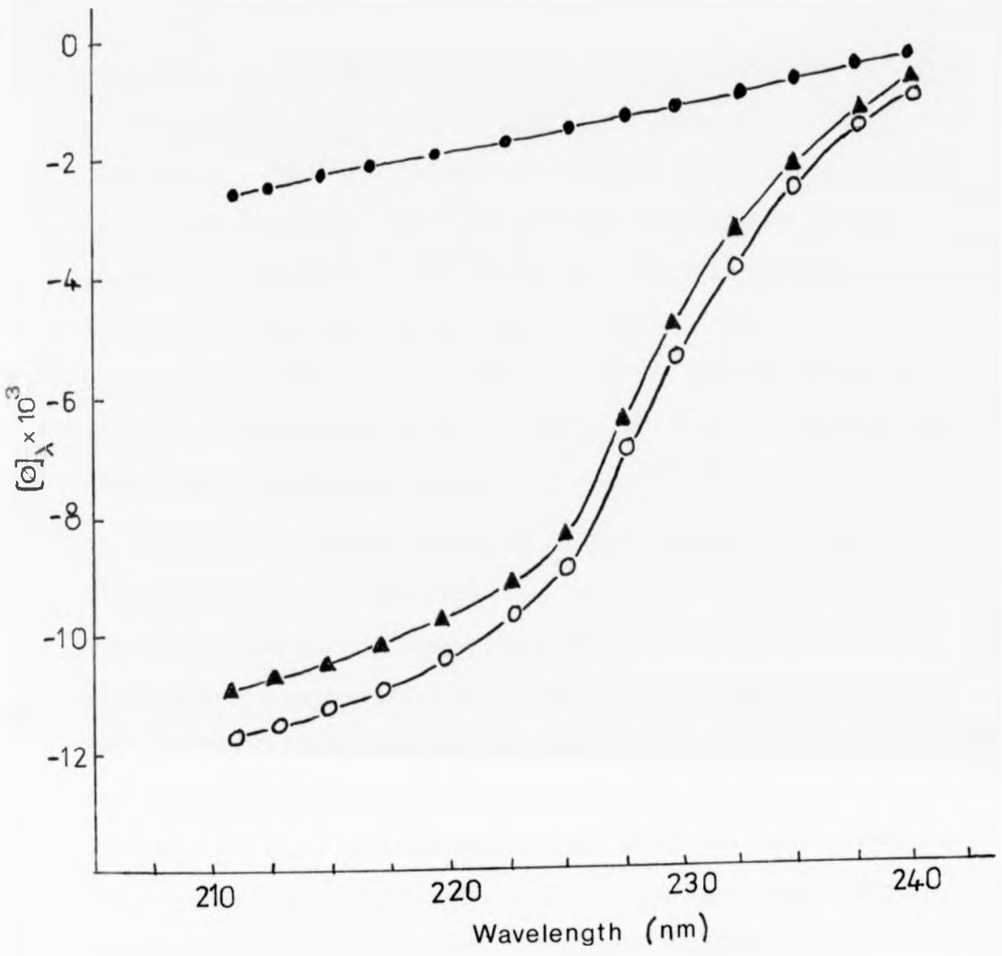


Fig 3.2 Plot of molar ellipticity against wavelength for the native enzyme (▲), fully renatured enzyme (0) and the denatured enzyme (●). Molar ellipticity values were calculated using the method described in Chapter 3. The values for the denatured enzyme were obtained in denaturing buffer at a protein concentration of 6.0 mg/ml. Apart from the path length which was 0.01mm, the values were determined in exactly the same manner as for the native and renatured species.

spectrum for the denatured enzyme on the other hand showed no such minima reflecting a marked absence of α-helical structure. This result is not unexpected of the denatured enzyme and confirms that the protein is unfolded in the presence of GdnHCl. The α-helical content for both native and fully renatured enzyme was calculated and the values are collected in Table 3.2. The values are almost identical and are in agreement with the value previously reported for rabbit muscle creatine kinase by Watts (1973).

The results obtained using polyacrylamide disc gel electrophoresis to estimate the homogeneity of the native and fully renatured enzyme samples are shown in Fig. 3.3. Both enzyme samples gave only one protein band in each case and the relative migration of both native and fully renatured enzyme was the same (relative to the dye bromophenol blue). From Fig. 3.3 it was estimated that both the native and the fully renatured enzyme were greater than 95% homogeneous. In particular no high molecular weight products were in evidence in the fully renatured enzyme. The results of the molecular weight determinations of the native and fully renatured enzyme, using SDS polyacrylamide gel electrophoresis, are collected in Table 3.2. The values are identical and are in agreement with the value reported for rabbit skeletal muscle creatine kinase by Yue et al. (1967). The results of the enzyme kinetic studies on the native and fully renatured enzyme are also collected in Table 3.2. The values are almost identical for the two enzyme samples and are in good agreement with the values reported previously by Milner-White and Watts (1971) using a pH-stat method for assaying the enzyme activity.

Table 3.2 Characterisation of creatine kinase in its native and fully renatured state

State	Km (mM)		Vmax	α-helix	Homogeneity
	ATP	Creatine	μmol.min.$^{-1}$mg^{-1}	content (%)	(%)
Native	0.40	10	150	31	95
Renatured	0.41	9.8	150	30	95

The kinetic constants were determined at 30°C and pH 9.0 as described in Chapter 3. The α-helix content was estimated from the c.d. spectra presented in Fig 3.2 and the homogeneity was estimated from the densitronic traces presented in Fig 3.3.

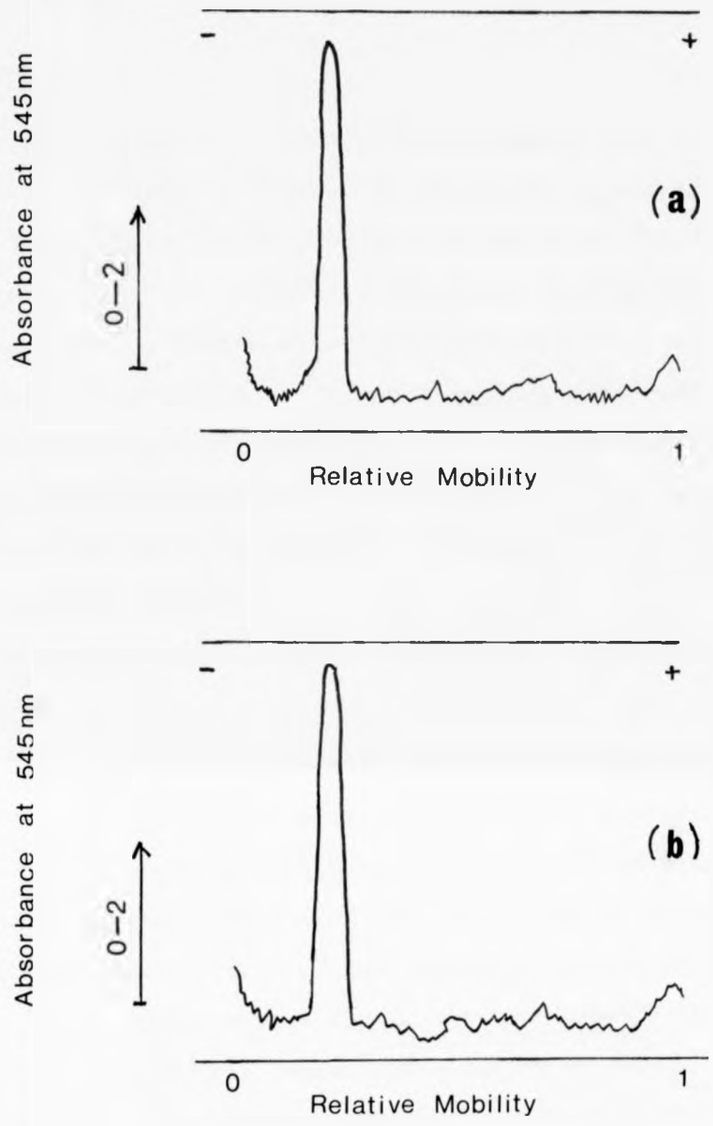


Fig 3.3 Scans at 545nm of Coomassie Blue-stained disc acrylamide gels run with samples (1.5μg) of native creatine kinase (a) and fully renatured enzyme (b). Electrophoresis staining and scanning were performed as described in Chapter 2.

DISCUSSION

The results reported here clearly suggest that the denaturation of creatine kinase by GdnHCl is completely reversible and that the product of renaturation, the fully renatured enzyme, is essentially identical with the native enzyme in terms of active site structure, molecular weight and overall conformation. It can therefore be argued with confidence that studies with matrix-bound derivatives involving denaturation-renaturation of matrix-bound subunits and re-association of matrix-bound subunits with added soluble subunits should be free from complications arising from incorrect refolding and re-association of the enzyme in solution.

In addition the results have revealed a number of important facets of the renaturation process of creatine kinase. It is evident that certain variables can have a decided influence on the in vitro renaturation of the enzyme and in particular the residual concentration of the denaturant is an important factor. In connection with the denaturant (GdnHCl) it was found that the available commercial supplies of GdnHCl were of inconsistent, often poor, quality and likely to lead to spurious results. This observation supports the conclusions regarding (GdnHCl) reagent quality and purity of Nazaki (1972) and it was therefore necessary to undertake a special purification (Chapter 2) of the reagent to ensure a reliable quality.

The regain of enzyme activity was found to be independent of protein concentration over the range

50 - 200 µg/ml. This would tend to suggest that the renaturation proceeds by an apparently first order process and since the renaturation profile is characterised by a rapid regain of enzyme activity, this would be consistent with single subunits of creatine kinase being enzymatically active. If the individual subunits were inactive and enzyme activity was dependent upon a rate limiting dimerisation step then second order kinetics would be expected for the renaturation process, and the rate of regain of enzyme activity would be expected to increase with increasing protein concentration.

Teipel and Koshland (1971) observed from several studies on various enzymes that the gross structural changes accompanying renaturation as measured by optical rotation were complete within one minute of initiation of renaturation. If this also holds for creatine kinase then the mechanism below might be applicable.

Upon renaturation there is an extremely fast refolding of the unfolded subunits to produce a refolded inactive subunit which then undergoes a first order process (involving a relatively small conformational change) to produce an active subunit. If an inactive subunit is formed initially

then it can have only a relatively short half life of 5 minutes (under these conditions) since 80% of the enzyme activity is regained in the first 10 minutes of renaturation (80% completion of a first order process corresponds to just over two half lives). It is interesting to speculate that the slow regain of the remaining enzyme activity (5%) observed in the renaturation profile might correspond to a dimerisation step in the above mechanism.

CHAPTER 4

GENERAL PROPERTIES

INTRODUCTION

The work presented in this Chapter is concerned with the evaluation and reproducibility of some of the routine methods involved in working with the matrix-bound derivatives, described in this Thesis, together with an examination of some characteristic properties of the enzyme and its matrix-bound derivatives.

If attempts to derive a matrix-bound subunit derivative are to be successful then it is essential that the oligomeric protein be attached to the gel particles via one subunit only (Chan, 1970). The distribution of potential reactive groups on the gel is governed by the amount of CNBr reagent used in the activation procedure (March et al., 1973) and therefore an appropriate amount of CNBr must be used which will generate spatially isolated reactive groups on the gel to ensure single subunit binding and in sufficient number to bind a reasonable amount of protein. Previous workers (Chan, 1970; Feldman et al., 1972; Bruch et al., 1976) have found that a level of 1 - 3 mg CNBr per ml of packed gel was suitable for purpose of binding proteins via one subunit only. However Chan et al. (1973a) discovered that this low level of activation was not suitable in the case of transaldolase as too little protein was bound for reliable work. In the case of transaldolase (Chan et al., 1973a) a compromise level of gel activation was used in which sufficient protein was bound to the gel, but 30% of all bound molecules were attached via both subunits. The influence of the CNBr activation level on the preparation of matrix-bound subunits of creatine kinase was studied in order to determine the optimum level of activation for the production of matrix-bound subunits.

The exact determination of the amount of immobilised protein is essential if one wishes to monitor changes in quaternary structure of matrix-bound proteins or estimate specific activities. Several methods are available for immobilised protein evaluation (Koelsch et al., 1975) namely: protein balance (estimation of protein recovered in final washings), amino acid analysis, modified Lowry method, spectrophotometric analysis and fluorophotometric analysis. Of these methods the modified Lowry method was selected for use in this present work because of its simplicity and reported accuracy (Koelsch et al., 1975). The remarkable reproducibility and accuracy of the method in comparison with the other methods mentioned above was initially attributed to a quantitative detachment of the protein from the matrix by the treatment of the gel sample with the alkaline Lowry reagents. However control experiments by Koelsch et al. (1975) revealed that the alkaline medium detached no more than 80% of the bound protein from the gel beads even after prolonged exposure to the alkaline reagent. Quantitative estimation of bound protein necessitates treatment of the protein-containing gels with the Folin-Ciocalteu reagent which presumably suggests that the reduced phosphomolybdate complex diffuses freely out of the matrix. A study was made to confirm the accuracy and reproducibility of the modified Lowry method in the present studies with matrix-bound creatine kinase.

To determine the activity of matrix-bound derivatives certain modifications to the assay techniques are usually required because of the particulate nature of the matrix-bound enzyme. In contrast to catalysis in homogeneous solution,

some means of maintaining a steady diffusion of substrates and products must be used (Mort et al., 1973). This has been achieved either by continuous stirring of the mixture (Hornby et al., 1966) or by continuous passage of the substrate solution through a stationary column of the matrix-bound enzyme as described by Lilly et al. (1966). However the latter method is complicated by surface effects, uniformity of flow and diffusion limitations (Chan, 1976) that can profoundly influence the activity measurements and therefore a continuous stirring procedure was used in this present work.

The spectrophotometric assay procedure of creatine kinase activity used by McLaughlin et al. (1972) was adapted to produce a direct spectrophotometric monitoring of the reaction mixture with continuous stirring, suitable for the measurement of immobilised enzyme activity. The arrangement is shown in Fig. 4.1 and consists of a thermostatted cuvette-holder mounted on top of a stirring motor. The reaction mixture is stirred continuously by placing a small magnet (0.4 cm in length) in the cuvette. At suitable intervals the cuvette is transferred to the cell compartment of a recording spectrophotometer and a trace of the absorbance is recorded for a few seconds (Mort et al., 1973). The main limitation of this method is the light scattering effect of the gel. At 340 nm a 1:10 (v/v) suspension of stirred Sepharose has an absorbance of 0.6. The concentration of gel used in an assay system depends largely on the activity of the immobilised enzyme. A study was made to evaluate the reproducibility of the assay method with the matrix-bound enzyme.

The influence of anions on creatine kinase activity has

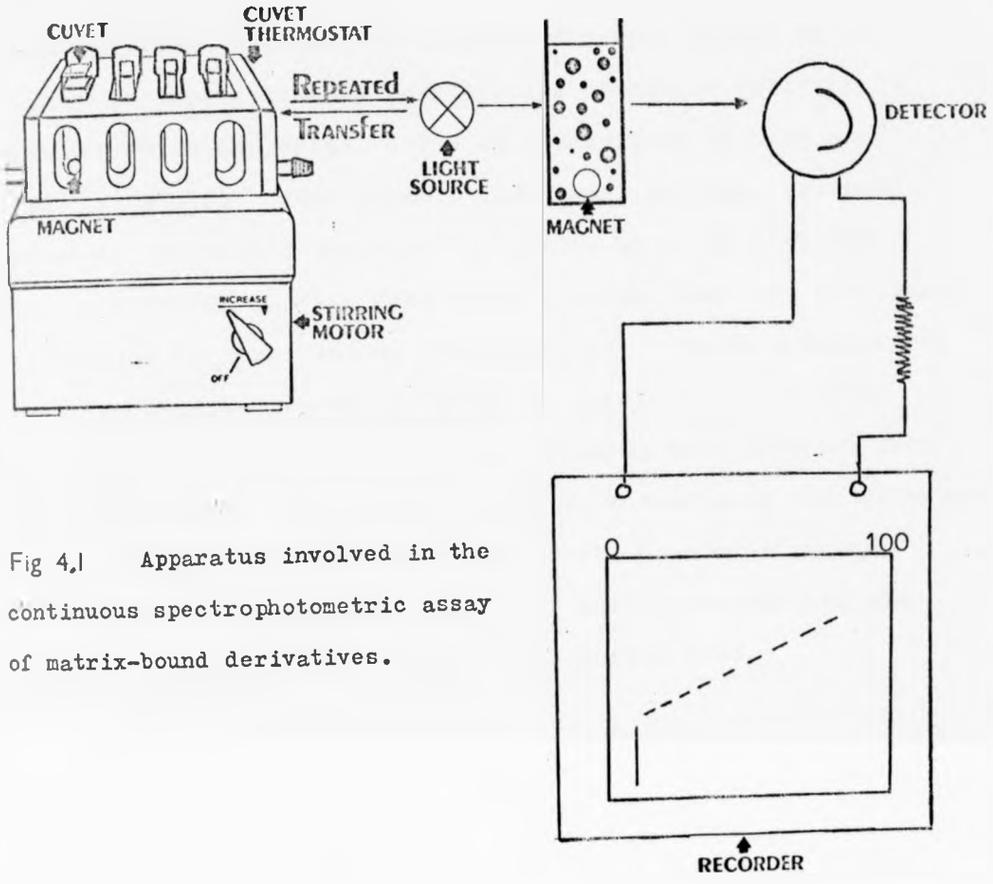


Fig 4.1 Apparatus involved in the continuous spectrophotometric assay of matrix-bound derivatives.

been outlined in Chapter 1 and the principal effect of anions is the reversible inactivation of enzyme activity (Milner-White and Watts, 1971) as a result of binding at the active site. One notable exception, acetate, has been shown to reversibly activate the enzyme by as much as 20%, and in addition acetate activation does not preclude subsequent inhibition by other anions (Watts, 1973). These observations have led Anosike and Watts (1976) to consider that acetate may exert its effect on an acetate binding site distinct from the active site. A study was made to investigate the influence of acetate on the activity of the matrix-bound derivatives to discover if the activation of the soluble enzyme was also observed in the various matrix-bound derivatives.

EXPERIMENTAL

Immobilisation of the enzyme

Creatine kinase was routinely coupled to CNBr-activated Sepharose 4B using the procedure described in Chapter 2. The preparation of matrix-bound subunits and the determination of protein contents were carried out as described in Chapter 2. The immobilisation procedure was studied under increasing levels of CNBr activation to establish the importance of the level of activation on the subsequent production of matrix-bound subunits. The routine method of preparing matrix-bound enzyme was employed except that various amounts of CNBr (2 - 500 mg) were used to activate the gel. From the different (in terms of protein content) matrix-bound dimers obtained at the increasing levels of activation, attempts were made, using the denaturation-renaturation procedure (Chapter 2), to produce the corresponding matrix-bound subunit derivative.

Protein determinations

Protein content estimations of the various matrix-bound derivatives was accomplished using a slightly modified version of the lowry method (Lowry et al., 1951) which is described in detail in Chapter 2. The linearity and reproducibility of the modified procedure was investigated using a sample of routinely prepared matrix-bound enzyme (400 µg protein/ml of gel). A standard suspension (1:1, v/v) of gel in phosphate buffer was prepared and aliquots were removed from the stirred suspension for protein determination. Protein contents were calculated by referring the observed absorbance at 625 nm to a standard protein calibration graph (Fig. 2.3).

All values for protein content of the matrix-bound derivatives are expressed as µg/ml of packed gel (Chapter 2).

Enzyme Activity

Full details of the assay procedure and the standard assay components for the determination of enzyme activity of the soluble enzyme and matrix-bound derivatives is given in Chapter 2. Enzyme activity values were determined for a set of matrix-bound derivatives prepared as described in Chapter 2. The protein contents of the routinely prepared derivatives were:

Matrix-bound enzyme (derivative A)	400 µg/ml
Matrix-bound subunit (derivative B)	200 µg/ml
Matrix-bound re-associated enzyme (derivative C)	390 µg/ml

The reproducibility and linearity (with respect to enzyme concentration) of the assay method was investigated using a sample of matrix-bound enzyme (400 µg/ml of packed gel). Three suspensions of gel in phosphate buffer (1:75, 1:50, 1:25, v/v) were prepared and various aliquots were removed from the stirred suspensions and assayed for enzyme activity using the standard assay procedure (Chapter 2).

Effect of acetate

The effect of acetate on the enzyme activity of the matrix-bound derivatives was studied using a set of derivatives prepared by the routine method described in Chapter 2. The protein contents of the derivatives were:

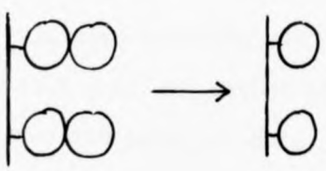
Matrix-bound enzyme	400 µg/ml
Matrix-bound subunit	200 µg/ml
Matrix-bound re-associated enzyme	390 µg/ml

The matrix-bound derivatives were prepared as suspensions (1:1, v/v) of gel in phosphate buffer. Enzyme activity was measured using the standard assay system described in Chapter 2 except that when considering the effect of acetate the assay system was altered by omitting the sodium acetate and replacing the magnesium acetate with magnesium sulphate (6 mM). A control experiment was devised to determine whether or not the coupling enzymes involved in the assay system (pyruvate kinase and lactate dehydrogenase) were affected by the acetate. In this experiment the standard assay system was used but in this case the reaction was not started by the addition of creatine kinase but by the addition of ADP (150 nmoles). The experiment was repeated but in the absence of acetate.

RESULTS AND DISCUSSION

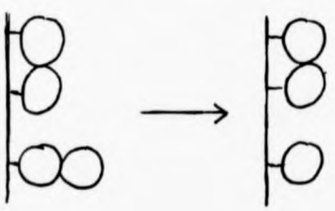
Immobilisation

The influence of the degree of gel activation on the subsequent preparation of matrix-bound subunits is shown in Table 4.1. It is clear, from these results, that although substantially more protein is coupled to the gel at the higher levels of activation, the bound protein is less suitable for the preparation of matrix-bound subunits. The percentage of attached dimer molecules bound via both subunits was calculated by subtracting the percentage protein content expected if no molecules were attached via both subunits (i.e. 50% in this case) from the observed percentage protein content for the "subunit" derivative. Multiplication of this percentage by two gives an indication of the percentage of dimeric molecules attached via both subunits. For example in the case of 100 mg CNBr/ml of gel 78% of the protein remained in the "subunit" derivative which means that 56% of the bound molecules were attached via both subunits.



50% remaining

(50-50) = 0%
0 x 2 = 0%
0% bound via both subunits



78% remaining

(78-50) = 28%
28 x 2 = 56%
56% bound via both subunits

RESULTS AND DISCUSSION

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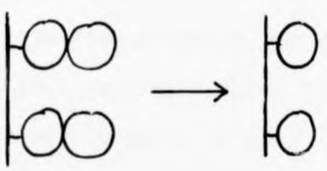
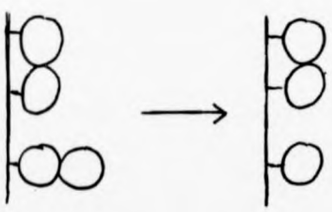


50%
remaining

(50-50) = 0%
0 x 2 = 0%
0% bound via both subunits



78%
remaining

(78-50) = 28%
28 x 2 = 56%
56% bound via both subunits

Table 4.1 The influence of the level of CNBr-activation on the production of matrix-bound subunits

CNBr-Activation mg/ml gel	Derivative A µg/ml	%	Derivative B µg/ml	%	Protein Attached via both Subunits (%)
2	290	100	145	50	0
5	400	100	200	50	0
50	600	100	350	58	16
100	720	100	560	78	56
250	925	100	860	92	84
500	980	100	950	97	94

The routine method of preparing the matrix-bound enzyme (derivative A) was employed except that varying amounts of CNBr were used to activate the gel. The matrix-bound subunit (derivative B) was prepared using the denaturation-renaturation procedure described in Chapter 2. The percentage protein attached via both subunits was calculated as described in Chapter 4. In each case the amount of creatine kinase added to the activated gel was 1.0 mg/ml of gel.

The technique of preparing matrix-bound subunits is fundamentally based on the ability to attach the protein molecule to the matrix via one subunit only in order that the non-covalently bound subunits can be removed by washing the gel with denaturing buffer. It can be seen from Table 4.1 that activation levels of 5 mg CNBr/ml of gel or less are suitable for coupling creatine kinase to Sepharose 4B via one subunit only. The level of activation used for all subsequent preparations of matrix-bound enzyme was 5 mg CNBr/ml of gel and this level of activation invariably provided matrix-bound enzyme with a protein content of 400 $\pm$ 20 µg/ml of packed gel.

Protein estimation

Typical results for protein contents of the matrix-bound derivatives are collected in Table 4.2. The matrix-bound subunit (derivative B) characteristically shows half the protein content of the matrix-bound enzyme (derivative A). A control experiment in which matrix-bound enzyme was extensively washed with denaturing buffer not containing GdnHCl showed that no protein was removed. The matrix-bound re-associated enzyme (derivative C) shows a high regain of protein after addition of denatured soluble subunits. Control experiments showed that neither derivative A nor washed activated/glycine-blocked Sepharose 4B (prepared by coupling glycine to CNBr-activated Sepharose 4B) could retain subunits under the same conditions of re-association.

The results obtained in the study of the reproducibility of the modified Lowry procedure are shown in Fig. 4.2. The results are presented as a graph of sample volume against

Table 4.2 Protein content and enzyme activity values for the matrix-bound derivatives

Matrix-Bound Derivative	Protein Content		Specific Activity (μmol/min/mg)	
	μg/ml	%	Acetate Absent	Acetate Present
Derivative A	400	100	50	98
Derivative B	200	50	48	50
Derivative C	380	95	50	80

The preparation of the matrix-bound derivatives is illustrated in Scheme 1.1 and is described in Chapter 2. Enzyme assays were performed in the absence and in the presence of acetate (0.1M) as described in Chapter 2. Soluble creatine kinase had a specific activity of 115 units/mg in the absence of acetate and 135 units/mg in the presence of acetate. Protein contents are expressed as μg/ml of packed gel.

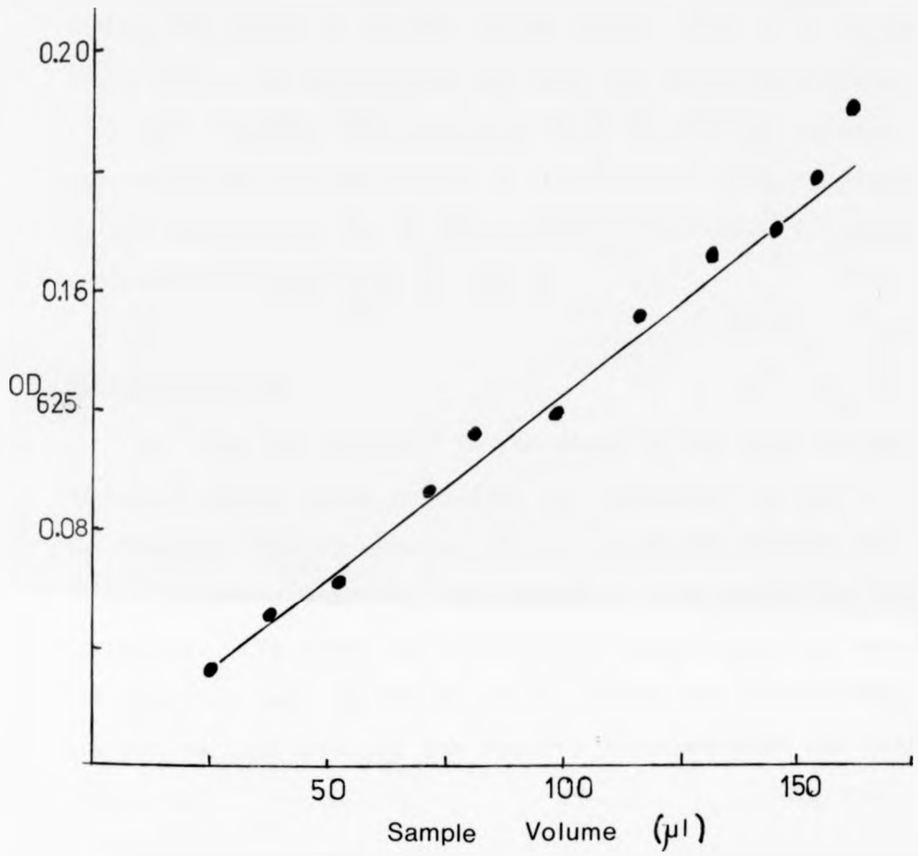


Fig 4.2 Plot of optical density at 625nm against sample volume of gel suspension (1:1, v/v) in phosphate buffer. A sample of matrix-bound enzyme (derivative A) (400 µg/ml of protein) was used in the experiments and protein contents were determined using the method described in Chapter 2.

observed absorbance at 625 nm. The graph clearly demonstrates that the observed absorbance is linear with the sample volume within the range of sample volume of 25 - 150 µl of suspension. Above 150 µl of suspension the observed absorbance deviates from the straight line obtained with the smaller volumes. Samples of gel suspension (1:1, v/v) were routinely restricted to the range 50 - 150 µl of suspension to ensure a linear and reproducible absorbance at 625 nm.

Enzyme activity

The results obtained in the study of the reproducibility of the standard assay procedure are presented in Table 4.3. The results clearly demonstrate good reproducibility and in the repeated samples, experiments 9 - 11, there is very good agreement. In addition this is also good linearity between the observed rate of change in absorbance and the enzyme concentration as indicated by the results obtained with the different dilutions.

Typical specific activity values for the matrix-bound derivatives are collected in Table 4.2. The specific activity values (in the absence of acetate) reported in Table 4.2 for the soluble enzyme and its matrix-bound derivatives are of the order 1.7 - 1.9 times greater than those reported by Bickerstaff and Price (1976a). This increase is solely due to a general improvement of the assay method involving greater amounts of coupling enzymes, higher assay temperature, higher concentrations of both substrates (ATP and creatine). As indicated in Table 4.2 creatine kinase is linked to Sepharose 4B with retention of approximately 50% of the specific activity

Table 4.3 Characterization of the standard assay procedure with respect to reproducibility and linearity with varying enzyme concentration

Experiment No.	Dilution Factor	Sample µl	Observed OD_{340} /min	Similarity	Average Value
1	1:25	10	0.0142	X	0.0138
2	1:50	20	0.0135	X	
3	1:25	30	0.0352	Y	0.0352
4	1:25	30	0.0348	Y	
5	1:50	60	0.0358	Y	
6	1:25	20	0.0220	Z	0.0224
7	1:50	40	0.0221	Z	
8	1:50	40	0.0229	Z	
9	1:75	60	0.0222	Z	
10	1:75	60	0.0225	Z	
11	1:75	60	0.0230	Z	

The procedure involved and the assay components used in the determination of enzyme activity are described in Chapter 2. A sample of matrix-bound enzyme (derivative A) (400 µg/ml) was used in the experiments.

of the native soluble enzyme (measured in the absence of acetate). This figure is comparable with the results of other studies on matrix-bound enzymes (Chan, 1970; Chan et al., 1973a; Grazi et al., 1973) and may possibly arise from a change in the microenvironment of the catalytic site upon immobilisation. The results show that not only are the matrix-bound subunits of creatine kinase active but these subunits possess a specific activity very similar to that of the matrix-bound enzyme (in the absence of acetate).

Effect of acetate on enzyme activity

The results obtained in the study of the acetate activation of the enzyme are collected in Table 4.2. The results show that the specific activity of the matrix-bound subunit (derivative B) is not increased significantly (by less than 5%) by the inclusion of acetate in the assay system, whereas the activities of both the matrix-bound enzyme (derivative A) and matrix-bound re-associated enzyme (derivative C) are increased considerably (by 95% and 70% respectively). These results suggest that acetate may exert its activating effect via a subunit interaction and not by a direct influence on the catalytic site of each subunit. If acetate exerts its effect via a conformational change in the enzyme (Watts, 1973), it would appear that this change is not generated to any great extent in the matrix-bound subunit derivative (Bickerstaff and Price, 1976b).

CHAPTER 5

CONFORMATION OF THE ACTIVE SITE

INTRODUCTION

The term immobilised enzyme suggests that the enzyme is restricted to some extent with regard to freedom of movement or orientation and depending on the nature of the enzyme this restriction may or may not have an influence on the catalytic properties of the enzyme. In order to establish whether or not the active site of the matrix-bound enzyme derivatives had altered properties from those of the native soluble enzyme, several aspects of active site topography were examined in detail. These studies included the kinetics of the enzyme catalysed reaction, pH optima, ADP binding and the inhibition kinetics of the reaction of the reactive thiol group with iodoacetamide. Although the reactive thiol group is not thought to be situated directly at the active site its importance in the topography of the active site (Keighren and Price, 1978) warrant its inclusion in this study.

The effects of immobilisation on the activity of enzymes vary depending on the method of immobilisation. In general it is found that agarose-bound enzyme derivatives exhibit somewhat decreased Vmax values and increased Km values compared to soluble enzyme values (Mosbach, 1976). In some cases substantial alteration of catalytic parameters have been reported (Katchalski et al., 1971) and it is significant that in such cases the enzyme activity was usually determined using a column or other flow system where surface effects, uniformity of flow and diffusion limitations can profoundly influence the observed activity (Chan, 1976). An alternative method of assay, involving continuous stirring

of the assay mixture containing an aliquot of gel suspension (Mort et al., 1973), used in this present work has proved reliable (Chapter 4) and in the cases where this assay method has been used (Chan, 1970; Chan et al., 1973a; Bruch et al., 1976) it has generally been found that Km values for matrix-bound derivatives were of the order 1.5 - 3.0 times greater than the corresponding values for the soluble enzyme. In view of the simplicity of the assay procedure it is likely that it does not contribute substantially to the altered parameters which probably arise from small changes in the microenvironment of the active site produced by immobilisation.

A particularly valuable method of checking the integrity of the active site of creatine kinase exists in the binding properties of the substrate ADP (James, 1976), and this property should be sensitive enough to reflect any adverse influence of the matrix. In a study by Price and Hunter (1976), it was demonstrated that the binding of ADP to the native soluble enzyme was hyperbolic in the absence of any ligands with a dissociation constant of 55 μM. However in the presence of Mg^{2+} + creatine + nitrate the binding of ADP was tightened considerably and the binding plots suggested the possibility of either negative interactions or two distinct binding sites (observed dissociation constants K_1 = 1.5 μM, K_2 = 18 μM).

In view of these interesting findings it would have been particularly valuable to repeat these experiments with the matrix-bound derivatives to ascertain the response of the matrix-bound subunit derivative. However application of the studies of Price and Hunter (1976) to the matrix-bound derivatives proved to be difficult due to the imposed limitation

on the protein contents of the matrix-bound derivatives (Chapter 4). It was found necessary (see Fig. 4.1) to limit the protein content of the matrix-bound enzyme to 400 µg/ml of gel to ensure that the subsequent matrix-bound subunit derivative would consist of 100% matrix-bound subunits. In binding studies it is essential, for accurate and reliable work, for the enzyme concentration to be of the same order as the dissociation content. The maximum protein concentration obtainable with the matrix-bound enzyme (derivative A) in binding experiments was 2.0 - 2.5 µM. However, while it was not possible to study ADP binding in the absence of added ligands, it was considered possible to study ADP binding to the matrix-bound enzyme (derivative A) in the presence of Mg^{2+} + creatine + nitrate. Studies with the matrix-bound subunit (derivative B) proved unreliable because of the lower protein concentration in this derivative.

The function of the single reactive thiol group per subunit, which is thought to be situated in close proximity to the active site, has commended much recent attention (der Terrossian and Kassab, 1976; Smith and Kenyon, 1974). A full discussion on the properties of the reactive thiol group is given in Chapter 7 and it now seems likely that the integrity of the thiol group on each subunit may be viewed as important in the conformational transitions which the enzyme undergoes on the formation of the catalytically active complex (Keighren and Price, 1978). In this context it was of interest to consider the reactivity of the thiol group in the matrix-bound derivatives and thereby discern any effect

imposed by the matrix. In addition ligand-induced conformational changes (monitored by the reactivity of the thiol group towards iodoacetamide) were also investigated to establish whether or not the conformational changes manifest in the formation of the "transition state analogue" complex (Watts, 1973) were dependent upon subunit interactions.

EXPERIMENTAL

Enzyme kinetics

Kinetic parameters for the substrates ATP and creatine were obtained using the standard assay system described (in detail) in Chapter 2. The studies were made by monitoring the effects on initial velocity of the enzyme reaction of varying in turn the concentration of one substrate in the presence of several fixed concentrations of the other substrate. The concentration of ATP was varied from 0.5 to 4 mM and the concentration of creatine was varied from 5 to 40 mM. When the ATP concentration was varied, the concentration of magnesium acetate was also varied to maintain the free concentration of Mg^{2+} at 1.0 mM (Storer and Cornish-Bowden, 1976).

The matrix-bound derivatives of creatine kinase were prepared as described in Chapter 2 and the protein contents of the derivatives were:

Matrix-bound enzyme (derivative A) 400 µg/ml of gel

Matrix-bound subunit (derivative B) 200 µg/ml of gel

Matrix-bound re-associated enzyme (derivative C) 390 µg/ml of gel

All the gels were prepared as suspensions (1:1, v/v) in phosphate buffer and were diluted 1:20 into phosphate buffer to give diluted suspensions from which convenient aliquots (0.05 — 0.1 ml) could be removed for direct assay of enzyme activity. In all cases the amount of enzyme added to the assay mixture was in the range 0.2 - 0.8 µg. Control experiments showed that the standard assay system gave proportional rates of changes in

absorbance with added protein up to 1.5 μg of protein. The soluble enzyme was dialysed against phosphate buffer and then further diluted into phosphate buffer as required.

Inhibition by iodoacetamide

Inhibition of the enzyme and its matrix-bound derivatives by iodoacetamide (IAM) was studied at 30°C in 0.1 M-glycine/NaOH at pH 9.0. Enzyme was allowed to equilibrate with any added substrates or ligands for 20 minutes in a stirred solution of total volume 2 ml. A control sample (0.1 ml) was transferred to a stirred solution of phosphate buffer containing 1.0 mM-dithiothreitol (DTT), and the reaction was then started by addition of iodoacetamide to the remainder of the enzyme. The progress of the inhibition reaction was monitored by removing samples at set times and diluting them into stirred solutions of phosphate buffer containing 1.0 mM-DTT. Samples were then assayed for residual enzyme activity by the standard assay procedure described in Chapter 2. The enzyme activity of these diluted samples did not change over a period of at least 3h at 20°C.

Matrix-bound derivatives were prepared as described in Chapter 2 and the protein contents of the derivatives were the same as those routinely obtained by the method. The gels were prepared as suspensions (1:1, v/v) of gel in phosphate buffer and for the purposes of these experiments the gels were washed with, and eventually re-suspended in 0.1 M-glycine/NaOH at pH 9.0. The soluble enzyme was dialysed against 0.1M-glycine/NaOH at pH 9.0. In all cases the enzyme concentration in the inactivation mixture was 1.5 μM (subunits) and the concentration

of IAM was 0.476 mM. Substrate-ligand concentrations used were: ADP 1.0 mM; magnesium acetate 10 mM; creatine 40 mM; $NaNO_3$ 0.1 M.

pH optimum

Enzyme activity was monitored over the pH range 7.5 - 10.5 under standard assay conditions, except that the following buffers were used: 0.1 M-Tris/acetate at pH 7.5 and 8.5; 0.1 M-glycine/NaOH at pH 9.0, 9.5 and 10.5. The pH of the assay mixture was checked before and after each assay and in no case did the pH alter by more than 0.1 pH unit. In the assay at pH 9.5 and 10.5 larger amounts of coupling enzymes (90 units of each enzyme) were required to ensure that the coupling reactions were not rate-limiting at these values.

ADP binding

Binding of ADP to the matrix-bound enzyme (derivative A) was studied at 20°C in a total volume of 2.5 ml in 0.1 M-Tricine/NaOH at pH 8.0. The matrix-bound dimer was washed and re-suspended (1:1, v/v) in 0.125 M-Tricine/NaOH at pH 8.0 containing 75 mM-creatine. A sample (2 ml) of this suspension was transferred to a hard glass centrifuge tube and then the substrates and ligands were added to the mixture. Thus 1 ml of gel was finally re-suspended in 2.5 ml total volume giving a final protein concentration of 2.0 μM (dimers). The mixture was allowed to equilibrate for 20 minutes (with gentle stirring) before addition of the radioactive ADP which had been prepared as described in Chapter 2. The stock solution of radioactive

ADP had a concentration of 0.5 mM and aliquots of the radioactive ADP were added to the enzyme mixture to give a final concentration of 1 - 40 μM. The suspension was stirred gently for a further 20 minutes at 20°C before samples (0.1 ml) of the suspension were removed for determination of the total radioactive ADP. The suspension was then centrifuged on an MSE Minor centrifuge at 2000 g for 5 minutes before a sample (0.1 ml) of the clear supernatant was removed for determination of soluble (unbound) radioactive ADP. The radioactivity of samples was measured as described in Chapter 2.

RESULTS AND DISCUSSION

pH optima

The effects of pH on the enzyme activities of the soluble enzyme and the various matrix-bound derivatives are shown in Table 5.1. In all cases there was a broad peak in the pH-activity profile with maximum activity being observed at pH 9.0. The results obtained for both soluble and matrix-bound derivatives are comparable with those reported by Noda et al. (1960).

Enzyme kinetics

A double reciprocal plot of the initial velocities observed for the matrix-bound enzyme at various concentrations of MgATP in the presence of fixed concentrations of creatine is shown in Fig. 5.1. Corresponding plots were obtained for the soluble and the other matrix-bound derivatives, and all showed the same intersecting pattern, characteristic of synergism in the binding of the two substrates to the enzyme (Watts, 1973). Kinetic parameters were estimated from secondary replots of the intercepts and slopes of the primary double reciprocal plots as described by Florini and Vestling (1957), and shown in Fig. 5.2. The kinetic parameters for the soluble enzyme and the matrix-bound derivatives are collected in Table 5.2. The results show that in the presence of 0.1 M-acetate the values of Vmax for the matrix-bound enzyme and the matrix-bound re-associated enzyme are very similar to that of the soluble enzyme. The matrix-bound subunit form has a lower Vmax (75% of the value of the soluble enzyme). There are some small

Table 5.1 Effect of pH on the activity of soluble and matrix-bound derivatives of creatine kinase

Buffer pH	Relative Enzyme Activity (%) Soluble Enzyme	Matrix-Bound Derivative A	B	C
7.5	78	74	72	72
8.5	98	96	94	92
9.0	100	100	100	100
9.5	88	92	80	88
10.5	67	52	60	68

The activity of the soluble and matrix-bound derivatives was determined in the following buffers : 0.1M-Tris/acetate at pH 7.5 and at pH 8.5; 0.1M-glycine/NaOH at pH 9.0, 9.5 and 10.5. The results in each case are expressed as a percentage of the maximum activity which in all cases was observed at pH 9.0.

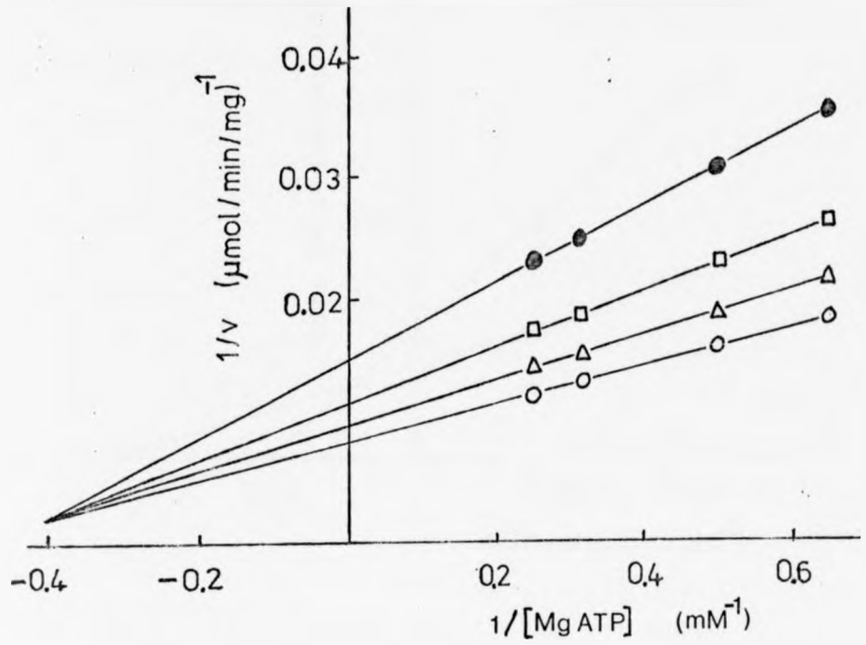


Fig 5.1 Double reciprocal plot data for the reaction catalysed by the matrix-bound enzyme (derivative A). The effect of varying the concentration of MgATP in the presence of several fixed concentrations of creatine was studied, and the concentrations of creatine were: O, 40mM; Δ, 20mM; □, 10mM; ●, 5mM.

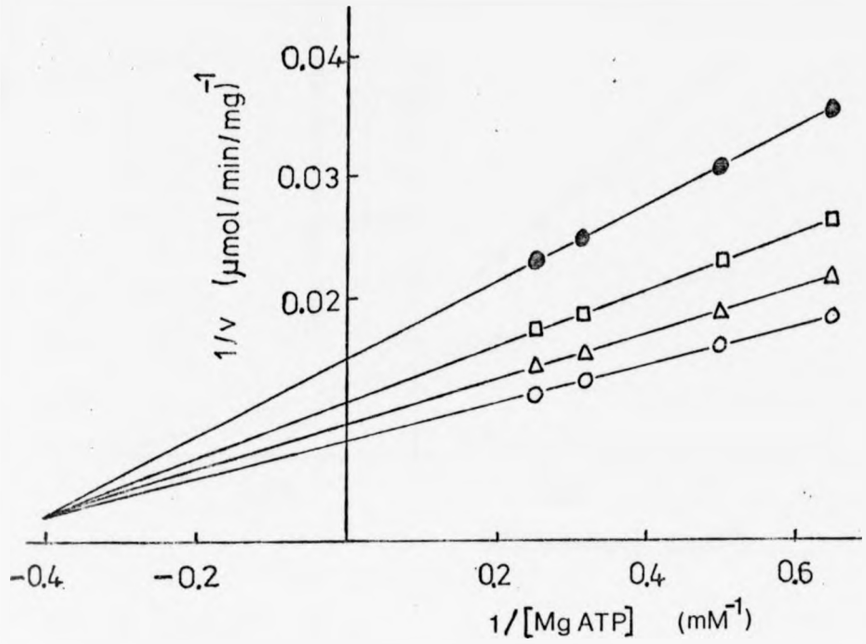


Fig 5.1 Double reciprocal plot data for the reaction catalysed by the matrix-bound enzyme (derivative A). The effect of varying the concentration of MgATP in the presence of several fixed concentrations of creatine was studied, and the concentrations of creatine were: ○, 40mM; △, 20mM; □, 10mM; ●, 5mM.

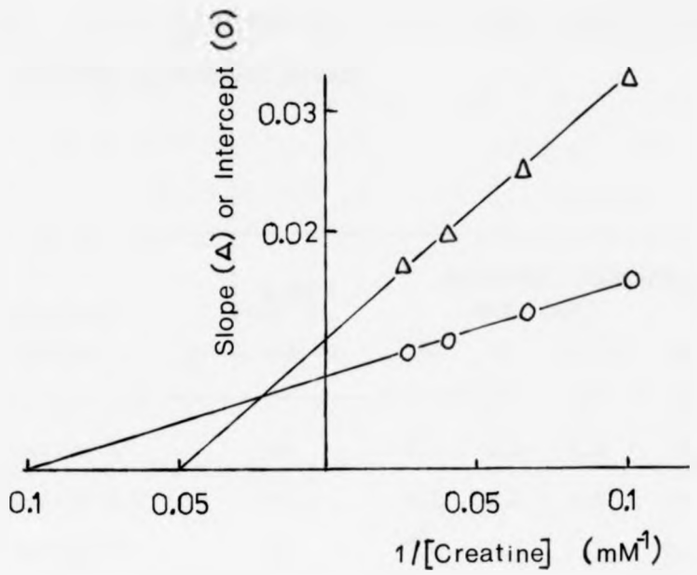


Fig 5.2 Secondary replot of the data presented in Fig 5.1 showing the variation of maximum initial velocity (O) and gradients (Δ) of the primary plot as a function of the reciprocal of the creatine concentration.

Table 5.2 Kinetic parameters for soluble and matrix-bound derivatives of creatine kinase

Matrix-Bound Derivative	Vmax μmol/min/mg	Apparent Kinetic Constants ATP (mM) Km	Ks	Ks/Km	Creatine (mM) Km	Ks	Ks/Km
Derivative A	145	0.9	2.4	2.6	5	13	2.6
Derivative B	115	1.2	2.4	2.0	17	32	1.9
Derivative C	142	1.0	2.5	2.5	8.5	25	2.9
Soluble Enzyme	150	0.4	1.1	2.8	10	28	2.8

The kinetic parameters were estimated from data of the type presented in Fig 5.1. When the concentration of ATP was varied the concentration of magnesium acetate was adjusted in order to maintain the free concentration of Mg^{2+} at 1.0 mM.

variations in the values of Km for MgATP with those for the matrix-bound derivatives being raised by approximately 2.5 fold compared with the soluble enzyme. The values of Km for creatine show some greater variation and it is interesting to note that the matrix-bound subunit derivative has a decreased Km for creatine.

It is also apparent that the ratio K_s/K_m for each substrate (which gives a measure of the extent of synergism in substrate binding) is decreased in all the matrix-bound derivatives compared with the soluble enzyme. The difference in the ratio K_s/K_m for the monomeric and dimeric matrix-bound derivatives is small and probably within experimental error. These studies show that immobilisation of the enzyme has not drastically affected the integrity of the active site, although small changes in its three-dimensional structure cannot be excluded. In addition the phenomenon of synergism in substrate binding does not appear to be dependent on the presence of the dimeric structure of the enzyme.

ADP binding

The results of the study of ADP binding to the matrix-bound enzyme are shown in Fig. 5.3 in the form of a Scatchard plot (Scatchard, 1949). It has been demonstrated by Price and Hunter (1976) that binding of ADP to soluble enzyme in the presence of Mg^{2+} + creatine + nitrate gives rise to a situation where the two binding sites on the enzyme are no longer equal and independent. Values for the dissociation constants were K_1 = 1.5 μM and K_2 = 18 μM (Price and Hunter, 1976). Values for the dissociation constants extrapolated from Fig. 5.3 are

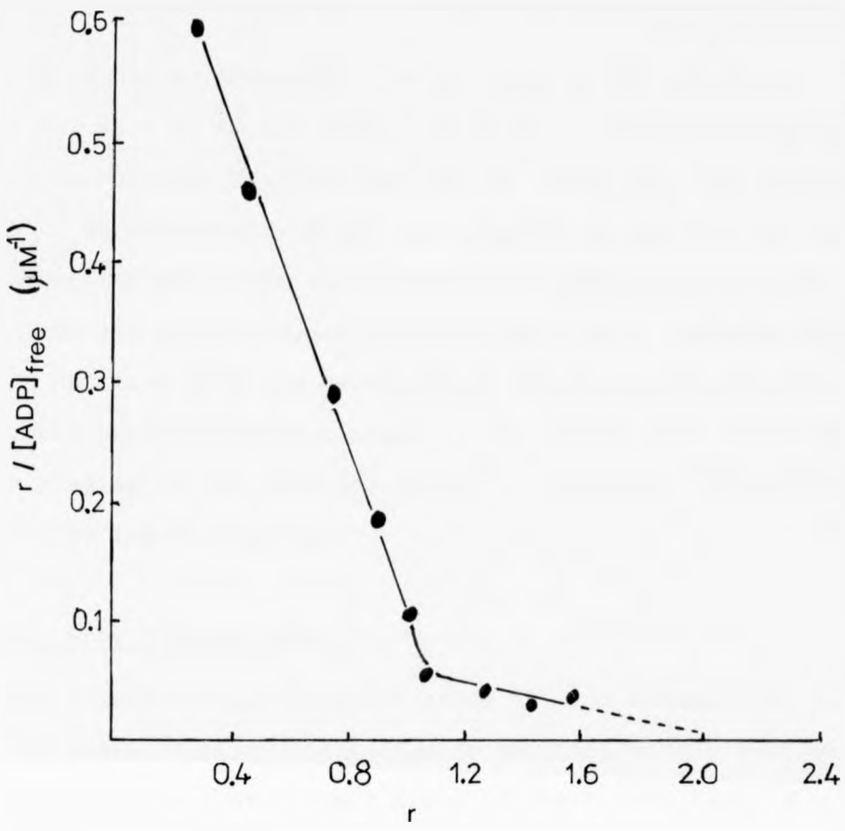


Fig 5.3 Scatchard plot for the binding of ADP to the matrix-bound enzyme (derivative A) in 50 mM-sodium Tricine buffer at pH 8.0. r represents the mcl ADP bound per mol creatine kinase dimer. The binding study was performed in the presence of 30 mM-creatine plus 10 mM-$NaNO_3$ plus 5 mM-magnesium acetate.

K_1 = 1.2 μM and an approximate value (only a few points on graph) for K_2 = 16 μM and these values are in excellent agreement with those obtained by Price and Hunter (1976) for the soluble enzyme. Unfortunately it was not possible to perform the same experiment reliably with the matrix-bound subunit derivative due to the low protein concentrations involved. However the results obtained with the matrix-bound dimer demonstrate that the subtle conformational changes at the active site involved in ADP binding in the presence of Mg^{2+} + creatine + nitrate are unaffected by immobilisation.

Inhibition by iodoacetamide

The reactivity of the thiol group in each subunit of creatine kinase towards iodoacetamide has been widely used as an index of the conformational state of the enzyme, and in particular as a means of detecting ligand-induced conformational changes in the enzyme (Watts, 1973; Milner-White and Kelly, 1976). The results of the studies on the reactivity of the thiol group of soluble creatine kinase and its matrix-bound derivatives are shown in Table 5.3. These results were obtained by measuring the rate of inactivation of the enzyme by iodoacetamide since it is known that the modified enzyme is completely inactive or very nearly so (Watts, 1973).

The data in Table 5.3 show that the reactivity of the thiol group in the various forms of the enzyme is quite similar, suggesting that immobilisation has not significantly affected the conformation of the enzyme in the region of the thiol group. The value of the rate constant for inhibition of the soluble enzyme by iodoacetamide is comparable with that reported by

Table 5.3 Second order rate constants (k) for inactivation of soluble and matrix-bound derivatives of creatine kinase by iodoacetamide

Reaction Mixture	Soluble Enzyme		Matrix-Bound Derivative A		B		C	
	k	(%change)	k	(%change)	k	(%change)	k	(%change)
E (enzyme only)	1100	-	800	-	1000	-	850	-
E + MgADP	1200	(+9)	900	(+12)	1000	(0)	1000	(+17)
E + MgADP + creatine	850	(-23)	600	(-25)	700	(-30)	600	(-30)
E + MgADP + creatine + NO_3^-	80	(-93)	50	(-94)	90	(-91)	70	(-92)

Inhibition of the soluble and the matrix-bound derivatives by iodoacetamide in the absence and in the presence of substrates or substrate complexes was studied at 30°C in 0.1M-glycine/NaOH at pH 9.0. The iodoacetamide concentration was 0.476 mM and the enzyme concentration was approximately 1.5μM (subunits) in all cases. Substrate concentrations used were : ADP, 1.0mM; magnesium acetate, 10mM; creatine, 40mM; $NaNO_3$, 0.1M.

Milner-White and Kelly (1976) under similar conditions. The effects of the ligand combinations Mg^{2+} + ADP and Mg^{2+} + ADP + creatine on the reactivity of the thiol group were also very similar for all the derivatives (Table 5.3). Of particular interest are the results obtained on addition of Mg^{2+} + ADP + creatine + nitrate (transition state analogue complex). In each case a decrease of more than 90% in the rate constant for the inactivation reaction was observed (Table 5.3).

These results indicate that the conformational responses of the various forms of the enzyme complexes do not require a dimeric structure. In this connection, it might be noted that the monomeric arginine kinase isolated from the lobster Homarus americanus is also capable of forming a transition state analogue complex on addition of Mg^{2+} + ADP + L-arginine + nitrate to the enzyme (Buttlaire and Cohn, 1974).

CHAPTER 6

STABILITY STUDIES

INTRODUCTION

One of the most important goals of enzymology is the elucidation of the principles which govern enzyme stabilisation. Clearly an understanding of these principles would enable the preparation of highly stable enzymes which would find extensive application in medicine, biochemical technology and chemical analysis (Martinek et al., 1977). It is therefore of considerable interest that immobilisation of enzymes often leads to altered stability properties in the matrix-bound enzyme (Mosbach, 1977). The stability properties of matrix-bound creatine kinase were examined with respect to storage at 4°C, thermal inactivation at 45°C and resistance to physical disruption by a denaturing agent (GdnHCl), in order to assess the influence of immobilisation on the stability of creatine kinase.

Perhaps the largest single factor which can affect the stability of a matrix-bound enzyme is the nature of the matrix. A matrix containing hydrophobic groups (e.g. polystyrene) might denature the protein in a manner analogous to a hydrophobic solvent. Moreover a hydrophobic matrix need not induce immediate denaturation; it might cause a slow inactivation during storage or modify sensitivity of the protein toward heating, pH or denaturants (Goldman et al., 1971). A hydrophilic matrix of positive or negative charge might lead to, under certain circumstances, an increase or a decrease in the stability of an immobilised enzyme due to electrostatic interaction between the protein and the matrix (Martinek et al., 1977). Clearly it is very difficult to establish a general pattern of the influence of immobilisation on enzyme stability

due to the very large variety of matrices with equally wide ranging properties. Therefore for the purpose of comparison with the present work, discussion of other studies on immobilised enzyme stability will be limited to those studies using Sepharose as the matrix.

In a number of cases where enzymes have been immobilised to Sepharose, increased stability towards heat and storage have been reported (Melrose et al., 1971). Recent studies (Miura et al., 1977; Fukui et al., 1975; Goheer et al., 1976; Lowe, 1977) also support this observation. Attempts to explain the apparent enhancement of stability normally result in rather incomplete conclusions regarding altered enzyme conformation or altered enzyme microenvironment. Indeed a satisfactory explanation of altered stability will not be possible until a quantitative description of the non-covalent forces which maintain proteins in their native state is available. It may very well be that a general mechanism of protein unfolding does not exist and that each case is unique.

It is beyond doubt, however, that inactivation of enzymes under the action of heating or denaturing agents involves considerable conformational changes in the protein molecule which lead to unfolding (Teipel and Koshland, 1971). If we therefore consider that unfolding is an indispensable step of enzyme de-stabilisation then the more tightly immobilised the protein is to the matrix, the more resistance there will be to unfolding. Gabel (1973) showed that the more linkages formed between trypsin and Sephadex, the greater the apparent stability of the immobilised enzyme. Therefore it seems likely that in some of the cases where increased stability has been observed,

the multipoint attachment of the protein to the gel has been a significant factor (Martinek et al., 1977). However it is not the sole factor in view of the observed increased stability in matrix-bound enzymes specifically coupled via one subunit by limited-point attachment (Chan et al., 1973a; Lowe, 1977; Fukui et al., 1975; Chan and Mosbach, 1976). The nature of the increased stability in such matrix-bound proteins is, as yet, unknown.

In this present study three important aspects of enzyme stability were investigated to provide information on the character of matrix-bound creatine kinase in terms of stability properties. The parameters studied were: resistance to physical disruption by a denaturant, resistance to thermal inactivation and long term storage at $4^{o}C$. It was hoped that these three parameters would provide a reasonably comprehensive view of the stability of matrix-bound creatine kinase.

EXPERIMENTAL

Inactivation by guanidine hydrochloride

The stability of the enzyme and its matrix-bound derivatives towards inactivation by GdnHCl was studied by incubating samples at 30°C in denaturing buffer in which the concentration of Tris/HCl and DTT were kept at 0.1 M and 5 mM respectively and the GdnHCl concentration varied over the range 0 - 1.0 M. After a 20 minute incubation period, during which the mixtures were kept continuously stirred, a sample was removed for direct assay of enzyme activity. The standard assay procedure was used (Chapter 2), and supplemented with 15 μg of trypsin in each assay to prevent renaturation within the assay mixture. Control experiments showed that neither residual denaturant nor the added trypsin affected the activity of native creatine kinase or the coupling enzymes (the controls were performed by adding the appropriate denaturant and/or trypsin to a standard assay mixture and observing the effect on the activity of native enzyme). The residual concentration of GdnHCl in the assay mixtures arising from the direct sampling procedure ranged from 0 - 0.01 M. It has been shown by Chan et al. (1973b) that incorporation of trypsin into the assay system effectively prevented renaturation within the assay system, and this has also been observed with creatine kinase. Direct addition of denatured enzyme to the standard assay system resulted in a steady increase in the rate of change of absorbance. Direct addition of denatured enzyme to the standard assay system + 15 μg trypsin produced no change

in absorbance and the integrity of the assay system was checked by the addition of native creatine kinase.

The matrix-bound derivatives were prepared as described in Chapter 2 and the protein contents of the matrix-bound derivatives were the same as those routinely obtained by that method (Table 4.2). The gels were prepared as suspensions (1:1, v/v) of gel in phosphate buffer and for the purposes of these experiments the gels were washed and eventually re-suspended in 0.1 M-glycine/NaOH at pH 9.0. The soluble enzyme was dialysed overnight against this buffer at 4°C. In all cases the enzyme concentration in the inactivation mixture was 30 μg/ml (0.73 μM subunits).

Stability towards thermal inactivation

The rates of thermal inactivation of the enzyme and its matrix-bound derivatives were studied at 45°C in 0.1 M-glycine/NaOH buffer at pH 8.5 (45°C). Buffer (2 ml) was equilibrated in a thermostatically controlled cuvette for 15 minutes before a small sample (0.1 ml) of enzyme was added. The mixture was then stirred continuously with a small magnetic stirring bar (0.4 cm in length) and samples (0.025 ml) were removed at set times and assayed directly for residual enzyme activity. The standard assay mixture was supplemented with 15 μg of trypsin as described in the preceding section. Matrix-bound derivatives were prepared as described in Chapter 2 and the protein contents of the derivatives were the same as those routinely obtained by that method. The gels were washed with and eventually re-suspended in 0.1 M-glycine/NaOH at pH 9.0. In all cases the enzyme concentration in the inactivation mixture was 30 μg/ml (0.73 μM subunits).

Stability towards storage at 4°C

The stability of the soluble enzyme and the matrix-bound enzyme upon storage at 4°C was studied. The matrix-bound enzyme (derivative A) was prepared by the method described in Chapter 2 and the protein content of the derivative was determined to be 400 µg/ml of packed gel. The matrix-bound enzyme was stored as a suspension (1:1, v/v) in phosphate buffer in a total volume of 5 ml. The soluble enzyme was dialysed against phosphate buffer and was stored as a solution in phosphate buffer at a concentration of 200 µg/ml in a total volume of 5 ml. At set times an aliquot (0.05 ml) was removed from the samples and diluted 1:40 into phosphate buffer before an aliquot was removed for direct assay of enzyme activity. In the case of the matrix-bound enzyme a control experiment was performed to reveal any soluble enzyme activity in the matrix-bound enzyme sample. This was accomplished by filtering the assay mixture + matrix-bound derivative gel sample through Whatman No. 1 paper and then replacing the assay mixture in the spectrophotometer to record any subsequent rate of change in absorbance. The standard assay procedure was used as described in Chapter 2.

RESULTS

Resistance to inactivation by heating and GdnHCl

In common with the results of studies on other enzymes immobilised on Sepharose (Chan et al., 1973a; Fukui et al., 1975; Lowe, 1977; Chan and Mosbach, 1976), it was found that immobilisation of creatine kinase led to an enhancement of stability towards either thermal inactivation (Fig. 6.1) or inactivation by GdnHCl (Fig. 6.2). In both types of study the order of stabilities observed was: derivatives A and C > derivative B > soluble enzyme. From Fig. 6.1 the first order rate constants for inactivation at 45^{o}C were calculated to be 0.028, 0.020 and 0.011 minute^{-1} for soluble enzyme, derivative B and derivatives A and C respectively. The concentrations of GdnHCl which cause a 50% loss of activity for these samples were 0.52, 0.57 and 0.64 M respectively for soluble enzyme, derivative B and derivatives A and C (Fig. 6.2.).

In connection with the studies on GdnHCl inactivation, it should be noted that with both the soluble enzyme and the matrix-bound derivatives it proved impossible, within the period studied (3 h) to achieve an equilibrium, limiting value of the residual enzyme activity at a given concentration of GdnHCl; a progressive loss of activity was observed over a period of several hours. For this reason experiments were conducted with a fixed incubation time (20 minutes) at a given concentration of GdnHCl, so the results presumably represent a combination of kinetic and thermodynamic aspects of the unfolding of the protein structure.

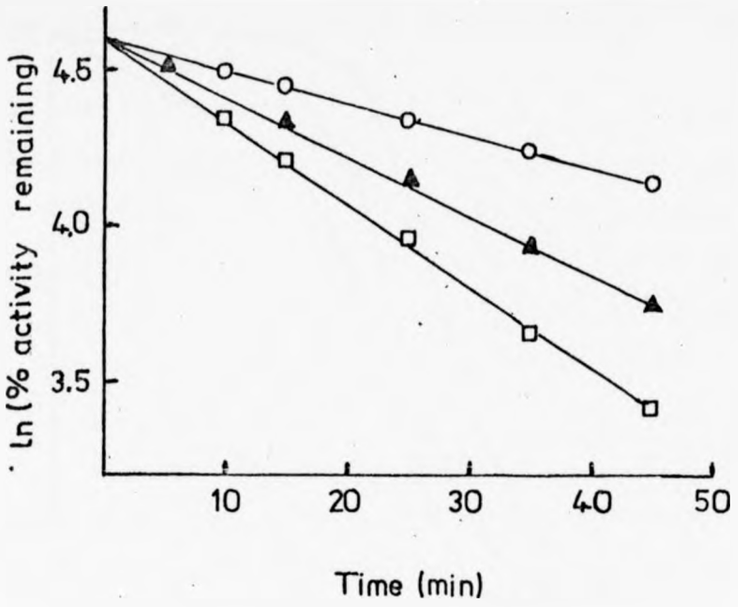


Fig 6.1 Semi-logarithmic plot comparing the rate of thermal inactivation of soluble and matrix-bound derivatives of creatine kinase : □ , soluble enzyme; ▲, matrix-bound subunit; O, matrix-bound dimer. The studies were made in 0.1M-glycine/NaOH at pH 8.5 at 45°C in a final volume of 2ml.

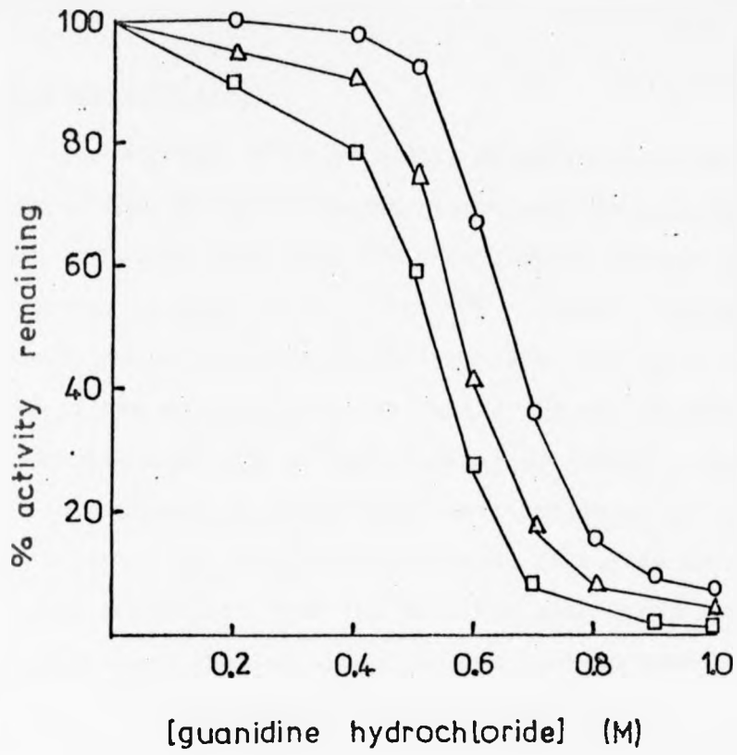


Fig 6.2 Effect of guanidine hydrochloride on the activity of soluble and matrix-bound derivatives of creatine kinase at pH 7.5 and 30°C : □, soluble enzyme; Δ, matrix-bound subunit; O, matrix-bound dimer. The concentration of guanidine hydrochloride-inactivated creatine kinase was approximately 30 μg/ml in each case and the period of incubation was 20 minutes. The standard assay system was used and supplemented with trypsin (15 μg) as described in Chapter 6.

Storage stability

The storage ($4^{o}C$) stability properties of the soluble enzyme and the matrix-bound enzyme are shown in Fig. 6.3, and it can be seen that the matrix-bound enzyme exhibits superior storage stability at $4^{o}C$. After a period of one month the soluble enzyme activity was reduced to a level 60% of the original activity while the matrix-bound enzyme displayed over 90% of the original activity. Control experiments designed to reveal any contribution of soluble enzyme activity in the matrix-bound enzyme (i.e. the extent of enzyme detachment from the gel) are also shown in Fig. 6.3. It was found that no appreciable soluble enzyme activity appeared in the matrix-bound enzyme samples indicating that there was no significant leakage of the enzyme from the matrix. The stability of the covalent linkage over long periods of storage has been observed by Gabel and Hofsten (1970) in other Sepharose-bound enzymes.

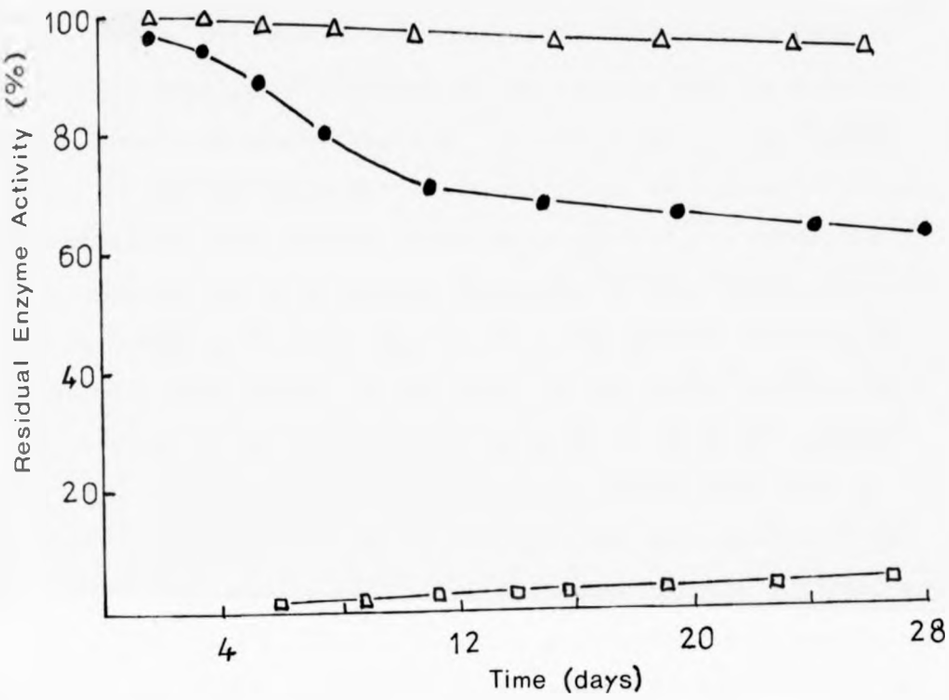


Fig 6.3 Stability of the soluble enzyme and the matrix-bound enzyme (derivative A) upon storage at 4°C in phosphate buffer ; ● , soluble enzyme; Δ, matrix-bound enzyme; □, soluble activity present in the matrix-bound derivative. The soluble activity present in the matrix-bound derivative was determined by filtering the assay mixture as described in Chapter 6.

DISCUSSION

As indicated earlier, the nature of the increased stability observed in the matrix-bound enzyme is difficult to specify and therefore any discussion of the subject must be coloured with a modicum of speculation. If we accept the postulate that the native conformation of a protein is the conformation in which the free energy of the molecule and its immediate surroundings is at a minimum (Martinek et al., 1977), then it seems possible that the influence of the matrix could be to lower the free energy of the matrix-bound enzyme with respect to the transition state of unfolding (i.e. slow the process down). This might be possible if the matrix were able to restrict the mobility of the backbone and side chains of the molecule thereby making it more difficult to unfold the protein. If the bonds of a protein molecule are envisaged as undergoing vibrational and rotational transitions then sensitive regions could be exposed briefly to the surrounding environment (which might lead to unfolding). The restriction imposed by the matrix as suggested above might decrease the frequency with which the sensitive regions become exposed.

It may well be that the covalent linkage itself is important rather than the matrix as a whole since it is apparent that glycoproteins often have stability properties superior to those of the corresponding carbohydrate-free protein (Pazur et al., 1970). Indeed artificial "glycosylation" of Acinetobacter asparaginase by coupling glycopeptide using glutaraldehyde gave enzyme derivatives which had greater heat stability than the native enzyme on heating at 45^{o}C (Holcenberg et al., 1975).

In the storage stability ($4^{o}C$) study it was demonstrated that there was no significant detachment of the enzyme from the matrix (Fig. 6.3) over the period of time studied (one month). This result does not support the observations of Tesser et al. (1972), that the matrix-bound ligands are slowly released into the aqueous environment. This apparent discrepancy can be reconciled in the light of the systematic investigation by Lasch and Koelsch (1978) to elucidate the factors involved in the leakage of ligands from matrices. Using leucine amino-peptidase as a model enzyme attached to CNBr-activated Sepharose 4B Lasch and Koelsch made an extensive study of both single and multipoint covalently bound enzyme derivatives. In considering the results obtained with the single-point attached enzyme, the most important factors contributing to the leakage of the enzyme were (a) nucleophilic buffers, (b) high pH and (c) high temperatures. In view of the work of Lasch and Koelsch (1978) the results obtained with matrix-bound creatine kinase are consistent since it was stored in a non-nucleophilic buffer (0.1 M-sodium phosphate at pH 8.0 containing 1.0 mM-EDTA) at low temperature.

CHAPTER 7

THIOL GROUP STUDIES

INTRODUCTION

The thiol groups of proteins are of considerable biochemical interest since they are among the most highly reactive of the amino acid side chains. Among the eight thiol groups per dimer contained in rabbit muscle creatine kinase only two are known to be readily accessible and highly reactive towards many thiol-blocking reagents (Kuby et al., 1962; Okabe et al., 1970). As the integrity of these two thiol groups (one per subunit) appeared to be necessary for enzyme activity, many extensive studies have been carried out concerning their chemical reactivity and functional rôle (Watts, 1973). In view of the intense interest in this aspect of the enzyme, a study was undertaken to compare some properties of the reactive thiol group(s) in the subunit and dimeric form of the enzyme in order to establish any relevance that the quaternary structure might have to the reactivity of these thiol groups.

Although the thiol groups reactive towards iodoacetamide etc. are probably situated near the active site (Milner-White and Watts, 1971) their importance has been based mainly on the observation that blocking these thiol groups will inactivate enzyme activity. Two groups of workers have recently shown that these reactive thiol groups cannot be directly involved in the catalytic mechanism, and is therefore to be regarded as a "non-essential" group. Smith and Kenyon (1974) and Smith et al. (1975) reported that the inhibition of creatine kinase by the variety of common thiol reagents was rather determined by the chemical properties of the blocking group introduced, such as bulk, charge, hydrophobicity and hydrogen-

bonding ability. Thus, when the reactive thiol groups were fully substituted with the relatively small, neutral non-hydrogen-bonding methane-thiol group (CH_3S-), the enzyme retained 20 - 30% residual activity. More recently der Terrossian and Kassab (1976) have reacted the two reactive thiol groups of rabbit muscle enzyme stoichiometrically with Nbs_2 and the resulting inactive mixed disulphide was subsequently substituted with cyanide, the smallest uncharged thiol-blocking group. The S-cyano derivative of creatine kinase retained 70% residual enzyme activity. In addition the S-cyano derivative was still able to react with a variety of thiol reagents with the further blocking of another two thiol groups per dimer. The bis-S-cyano derivative of creatine kinase retained 50% residual activity and it was concluded by der Terrossian and Kassab (1976) that the two cyanylated thiol groups per subunit were not essential for the catalytic activity of the enzyme. The presence of a second pair of accessible thiols per dimer in the enzyme is supported by Smith et al. (1975).

Although these elegant studies clearly indicate that the thiol group per subunit can no longer be regarded as essential for the catalytic activity of the enzyme, its exact rôle is still uncertain. Some progress in the elucidation of its function has been made by Keighren and Price (1978) using the 2-mercuri-4-nitrophenol (MNP) moiety as a reporter group to examine the ligand-induced conformational changes of various MNP derivatives of the soluble enzyme, and in particular to study those conformational changes that are associated with formation of the "transition state analogue" complex. The results obtained by Keighren and Price (1978) suggest that

the integrity of the reactive thiol group is required for conformational changes in the enzyme that are associated with formation of the catalytically active complex.

In this present section a study was made to compare the reactivity of the thiol groups of the monomeric and dimeric derivatives of matrix-bound creatine kinase in order to discover any influence that the quaternary structure might have on the reactivity of the thiol group on each subunit. In addition an investigation of the ability of thiol-blocked subunits to re-associate and re-form the dimeric structure was undertaken so as to assess any contribution that the thiol group might exert in the process of re-association of subunits.

EXPERIMENTAL

Determination of reactive thiol groups

The numbers of reactive thiol groups present in the enzyme and its matrix-bound derivatives were measured by using Nbs_2 assuming an absorption coefficient of 13.6 litre $mmole^{-1}$ cm^{-1} for the Nbs^{2-} anion at 412 nm (Ellman, 1959). Nbs_2 (final concentration 0.25 mM) was added to a gently stirred suspension of 1.0 ml of gel in 1.0 ml of phosphate buffer, and the stirring continued for 15 minutes at 20°C. The suspension was then centrifuged at 2000 g for 5 minutes and the absorbance of the supernatant measured at 412 nm. Control experiments showed that small corrections were necessary for the matrix-bound subunit and matrix-bound re-associated enzyme derivatives to take account of the reaction of Nbs_2 with residual DTT from the renaturing buffer. The correction was always less than 10% of the total observed absorbance. In every case there was no further reaction with Nbs_2 over an additional 30 minute period.

The numbers of reactive thiol groups were also determined by using iodo[1-C^{14}]acetamide. A small sample of the stock radioactive IAM (see Chapter 2) was added to a gently stirred suspension of 1.0 ml of gel in 1.0 ml of 0.1M-glycine/NaOH at pH 9.0 to give a final concentration of IAM of 0.476 mM. The mixture was stirred at 30°C for 15 minutes before the reaction was stopped by the addition of 0.5 ml of phosphate buffer containing 10 mM-DTT. The gel was then transferred to a sintered glass tube (1 cm x 10 cm) and washed extensively with phosphate buffer (200 ml) to remove unchanged radioactive

IAM. Radioactivity of matrix-bound derivatives was determined after hydrolysis of a 0.2 ml sample of a 1:1 (v/v) gel suspension in standard phosphate buffer in 0.5 ml of 12 M-HCl as described in Chapter 2. Control experiments showed that the presence of neither Sepharose nor enzyme affected the observed radioactivity (c.p.m.) of a standard solution of iodo [1-C^{14}]acetamide (2300 c.p.m.) and that more than 99.5% of the unchanged radioactive IAM (2290 c.p.m.) was removed from the gel by washing with standard phosphate buffer as described. Small corrections were necessary, however, with the matrix-bound subunit and matrix-bound re-associated enzyme to account for the reaction of radioactive IAM with residual DTT from the renaturing buffer. The correction was always less than 15% of the total observed radioactivity. The incorporation of iodo [1-C^{14}]acetamide into soluble enzyme was monitored by the general method previously described by Griffiths et al (1975). In all cases there was no further incorporation of radioactivity after an additional 45 minute period of incubation with the radioactive IAM.

Preparation of various thiol-blocked enzyme derivatives

The preparation of the various thiol-blocked enzyme derivatives by reaction with IAM is illustrated in Scheme 7.1. Derivative E, the thiol-blocked matrix-bound enzyme was routinely prepared by reacting the matrix-bound enzyme (derivative A) with excess IAM as described above (in the section dealing with the determination of the number of thiol groups) using radioactive IAM. Further derivatives were produced by subjecting derivative E to the denaturation-

renaturation sequence analogous to that used (Chapter 2) for the preparation of the matrix-bound subunit (derivative B) from the matrix-bound enzyme (derivative A). This process yielded a mono-thiol-blocked matrix-bound subunit (derivative F) form (Scheme 7.1). A di-thiol-blocked matrix-bound subunit (derivative G) was obtained from either derivative F or directly from derivative B by reaction with an excess of IAM as described earlier.

The rate of incorporation of radioactive IAM into the soluble enzyme, matrix-bound enzyme (derivative A), matrix-bound subunit (derivative B) and the mono-thiol-blocked matrix-bound subunit (derivative F) was also investigated. The procedure was exactly the same as that described earlier for the determination of the number of thiol groups by radioactive IAM incorporation except that the reaction was stopped at various time points (1 - 15 minutes) by the addition of 0.5 ml of phosphate buffer containing 10 mM-DTT and the amount of radio-activity incorporated at that time point was then determined.

Re-association of thiol-blocked matrix-bound subunits

This study involved the matrix-bound derivatives B, F and G (Scheme 7.1) described above. The derivatives were re-suspended in renaturing buffer and to a gently stirred suspension (1:5, v/v) of gel in buffer was added small aliquots (0.05 ml) of a solution of denatured soluble enzyme. The procedure and conditions of renaturation were exactly the same as for the preparation of the matrix-bound re-associated enzyme (Chapter 2). The experiment was repeated with one modification which was that the denatured soluble enzyme had

been previously reacted (prior to denaturation) to its limiting extent, of one thiol group per subunit, with iodoacetamide. The IAM-blocked soluble enzyme was prepared by adding IAM (final concentration 3 mM) to a solution of native enzyme (final concentration 0.25 mM) in phosphate buffer and allowing the mixture to stand at 20°C for 30 minutes. The mixture was then dialysed overnight against phosphate buffer to remove unreacted IAM before the IAM-blocked enzyme was incubated with denaturing buffer. The levels of renaturation were monitored by determining the protein contents before and after each experiment as described in Chapter 2.

Re-association of thiol-blocked soluble subunits

Denaturation of the soluble enzyme was achieved by incubating the native enzyme (5 mg/ml) with a modified denaturing buffer consisting of 0.1 M-Tris-HCl at pH 7.5 containing 6M-GdnHCl and 2 mM-EDTA, for 1.0 h at 20°C. Renaturation was accomplished by diluting (1:30) a sample of denatured enzyme into phosphate buffer. The renaturation profile was determined exactly as before using the standard assay procedure. The renaturation profile was also determined in terms of the disappearance of thiol groups reactive towards Nbs_2 during the period of renaturation. Renaturation was initiated then successive aliquots (0.1 ml) were removed at set times and diluted into phosphate buffer containing 0.1 mM-Nbs_2. The mixture was rapidly mixed (by inversion of the test tube) and then after 2 minutes standing at 20°C the absorbance of the solution was measured at 412 nm.

Renaturation of soluble enzyme was interrupted by the

addition of IAM in an attempt to produce a partially renatured thiol-blocked enzyme and this was accomplished by adding IAM (final concentration 0.3 mM) to the renaturing enzyme solution (0.005 mM) during the renaturation period. Gel filtration studies on the thiol-blocked product were performed essentially as described in Chapter 2. A Sephadex G-100 column (32 cm x 1.5 cm) was equilibrated with phosphate buffer at $4^{o}C$ and fractions (1.0 ml) were collected for protein estimation. The elution volumes for several proteins of known molecular weight were determined and a graph of relative elution volume against log molecular weight constructed (Chapter 2).

RESULTS AND DISCUSSION

Determination of reactive thiol groups

The numbers of reactive thiol groups in the matrix-bound derivatives of creatine kinase were initially determined by using Nbs_2 . This reaction is easily applicable to such derivatives since the yellow Nbs^{2-} anion product is released into solution and can be readily measured spectrophotometrically in the supernatant after the gel suspension has been centrifuged (2000 g for 5 minutes). The results obtained with the various matrix-bound derivatives and the soluble enzyme are shown in Table 7.1. The results clearly demonstrate that the matrix-bound enzyme (derivative A) and the matrix-bound re-associated enzyme (derivative C) in common with the soluble enzyme possess one reactive thiol group per subunit, whereas two reactive thiol groups per subunit are present in the matrix-bound subunit (derivative B).

The absorbance values obtained in the reaction with Nbs_2 were relatively small (in the region 0.10) at the protein concentrations obtainable with the matrix-bound derivatives and therefore a check on these results was performed by using iodo [1-C^{14}]acetamide as a thiol-modifying reagent. The results are also collected in Table 7.1 and they confirm the pattern observed with Nbs_2 i.e. one reactive thiol group per subunit for dimeric forms of the enzyme and two reactive thiol groups per subunit for the matrix-bound subunit derivative. In addition it should be noted that the derivatives that had been reacted with radioactive IAM to the limiting extents shown in Table 7.1 did not react further with added Nbs_2 ,

Table 7.1 Estimation of reactive thiol groups in soluble and matrix-bound derivatives of creatine kinase

Matrix-Bound Derivative	Protein Concentration (μM-subunits)	Nbs^{2-} Released (μM)	[1-^{14}C] Acetamide Incorporated (μM)	No. Thiols Reacted / Subunit
Derivative A	5.8	6.1	6.2	1.06
Derivative B	2.9	6.2	6.4	2.17
Derivative C	5.6	6.2	6.3	1.11
Soluble Enzyme	5.8	5.5	5.7	0.96

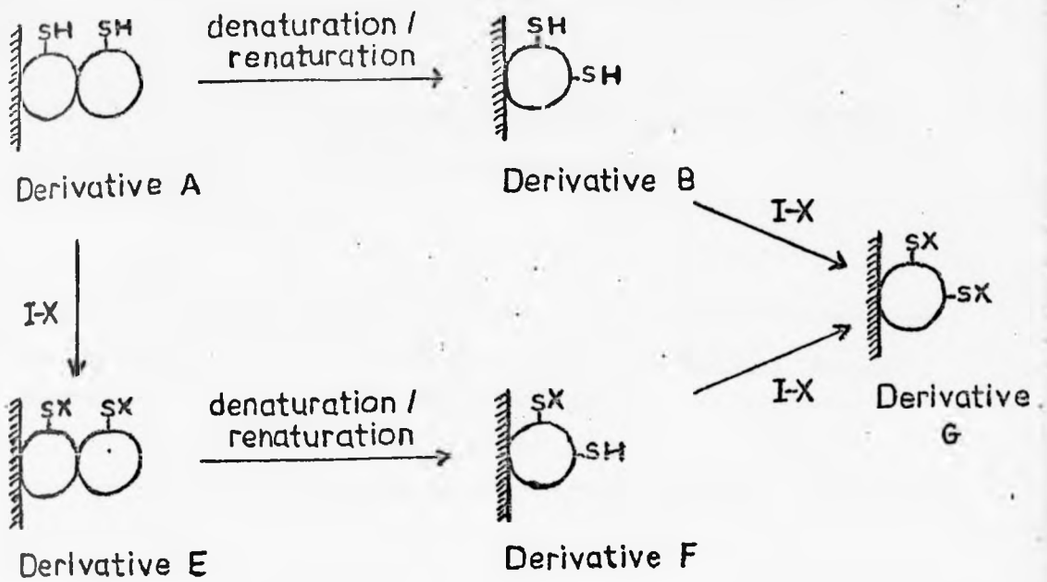
Reactive thiol groups were estimated by two methods ; release of the anion Nbs^{2-} and incorporation of [1-^{14}C] acetamide. The number of thiol groups reacted/subunit is presented above as an average of the very similar values obtained by these two methods. The protein concentration refers to the final concentration of protein in the reaction mixture and not to the protein content of the packed gel.

indicating that the two reagents are reacting with the same thiol group(s) on the enzyme.

Further experiments with thiol-blocked matrix-bound derivatives

The interconversions of the various carbamoylated enzyme derivatives are illustrated in Scheme 7.1. Derivatives A and B represent the matrix-bound enzyme and the matrix-bound subunit form respectively; derivative E is the carbamoylated matrix-bound dimer (reacted to the limiting extent of one thiol group per subunit); derivative F is the mono-carbamoylated matrix-bound subunit form obtained by a denaturation-renaturation sequence analogous to that used in the preparation of derivative B from derivative A (Chapter 2); derivative G is the di-carbamoylated matrix-bound subunit form obtained either from derivative F or directly from derivative B by reaction with IAM in a fashion analogous to that described in the Experimental section. The protein contents of derivative E and derivative F were determined and found to be 400 μg/ml of gel and 205 μg/ml of gel respectively. In addition derivative F was found to possess 53% of the radioactivity present in derivative E and these results confirm that in derivative E the radioactive IAM had reacted uniformly with the two subunits of the matrix-bound dimer.

The results obtained in the study of radioactive IAM incorporation into the various enzyme derivatives are collected in Table 7.2. The inactivation of soluble enzyme and matrix-bound enzyme (derivative A) is accompanied by a proportional incorporation of one acetamide moiety per subunit, whereas inactivation of the matrix-bound subunit (derivative B) is



Scheme 7.1 Scheme depicting the relationship between the various carbamoylated matrix-bound derivatives of creatine kinase. Derivatives A and B were obtained as described in Chapter 2. Derivative E was prepared by treating derivative A with a large excess of iodoacetamide (I-X). Derivative F was then derived from derivative E by the denaturation-renaturation procedure outlined in Chapter 2. Derivative G was obtained either from derivative F or directly from derivative B by reaction with a large excess of iodoacetamide

Table 7.2 Rate of incorporation of [1-^{14}C] Acetamide into soluble and matrix-bound derivatives of creatine kinase

Matrix-Bound Derivative	Time (min)	[1-^{14}C] Acetamide Incorporated/Subunit (μM)	(%)	Residual Enzyme Activity (%)
Derivative A (4.2μM)	1	2.4	58	60
	2	3.1	74	43
	3	3.8	28	28
	15	4.4	105	2
Derivative B (2.1μM)	1	1.4	69	55
	2	2.3	110	44
	3	2.8	135	28
	15	4.4	210	2
Soluble Enzyme (4.5μM)	1	2.0	45	58
	2	2.7	61	41
	3	3.6	80	25
	15	4.3	95	2

The protein concentration of both the soluble and the matrix-bound derivatives indicated above is the final concentration of protein in the inactivation mixture and is presented as μM-subunits. The incorporation of [1-^{14}C] acetamide is presented as a percentage relative to the concentration of protein.

accompanied by incorporation of two acetamide moieties per subunit. From the studies of the rate of radioactive incorporation of IAM in the matrix-bound subunit derivatives B and F it appears that the two thiol groups per subunit are of comparable reactivity, i.e. there is no rapid reaction of one thiol group followed by a slower reaction of the second. The rate of inactivation of the enzyme by IAM (Table 7.2) is similar in the soluble enzyme, matrix-bound subunit and matrix-bound enzyme forms, and therefore it seems likely that reaction of only one thiol group (the same one as in the dimeric forms) is required for the inactivation of the matrix-bound subunit form. This would tend to suggest that reaction of the second thiol group does not affect the enzyme activity and this possibility is supported by the observation that the mono-carbamoylated matrix-bound subunit (derivative F) is enzymically inactive.

The work reported above clearly demonstrates the appearance of the second thiol group per subunit in the monomeric derivative of immobilised creatine kinase which presumably must be masked in the dimeric form to attack by common thiol-blocking reagents such as iodoacetamide and Nbs_2. It is tempting to suggest that this second thiol group may be situated at the intersubunit region of the enzyme and is masked by the physical presence of the complementary subunit. Indeed the appearance of additional accessible thiol groups upon subunit dissociation has been observed in another enzyme by Lazurus et al. (1966) who demonstrated that yeast hexokinase consisted of two associated identical subunits joined by non-covalent forces and that subunit association was responsible for the sequestration of thiol groups into hydrophobic regions.

Re-association of thiol-blocked matrix-bound subunits

These studies were designed to investigate the possibility that one or both of the accessible thiol groups on each subunit might be involved in the process of subunit re-association. The study considered the ability of various matrix-bound subunit derivatives to re-associate with both native soluble subunits and thiol-blocked soluble subunits. The results are collected in Table 7.3 and in the case of the matrix-bound subunit (derivative B) it was found that (as normal) the derivative could re-associate with added subunits of the native soluble enzyme to re-form the dimeric structure. However the matrix-bound subunit (derivative B) was not able to re-associate with added subunits of thiol-blocked soluble enzyme (prepared as described in the Experimental section). In the cases of the mono-carbamoylated matrix-bound subunit (derivative F) and the di-carbamoylated matrix-bound subunit (derivative G), re-association did not occur with either the added native subunits or the added thiol-blocked subunits. These results strongly suggest that both the thiol groups may be involved either singly or together in the process of subunit re-association or in the stability of the inter-subunit region. If this conclusion is valid then it should be possible to arrest the process of re-association in the soluble enzyme by blocking the thiol groups during renaturation and this aspect is examined in the following section.

Re-association of thiol-blocked soluble subunits

The renaturation of the soluble enzyme has been described in Chapter 3 and it has been demonstrated that creatine kinase renatures from a denatured state very rapidly to regain 80%

Table 7.3 Re-association of the various matrix-bound subunit derivatives with soluble subunits of native enzyme and thiol-blocked enzyme

Matrix-Bound Derivative	Protein Content µg/ml	Re-association with Soluble Subunits: Native µg/ml	Re-association with Soluble Subunits: Thiol-Blocked µg/ml
Derivative B	200	385	200
Derivative F	205	205	205
Derivative G	200	200	200

The procedure and conditions of renaturation were exactly the same as for the preparation of the matrix-bound re-associated enzyme (derivative C). The thiol-blocked subunits were prepared as described in Chapter 7.

of the original activity in 15 minutes or so with full regain of enzyme activity after a 3 h period of renaturation at 20°C followed by an overnight period at 4°C. In view of the results above, the time course of the disappearance of the thiol groups reactive towards Nbs_2 during renaturation of the soluble enzyme was investigated. The results are depicted in Fig. 7.1 and they demonstrate a rapid decline in the number of thiol groups from 4 per subunit to 2 per subunit in the first 10 minutes or so of renaturation, whereas the subsequent decline from 2 per subunit to 1 per subunit takes place over a further 60 - 70 minutes. It is tempting to suggest that the initial process of renaturation is a rapid refolding of subunits to give correctly folded subunits (possessing two reactive thiol groups per subunit, by analogy with the matrix-bound subunit), and that this is followed by a slow re-association process to give the dimeric form with one reactive thiol group per subunit.

The time course of the regain of enzyme activity under the same conditions shows that 75 - 80% of the final activity is regained in the first 10 - 15 minutes, and it is noteworthy that the matrix-bound subunit (derivative B) has a Vmax approximately 80% of that of the matrix-bound enzyme (derivative A) (Chapter 5). Earlier results described in Chapter 3 have shown that the regain of enzyme activity over the first 10 - 15 minutes of renaturation is independent of protein concentration in the range 50 - 200 µg/ml, i.e. that the regain of activity occurs in a first order process with respect to protein. These results would be consistent with the hypothesis that the early phase of the renaturation process involves the conversion of unfolded subunits to correctly folded active subunits.

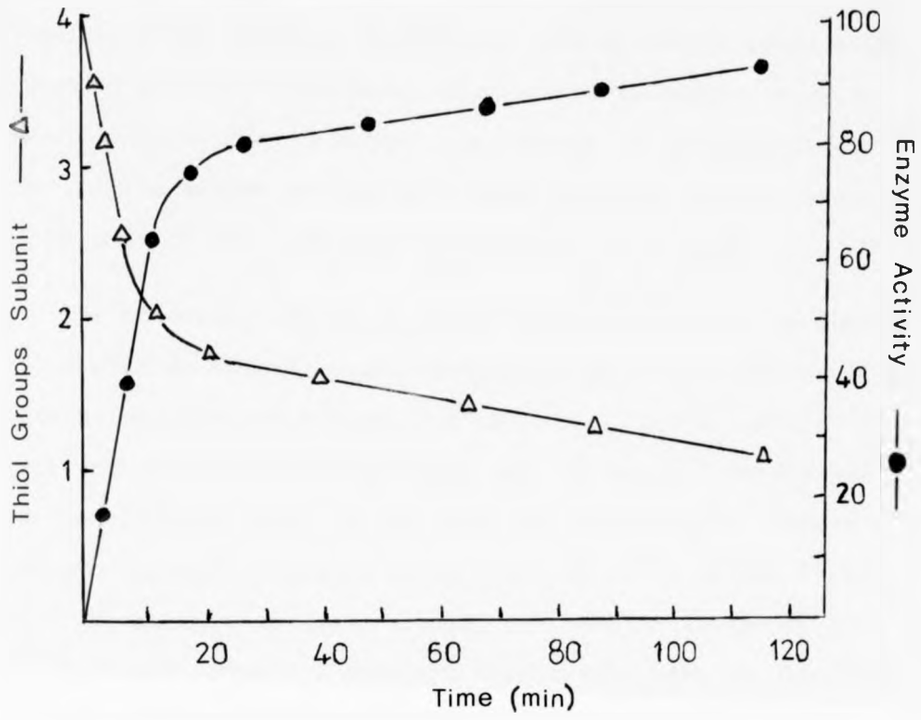


Fig 7.1 Renaturation of soluble creatine kinase (133 ug/ml) in 0.1M-sodium phosphate buffer at pH at 8.0 containing 1.0mM-EDTA at 20°C. The enzyme was denatured with 6M-guanidinium hydrochloride as described in Chapter 7 ; Δ, represents the number of thiol groups/subunit; ●, represents the percentage regain of enzyme activity.

If this conclusion is valid and it is assumed that the results of the previous section on re-association of matrix-bound subunits can be extrapolated to the soluble enzyme, then it should be possible to arrest the process of re-association in the soluble enzyme by adding a thiol-blocking reagent after 10 minutes of the renaturation process had elapsed.

The molecular weight of the product obtained after adding iodoacetamide (final concentration 0.3 mM) to a solution of renaturing creatine kinase (2.5 μM) after 10 minutes of renaturation was estimated using gel filtration (Chapter 2). The results are shown in Fig. 7.2 and the molecular weight of the product was estimated to be 55000 which is rather higher than the subunit molecular weight of creatine kinase of 41000 (Watts, 1973). Although this result does not confirm that the product is thiol-blocked single subunits, it is sufficiently displaced from the dimeric molecular weight of 82000 to be of significance. Future work might refine the procedure by improving the resolution of the gel filtration method and using a faster reacting thiol-blocking reagent such as Nbs_2.

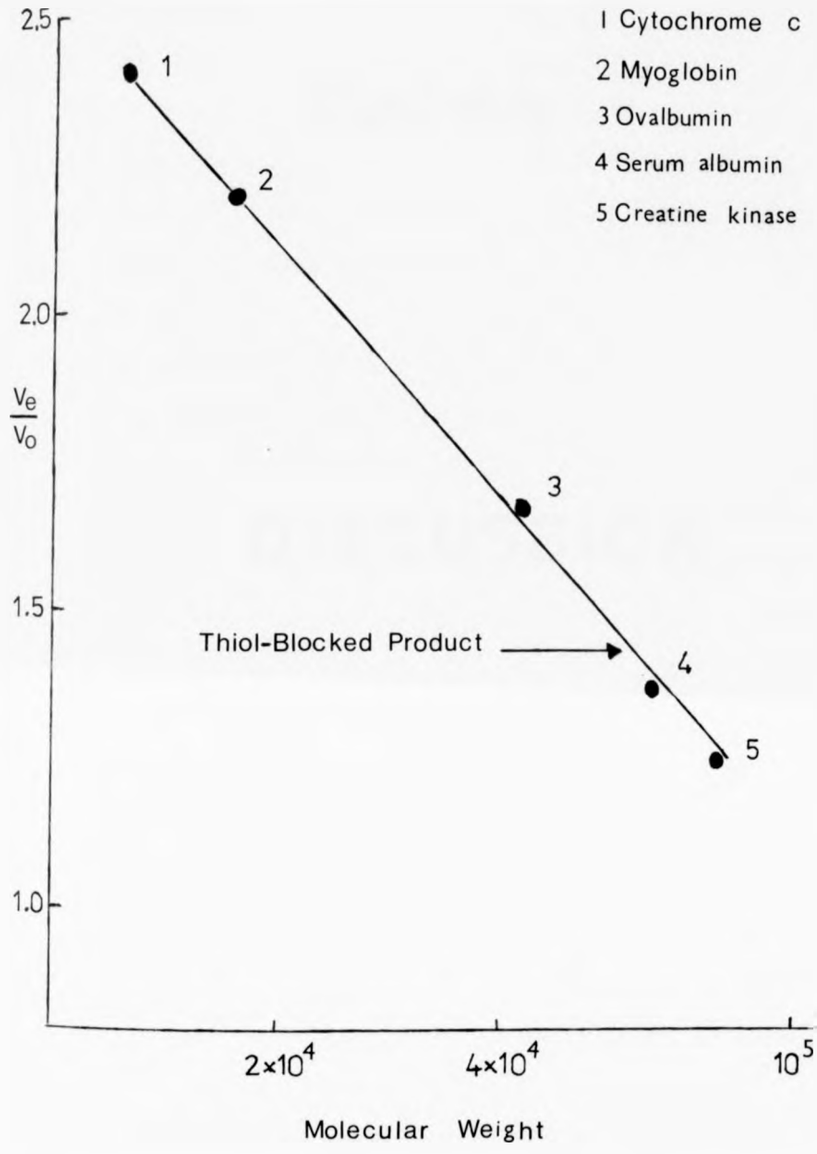


Fig 7.2 Estimation of the molecular weight (by gel filtration) of the thiol-blocked product of renaturing soluble enzyme. The product was obtained by adding an excess of iodoacetamide to the soluble enzyme during renaturation as described in Chapter 7.

CHAPTER 8

DISCUSSION

DISCUSSION

Perhaps the single most important result to be derived from these studies is that the matrix-bound subunit form of creatine kinase is catalytically active. This point is of some interest in view of the widespread occurrence of oligomeric enzymes with identical subunits. Many such enzymes display Michaelis-Menten kinetics indicating no apparent cooperativity among the active sites. The advantage conferred by the quaternary structure is not immediately obvious and this also seems to be the case with creatine kinase. The activity of the isolated subunits of creatine kinase is of interest in view of the fact that the related enzyme arginine kinase occurs in a monomeric form in a number of species, in particular lobster (Morrison, 1973). Previous work has indicated that lobster arginine kinase bears a notable similarity to the creatine kinase in terms of molecular weight and amino acid sequence around the reactive thiol group (Morrison, 1973).

The results obtained in this present study show that there are considerable similarities between the soluble enzyme and the matrix-bound enzyme in terms of:

(1) the parameters (Km and Vmax) of the enzyme catalysed reaction

(2) the reactivity of the reactive thiol group towards IAM

(3) changes in the reactivity of the reactive thiol group in the presence of combinations of substrates and ligands

(4) the binding of the substrate ADP (in the presence of Mg^{2+} + creatine + NO_3^-).

The cumulative inference from this data is that neither the

conformation of the enzyme (at the active site and at the reactive thiol group) nor the substrate-induced conformational changes (monitored by changes in the reactivity of the thiol group) are grossly altered upon immobilisation. Extrapolation of results obtained with the matrix-bound enzyme derivatives in comparison with soluble enzyme can thus be made with reasonable confidence. Some small changes in the degree of synergism of substrate binding, and in the extent of acetate activation, however, do indicate that some small perturbations in 3-dimensional structure do occur upon immobilisation. The observed similarities between the matrix-bound enzyme and the matrix-bound subunit forms of the enzyme demonstrate that the dimeric structure is of little importance in almost all of the properties discussed above.

A significant result was the appearance of the second thiol group in the matrix-bound subunit derivative, particularly in view of the results of der Terrossian and Kassab (1976) in which the two reactive thiol groups per dimer as well as two other thiol groups, normally non-accessible were substituted with cyanide, the smallest uncharged thiol-blocking group. However, it has not been demonstrated in this present work that the second thiol group per subunit found in the matrix-bound subunit and the extra accessible thiol-group per subunit observed by der Terrossian and Kassab are one in the same. Further work employing cyanide to substitute the thiol groups of the matrix-bound derivatives will be necessary to resolve this issue.

One of the chief limitations of the immobilised enzyme approach is that few physicochemical studies of the immobilised

monomers can be made. The particulate state of the bound enzyme precludes any examination of its hydrodynamic properties. Spectroscopic techniques are handicapped by the light-scattering effects of the gel particles although spectroscopic methods of assays are not affected significantly by light scattering, owing to the low amounts of matrix-bound derivative required. Clearly such difficulties would be overcome by the preparation of a solution of isolated soluble subunits. Attempts to produce single soluble subunits by blocking the reactive thiol groups with iodoacetamide during the renaturation of the soluble enzyme proved more difficult than was first anticipated. It is highly probable that the rapid renaturation is faster than the modification by the thiol-blocking agent (iodoacetamide) and future experiments with Nbs_2 as the thiol-blocking reagent might be more worthwhile. Alternatively it might be profitable to slow the renaturation process by conducting the experiments at $4^{o}C$ or in the presence of sucrose. In view of the fact that re-association can be prevented by blocking the thiol groups of the matrix-bound subunits (Chapter 7) it would be particularly useful to reversibly immobilise the enzyme. It would then be possible to prepare the thiol-blocked subunits and subsequently release them from the matrix (see Scheme 8.1). In a study on pig brain guanase Rossi et al. (1977) used covalent chromatography to study the rôle of the thiol groups in this enzyme. The enzyme was coupled to thiol-Sepharose 4B by a thiol disulphide interchange reaction between the disulphide groups of the Sepharose reagent and the thiol groups of the enzyme. The coupling is reversible under reducing conditions and the enzyme was eluted with a cysteine gradient.

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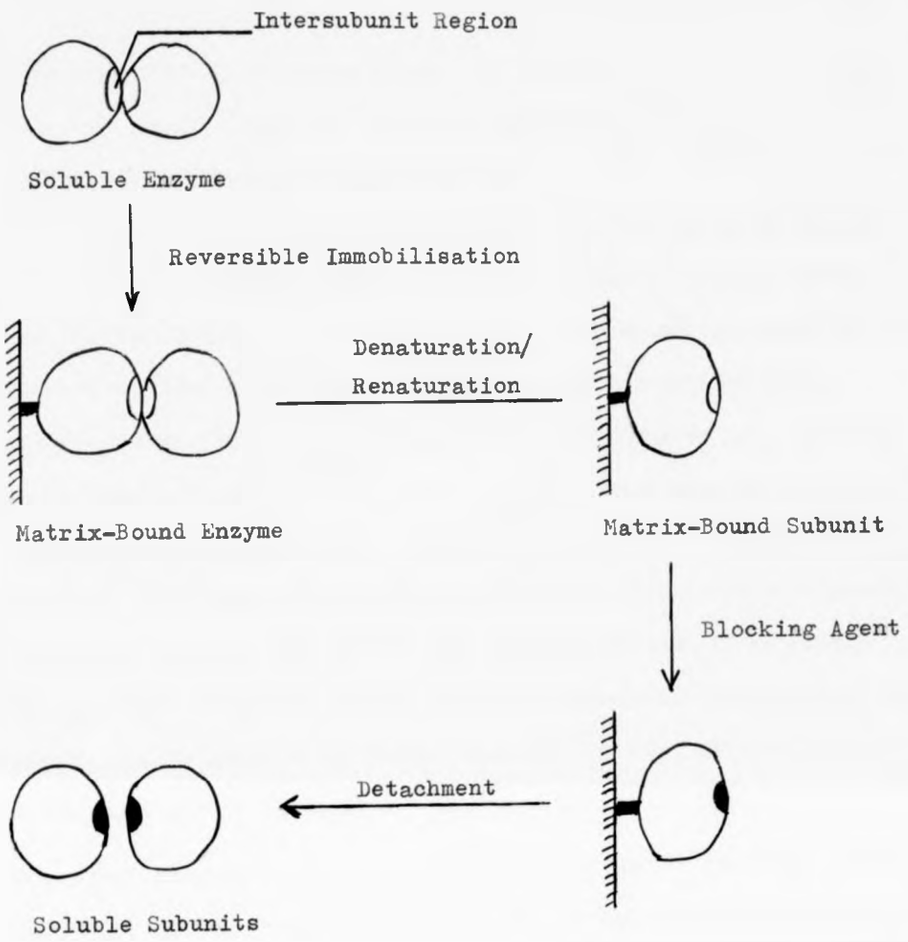

Scheme 8.1 Proposed scheme for the stabilization of dissociated subunits by chemical modification at the intersubunit region.

A noteworthy difference observed between the matrix-bound monomer and dimer forms of creatine kinase is the greater stability of the dimeric form over the subunit form, with regard to resistance to inactivation by heat or a denaturant. This result may be explained by the favourable subunit interactions in the dimer which may tend to reduce any disruptive influences on the individual subunits. The results are comparable with the pattern observed by Anosike et al. (1975) in their study on dimeric arginine kinase from Holothuria forskali (sea cucumber) and monomeric arginine kinase from Homarus vulgaris (lobster). In common with the arginine kinases the differences in stability between the dimer and monomer forms of creatine kinase are only observed under non-physiological conditions and therefore the relevance of these results to the enzyme in the physiological state is open to question.

The work presented in this Thesis vindicates the principle of employing matrix-bound enzyme derivatives for fundamental studies on the properties of enzymes. Not only do the methods employed in this present work provide valuable information on the subunit behaviour of the enzyme they also provide a basis for the understanding of the in vivo properties of the enzyme. It is now becoming apparent that one of the most important factors affecting metabolic regulation in the cellular microenvironment is the interaction between enzymes and cellular structures (Masters, 1978). In a study on lactate dehydrogenase (LDH) it was found that the isoenzymes exhibited different adsorption properties (Ehman and Hultin, 1973). LDH-5 bound readily to the particulate matter in skeletal muscle, whereas LDH-1 binds little if at all under a variety of experimental

conditions. Furthermore, the soluble enzyme was strongly inhibited by high pyruvate concentrations, whereas the bound form of the enzyme showed no inhibition. In this case the authors favoured the view that particulate association preserves the tetrameric integrity of the enzyme.

A large proportion of the aldolase, pyruvate kinase and phosphofructokinase of nervous tissue have also been found to be associated with particulate fractions (Masters, 1978). Indeed fractional extraction of heart muscle has revealed that about 30% of cellular creatine kinase activity is located in mitochondria and about 20% is bound to myofibrils (Saks et al., 1975) and the bound enzyme has been found to be electrophoretically identical with the MM isoenzyme. Kinetic parameters for the particulate enzyme have been determined, and Km values for creatine and ATP of 15.5 and 0.95 mM respectively, reported (Saks et al., 1975). These values are not unlike the corresponding values obtained with the matrix-bound derivatives in this present work. In view of the fact that a form of creatine kinase exists in a bound state then it is possible that use of matrix-bound derivatives may represent a useful approach to the study of the <u>in vivo</u> properties of the enzyme.

APPENDICES

APPENDIX I

Calculations Involved in the Preparation of Creatine Kinase

1. Calculation of the amount (Z) of NH_4Cl required to give a final concentration of 0.1 M

   Volume of supernatant = S_1ml

   Molecular weight of NH_4Cl = 53.5

   $\therefore$ 5.35 g/l = 0.1 M

   and $(S_1/1000) \times 5.35 = Z$ g to be added

2. Calculation of the volume (Y) of $MgSO_4$/Tris solution required to give a final concentration of 30 mM

   Volume of supernatant = S_2ml

   $(S_2 + Y)(0.03) = Y.2$

   $\therefore S_2.0.03 + Y.0.03 = Y.2$

   $\therefore Y = (S_2.0.03) \div 1.97$

3. Calculation of the volumes of ethanol required to give final concentrations of 36% and 50%

   Combined volume of magnesium acetate used in extraction = A

   Combined volume of supernatants after extraction = B

   % alcohol already present = $\frac{(B-A)}{B}.60 = M$

   Volume required to give 36% = $\frac{(36-M)}{64}.B = N$

   Volume required to give 50% = $\frac{(B+N)}{50}.14 = P$

APPENDIX II

Reagents and Solutions for SDS Acrylamide Gel Electrophoresis

Gel buffer: 7.8 g $NaH_2PO_4.H_2O$
38.6 g $Na_2HPO_4.7H_2O$
2.0 g SDS
Water to 1.0 litre

Acrylamide solution (22%): 22.2 g acrylamide
0.6 g methylenebisacrylamide
Water to 100 ml

Temed solution used neat

Ammonium persulphate solution 15 mg/ml (prepared fresh)

Gel solutions (approx. 12 gels) are prepared by mixing
10.1 ml of acrylamide solution
3.4 ml of distilled water
15.0 ml of gel buffer
1.5 ml of ammonium persulphate solution
0.045 ml of Temed.

Reservoir buffer : 1 part gel buffer + 1 part water

Protein sample buffer: 1 part gel buffer + 1 part water +
0.01 ml mercaptoethanol per 10 ml

Stain solution: 1.25 g Coomassie Brilliant Blue
227 ml methanol
46 ml glacial acetic acid
227 ml distilled water

Destain solution : 50 ml methanol
75 ml glacial acetic acid
875 ml distilled water

Tracking dye: 5 mg bromophenol blue
5 ml water
5 ml sample buffer

APPENDIX III

Reagents and Solutions for Disc Acrylamide Gel Electrophoresis

A. 24.0 ml 1.0 M-HCl
18.2 g Tris
0.23 ml Temed (neat)
Water to 100 ml (pH 8.9)

B. 0.8 g bisacrylamide
30.0 g acrylamide
Water to 100 ml

C. 0.15 g amonium persulphate
Water to 100 ml (prepared fresh)

Gel solutions (approx. 12 gels) are prepared by mixing
7.5 ml of A
7.5 ml of B
15.0 ml of C

Reservoir buffer 50X concentrated : 30.0 g Tris
144.0 g glycine
Water to 1000 ml

Use 20 ml/litre final buffer (pH 8.3)

Stain solution : 1.25 g Coomassie Brilliant Blue
554 ml distilled water
46 ml glacial acetic acid

Destain solution: 554 ml distilled water
46 ml glacial acetic acid

Tracking dye: 5 mg bromophenol blue
10 ml water

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PUBLISHED WORK

EVIDENCE FOR ACTIVE SUBUNITS OF MATRIX-BOUND CREATINE KINASE

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1. Introduction

Creatine kinase (EC 2.7.3.2.) which catalyses the transfer of a phosphoryl group between ATP and creatine consists of two very similar, if not identical, subunits of mol wt. 41 000, each possessing a highly reactive thiol group [1]. The dimeric enzyme is readily dissociated in denaturing media such as 6 M guanidinium hydrochloride (Gdn HCl) or 8 M urea [2]. In considering the role of the subunits of the enzyme it is of value to determine whether or not individual subunits can show enzymatic activity. There have been two principal approaches to the study of the activity of subunits of oligomeric enzymes. The first approach involves an analysis of the kinetics of regain of activity during re-association of the dissociated enzyme [3]. The second approach involves preparation of matrix-bound subunits which are unable to re-associate upon removal of the denaturing agent. This latter method was used successfully by Chan to study isolated subunits of aldolase [4] and transaldolase [5] and was adopted here for the study of creatine kinase. In the present communication we outline a method for attaching rabbit muscle creatine kinase to Sepharose and present evidence to show that individual subunits of the matrix-bound enzyme are active.

2. Materials and methods

Creatine kinase was isolated from rabbit skeletal muscle as described by Milner-White and Watts [6]. Freshly prepared enzyme had a specific activity (assayed in the forward direction ATP + creatine → ADP + phosphocreatine) of 55–60 units/mg under the conditions described by McLaughlin et al. [7] and 130–140 units/mg under the conditions described by Milner-White and Watts [6]. The enzyme preparations were judged to be more than 90% homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis [8].

Sepharose 4B (Pharmacia) was activated according to the procedure of March et al. [9] but using 5 mg CNBr per ml of packed gel. After activation, the gel was washed successively with 0.1 M sodium bicarbonate at pH 9.0, distilled water, and finally the coupling buffer (10 mM sodium phosphate at pH 8.0 containing 1 mM EDTA). To 10 ml of activated gel was added 10 mg of enzyme in 8 ml of coupling buffer, and the mixture was stirred at 4°C for 18 h. Excess soluble protein was then removed by washing the gel alternately with coupling buffer containing 1 M NaCl and coupling buffer containing no NaCl, until no protein could be detected in the washings. A volume of 0.1 M sodium glycine at pH 8.0, equal to the volume of packed gel was then added to the matrix-bound derivative and the mixture was left to stand at 20°C for 2 h to allow the complete blocking of remaining activated groups on the Sepharose. After washing to remove excess glycine, the gel was suspended in coupling buffer. Dissociation of the matrix-bound enzyme by Gdn HCl and subsequent re-association with added subunits of soluble enzyme were performed essentially according to the procedures described by Chan [4].

The concentration of soluble enzyme was determined either spectrophotometrically at 280 nm using the published value for the extinction coefficient [6], or by the method of Lowry et al. [10] using bovine serum albumin as a standard. The two procedures gave identical results. Protein concentrations of matrix-bound derivatives were determined using a slightly modified version of the Lowry method used by Havekes

et al. [11]. The mixture of matrix-bound enzyme and alkaline copper tartrate reagent was stirred for 10 min prior to addition of the Folin-Ciocalteau reagent. After the addition and a further 30 min stirring, the mixture was filtered before the absorbance was determined. With this procedure, protein determinations were highly reproducible and the absorbance was linear with protein content at least up to 45 μg of protein.

Creatine kinase activity was assayed using the coupled assay system described by McLaughlin et al. [7]. The activity of matrix-bound derivatives was determined by addition of a small aliquot (10–100 μl) of suitably diluted suspension to the assay mixture, which was maintained at 25°C and continuously stirred using an apparatus described by Mort et al. [12]. At suitable (2 min) intervals the cuvette containing the mixture was placed in a spectrophotometer to record the absorbance at 340 nm for a few seconds. Under these conditions the observed activity was proportional to the amount of enzyme added to the assay mixture (0.2–0.8 μg enzyme). The contribution of any remaining soluble enzyme to the observed activity was checked by filtration of the mixture and found to be less than 2% in all cases. Pipetting of gel suspensions was found to be most accurately and conveniently carried out using a variable automatic pipette with the plastic tips cut so as to increase the size of the aperture.

3. Results and discussion

As shown in table 1 creatine kinase could be linked to Sepharose 4B with retention of approx. 50% of the specific activity of the soluble enzyme. This figure is comparable with the results of other studies on matrix-bound enzymes [4,5,13,14] and could possibly arise either from distortion of the enzyme or a change in the microenvironment of the catalytic site upon immobilisation. Further investigation of the kinetic and binding properties of the matrix-bound enzyme may help to clarify this point.

The preparation of matrix-bound subunits of creatine kinase involves treatment of the matrix-bound enzyme with a denaturing agent, followed by washing to remove dissociated subunits. The ability of soluble creatine kinase to renature upon removal of denaturing agent was checked under conditions similar to those used in the study of matrix-bound derivatives. A sample of enzyme which had been incubated at a concentration of 0.6 mg/ml in denaturing buffer (0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol and 6 M Gdn HCl) for 30 min at 20°C was diluted 10-fold into renaturing buffer (0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol). At known times, aliquots were then removed for assay. As shown in fig.1 about 80–85% of the original activity of the enzyme before treatment with Gdn HCl could be recovered after 1 h. These results were comparable with those previously obtained [2] under slightly different conditions.

Extensive washing of matrix-bound enzyme (derivative A) with the above denaturing buffer yielded a derivative which had very nearly half the protein content of derivative A (table 1). A control experiment showed that treatment with the same buffer without Gdn HCl had no effect on the protein content or activity of derivative A. These results show that the enzyme is bound to the matrix almost exclusively

Table 1
Activity and protein content of matrix-bound creatine kinase derivatives

Matrix-bound Derivative	Protein content μg/ml	%	Activity U/ml	%	Specific activity U/mg	%
Derivative A	400	100	11.2	100	28	100
Derivative B	210	52.5	5.4	48	25.7	92
Derivative C	380	95	10.4	93	27.4	98

Derivative A was prepared by coupling creatine kinase to Sepharose 4B. Derivative B was prepared by washing Derivative A with denaturing buffer (containing guanidinium hydrochloride). Derivative C was prepared by adding dissociated soluble enzyme to Derivative B. The accuracy of protein content and activity determinations was ± 5%. Soluble creatine kinase had a specific activity of 55–60 U/mg.

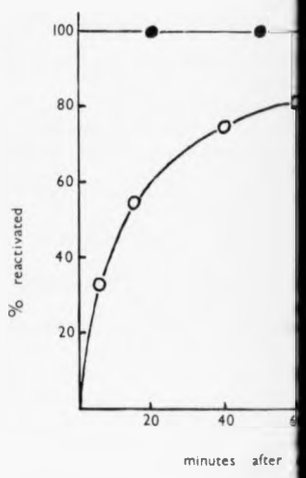

Fig.1. Reactivation of soluble creatine k
guanidine-HCl, in 0.1 M Tris-HCl at pH
12.5 mM dithiothreitol. At zero time th
solution was diluted 10-fold into the alt
guanidine-HCl and assayed at the stated
control experiment in which the enzym
0.1 M Tris-HCl at pH 7.5 containing 12
showed no loss of activity (•).

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the enzyme are active.

Stronger evidence for the prese
subunits in derivative B was provid
to re-associate with added 'subunit
creatine kinase (see scheme in fig.2
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An excess (1 mg) of the dissociated

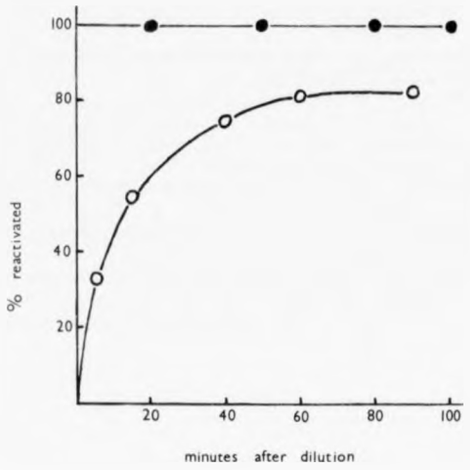

Fig.1. Reactivation of soluble creatine kinase from 6 M guanidine-HCl, in 0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol. At zero time the denatured enzyme solution was diluted 10-fold into the above buffer without guanidine-HCl and assayed at the stated times (○). A parallel control experiment in which the enzyme was incubated in 0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol showed no loss of activity (●).

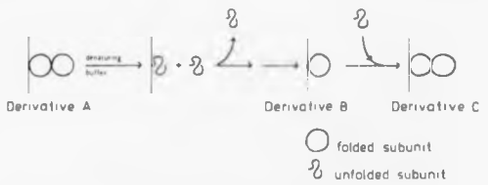

Fig.2. Scheme demonstrating the relationship between the derivatives of matrix-bound creatine kinase. Derivative A represents matrix-bound enzyme. Successive treatment of Derivative A with denaturing and renaturing buffer yielded Derivative B. Derivative C was derived from Derivative B by re-association with 'added' subunits of soluble enzyme.

via one rather than via both subunits (since in the latter case there would be no protein loss from derivative A after treatment with Gdn HCl). Following the washing of derivative A with denaturing buffer, the gel was suspended in the renaturing buffer, stirred for 1 h at 20°C, washed with coupling buffer and finally suspended in coupling buffer. The resulting product (derivative B) was found to have a specific activity of 92% compared with derivative A (Table). These data would suggest that matrix-bound subunits of the enzyme are active.

Stronger evidence for the presence of matrix-bound subunits in derivative B was provided by its ability to re-associate with added 'subunits' of soluble creatine kinase (see scheme in fig.2). In this procedure small aliquots of a solution (2 mg/ml) of soluble enzyme (which had been incubated in the denaturing buffer for 1 h at 20°C) were added to a well stirred suspension of derivative B in renaturing buffer (1:4). An excess (1 mg) of the dissociated soluble enzyme was added in this way over a period of 90 min. After the addition, the mixture was stirred for a further 2 h at room temperature to allow renaturation to occur. The product was then washed and re-suspended in coupling buffer, to yield derivative C (table 1). The results of the experiment show that the protein content and activity are restored to very nearly the original values of derivative A. Control experiments showed that neither derivative A nor washed non-activated Sepharose 4B could retain added subunits under the same conditions.

Taken together, the results shown in table 1 indicate that matrix-bound subunits of creatine kinase can be prepared and that these subunits possess a specific activity very similar to that of matrix-bound enzyme. The observation of active subunits of creatine kinase is of particular interest since the related enzyme arginine kinase occurs in a monomeric form in a number of species, particularly lobster [15]. Previous work has indicated that lobster arginine kinase bears a notable similarity to the creatine kinase subunit in terms of molecular weight and amino acid sequence around the rapidly reacting thiol group [15]. We plan to compare the properties of matrix-bound creatine kinase in the dimeric form and in the subunit form to assess the importance of the dimeric structure of the soluble enzyme.

Acknowledgement

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The Influence of Acetate on Matrix-Bound Creatine Kinase

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In considering the function of subunits in proteins, a comparison of the properties of the active subunits with those of the oligomeric protein should lead to a greater understanding of subunit interactions in proteins. This present communication describes the preparation of active subunits of matrix-bound creatine kinase and discusses the influence of the activator acetate on the activity of these subunits.

Creatine kinase (EC 2.7.3.2) was isolated from rabbit skeletal muscle as described by Milner-White & Watts (1971) and covalently bound to Sepharose 4B. The Sepharose was activated by the procedure of March *et al.* (1974), but by using 5 mg of CNBr/ml of packed gel. After activation the gel was washed successively with 0.1M-$NaHCO_3$, pH 9.0, water and finally the coupling buffer (10mM-sodium phosphate, pH 8.0, containing 1mM-EDTA). To 10ml of activated gel was added 10 mg of enzyme in 8ml of coupling buffer, and the mixture stirred for 18h at 4°C. Excess of soluble protein was then removed by washing the gel alternately with coupling buffer containing 1M-NaCl and coupling buffer containing no NaCl, until no protein could be detected in the washings. The gel was then treated with glycine/NaOH solution to block remaining activated groups on the Sepharose. After washing to remove excess of glycine, the gel was suspended in coupling buffer.

Extensive washing of matrix-bound enzyme (derivative *A*) with denaturing buffer (0.1M-Tris/HCl, pH 7.5, containing 12.5mM-dithiothreitol and 6M-guanidinium chloride) followed by resuspension and stirring, for 3h, in renaturing buffer (0.1M-Tris/HCl, pH 7.5, containing 12.5mM-dithiothreitol) yielded derivative *B*, which has been shown (Bickerstaff & Price, 1976) to be a derivative consisting of matrix-bound subunits. In the preparation of the matrix-bound renatured enzyme (derivative *C*), small samples of a solution (2mg/ml) of soluble enzyme (which have been incubated in denaturing buffer for 1h at 20°C) were added to a well-stirred suspension of derivative *B* in renaturing buffer. After the addition of 1mg excess, the mixture was stirred at 20°C for a further 2h to allow renaturation to occur. The product was then washed and resuspended in coupling buffer to yield derivative *C*.

Protein concentrations of matrix-bound derivatives were determined by using a slightly modified version of the Lowry method used by Havekes *et al.* (1974), which is described in Bickerstaff & Price (1976). Creatine kinase activity was assayed by using a coupled assay system as described by McLaughlin *et al.* (1972). The activity of matrix-bound derivatives was determined by addition of a small sample of suitably dilute suspension to the assay mixture, which was maintained at 30°C and continuously stirred by using an apparatus described by Mort *et al.* (1973). At suitable intervals the cuvette was placed in a spectrophotometer to record E_{340} for a few seconds. The assay mixture contained glycine buffer, pH 9.0 (100mM), creatine (40mM), phosphoenolpyruvate (1mM), ATP (4mM), NADH (133μM), $MgSO_4$ (6mM) and 24 units each of pyruvate kinase and lactate dehydrogenase in a final volume of 3ml. When considering the effect of acetate the $MgSO_4$ was omitted and replaced by magnesium acetate (5mM) and also sodium acetate (100mM).

Table 1 shows the results obtained in the presence and absence of acetate in the assay system. It shows that the specific activity of the matrix-bound subunit (derivative *B*) has not increased appreciably, whereas both the matrix-bound enzyme (derivative *A*) and the matrix-bound renatured enzyme (derivative *C*) have increased considerably. These results may indicate that acetate exerts its activating effect via a subunit interaction, since if the acetate activated each subunit equally then one might expect that the percentage increase in specific activity for the matrix-bound subunit would be one-half that for the matrix-bound dimer, i.e. 50%. Also if the acetate were selective and only activating one subunit, then assuming an even distribution of preferred and non-preferred matrix-bound subunits one would still expect a 25% increase in specific activity. Therefore acetate seems not to produce a direct influence on the catalytic site of each subunit, but to participate in a conformational change in the dimeric structure, which facilitates increased activity. The absence of a dimeric structure in the matrix-bound subunit

Table 1. *Protein content and specific activities of matrix-bound derivatives assayed in the absence and in the presence of acetate*

The matrix-bound derivatives were prepared as described in the text. The specific activity of the soluble enzyme assayed in the presence of acetate was 135 units/mg and in the absence of acetate was 115 units/mg. All assays were performed at 30 °C.

Matrix-bound derivatives	Protein content		Specific activity (units/mg)		Increase in specific activity (%)
	(μg/ml)	(%)	Acetate absent	Acetate present	
Derivative *A*	400	100	50	98	96
Derivative *B*	200	50	47.5	50	5.3
Derivative *C*	372	93	48	80	67

derivative would result in no activation. These results would therefore seem to indicate the importance of the dimeric structure in the activation of the enzyme by acetate.

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Reversible Denaturation of Rabbit Muscle Creatine Kinase

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It was previously demonstrated (Bickerstaff & Price, 1976) that matrix-bound subunits of rabbit muscle creatine kinase can reassociate with added subunits of soluble enzyme to re-form a matrix-bound dimer. Although it was demonstrated that renaturation of the soluble enzyme was possible, it seemed desirable to use more sensitive tests to show whether the product of renaturation is identical with the native enzyme or is a new species derived from incorrectly folded or reassociated subunits.

Creatine kinase was isolated from rabbit skeletal muscle as described by Milner-White & Watts (1971) and the enzyme preparations were judged to be at least 95% homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Enzyme activity was assayed in the forward direction (phosphocreatine synthesis) by using a coupled-assay system involving pyruvate kinase and lactate dehydrogenase as coupling enzymes (Bickerstaff & Price, 1976). All assays were performed at 30°C in 0.1M-glycine/NaOH at pH 9.0. Denaturation was achieved by incubating the native enzyme (final concn. 5mg/ml) with denaturing buffer consisting of 0.1M-Tris/HCl, 12.5mM-dithiothreitol and 6M-guanidinium chloride at pH 7.5 for 2h at 25°C. Renaturation was accomplished by diluting a sample of the denatured enzyme 1:30 into renaturing buffer containing 0.1M-Tris/HCl and 12.5mM-dithiothreitol, pH 7.5. To determine the renaturation profile, renaturation was initiated, then successive portions were removed at set times for assay of enzyme activity at that time. Samples removed from the renaturing medium for assay were first diluted 1:20 into an intermediate buffer consisting of 10mM-sodium phosphate, pH 8.0, containing 1.0mM-EDTA, from which a subsequent portion was removed for assaying enzyme activity. Control experiments received the same treatment and dilutions, but with the exception that the guanidinium chloride was omitted.

Kinetic parameters for ATP and creatine were obtained by using the assay procedure described above. The studies were made by monitoring the effects on the initial velocity of the enzyme reaction of varying in turn the concentration of one substrate in the presence of several fixed concentrations of the other substrate. The concentration of ATP was varied from 0.5 to 4mM, and the concentration of creatine was varied from 5 to 40mM. When the ATP concentration was varied, the magnesium acetate concentration was also varied to maintain the free Mg^{2+} concentration at 1.0mM (Watts, 1973). The kinetic constants were determined from secondary re-plots of the vertical intercepts and the slopes of the primary double-reciprocal plots as indicated in the graphical methods of Florini & Vestling (1957).

C.d. (circular dichroism) studies in the 210–260nm spectral range were obtained by using a Cary model 60 spectropolarimeter with a model 6003 c.d. attachment. The cell compartment was maintained at 27°C and the instrument was calibrated with D-10-camphorsulphonic acid. The slit width was programmed to yield constant energy over the

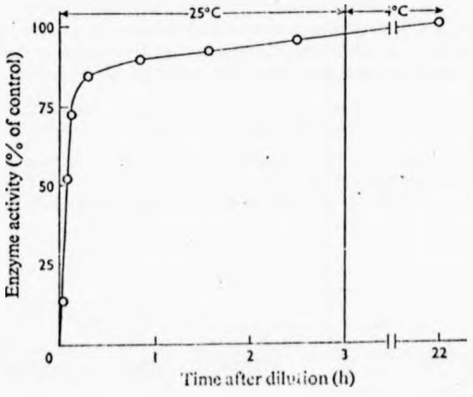

Fig. 1. *Renaturation profile of rabbit muscle creatine kinase*

The concentration of enzyme was 0.167mg/ml in 0.1M-Tris/HCl buffer containing 12.5mM-dithiothreitol at pH 7.5. The temperature of renaturation was 25°C for the first 3h, followed by 19h at 4°C. Samples were removed at the times indicated and assayed for enzyme activity as described in the text.

Table 1. *Characterization of rabbit muscle creatine kinase in its native and fully renatured state*

The kinetic parameters were determined at 30°C and at pH 9.0. All other conditions are as described in the text.

State	K_m (mM) ATP	K_m (mM) Creatine	$V_{max.}$ (μmol/min per mg)	α-Helix content (%)	Homogeneity (%)
Native	0.40	10	150	31	≥95
Renatured	0.41	9.8	150	30	≥95

range used, which was 0–0.4° when using a 1.0mm-path-length cell and a scan speed of 5nm/min. The c.d. spectra for both native and fully renatured enzyme were determined in the renaturation buffer at a protein concentration for both of 0.167mg/ml. The overall shape of the c.d. spectra of both native and fully renatured enzyme was similar over the whole range studied and the features of the c.d. spectra suggested that both had appreciable α-helical content. From the respective c.d. spectra the mean residue ellipticity at 224.5nm ($[\theta]_{224.5}$) was calculated for both the native and the fully renatured enzyme. By using the $[\theta]_{224.5}$ reference value for α-helix of Chen *et al.* (1974) the corresponding α-helix content was calculated for the native and fully renatured enzyme.

A test was made on the homogeneity of the native and the fully renatured enzyme by performing polyacrylamide-gel electrophoresis at pH 7.5, essentially as described by Davis (1964), and by thin-layer gel filtration with Sephadex G-200 in 10mM-sodium phosphate buffer, pH 8.0, containing 1.0mM-EDTA.

The renaturation profile is shown in Fig. 1, and the regain of enzymic activity is taken as an indication of the extent of renaturation. The graph shows a rapid regain of enzyme activity to 95% after 3h, followed by a slower regain of the remaining (5%) enzyme activity over a period of 19h at 4°C. The fully renatured enzyme was obtained as a routine after a 3h period of renaturation at 25°C, followed by an overnight period of renaturation at 4°C. This result clearly demonstrates that denatured enzyme can be completely renatured readily in solution. To establish whether or not the structure of the fully renatured enzyme is altered in any way from that of the native enzyme, several properties were examined. The results of these studies are summarized in Table 1. The kinetic parameters obtained for the fully renatured enzyme are essentially the same as those obtained for the native enzyme. In addition the results obtained here for the native enzyme agree well with those obtained by Milner-White & Watts (1971). The results of the c.d. studies also demonstrate that the fully renatured enzyme has, within experimental error, the same α-helix content as the native enzyme, which is also in agreement with the values reported by Watts (1973). Polyacrylamide-gel-electrophoretic and thin-layer-gel-filtration studies showed that both the native and fully renatured enzyme migrated similar distances as a single species. In particular, no high-molecular-weight aggregated material was present in the fully renatured enzyme.

Taken together, these results suggest that denaturation is completely reversible and that the product of renaturation, the fully renatured enzyme, is essentially identical with the native enzyme in terms of active-site structure, molecular weight and overall conformation. It can therefore be argued that studies with matrix-bound creatine kinase involving denaturation and renaturation of matrix-bound subunits and reassociation of matrix-bound subunits with added subunits of soluble enzyme should be free from complications arising from incorrect refolding and reassociation of the enzyme in solution.

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REVIEW

CREATINE KINASE: A REVIEW OF SOME RECENT WORK ON THE MECHANISM AND SUBUNIT BEHAVIOUR OF THE ENZYME

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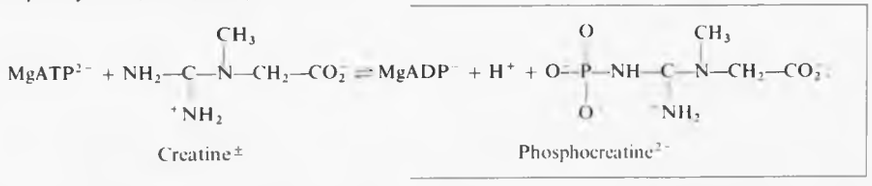
INTRODUCTION

The aim of this article is to describe some recent work on the enzyme creatine kinase (EC2.7.3.2) which has led to a greater understanding of the mechanism of action and subunit behaviour of the enzyme. Work on all aspects of the enzyme up to 1972 has been reviewed (Watts, 1973) and the reader is referred to that review for further background information. The primary function of the enzyme, which catalyses the reaction shown below, seems to be associated with regeneration of ATP in conjunction with contractile or transport systems (Watts, 1973).



$$MgATP^{2-} + \text{Creatine}^{\pm} \rightleftharpoons MgADP^{-} + H^{+} + \text{Phosphocreatine}^{2-}$$

There has recently been considerable interest in clinical assays of creatine kinase activity (Szasz *et al.*, 1976), as it has been proposed as a measure of myocardial infarct size (Sobel, 1976; Sobel *et al.*, 1977) and as an indication of muscular dystrophy and other disorders (Sherwin *et al.*, 1969; Roy, 1974). Other recent articles have dealt with various physiological aspects of the enzyme, such as its rôle in muscle (Berson, 1976; Seraydarian & Abbott, 1976; Mani & Kay, 1976) and adipose tissue (Berlet *et al.*, 1976), and changes in isoenzyme patterns during development (Morris *et al.*, 1976). The work reviewed in this article will however refer to the enzyme isolated from rabbit skeletal muscle, as it is this form which has been the most intensively studied.

STRUCTURAL FEATURES OF THE ENZYME

Creatine kinase consists of two very similar, if not identical, subunits of mol. wt 41,000 daltons (Watts, 1973). Dissociation of the dimer can be brought about by agents such as urea, guanidinium chloride and sodium dodecylsulphate (Yue *et al.*, 1967). Only very limited sequence information is available, accounting for less than 15% of the total amino acid content of the molecule (Watts, 1973; Roy, 1974). Particular attention has been focussed on the sequence of a peptide containing a reactive thiol group, and there appears to be considerable sequence homology between this peptide and the corresponding peptide from other isoenzyme forms of creatine kinase (Watts, 1973) and from arginine kinase isolated from lobster (Morrison, 1973).

Some preliminary X-ray diffraction measurements on the enzyme have been made (McPherson, 1973). Of the three crystal forms studied, two appeared to possess an asymmetric unit consisting of two molecules (i.e. 160,000 daltons), whereas in the third (orthorhombic) form, the asymmetric unit consisted of a single protein subunit. From the unit cell dimensions, it was calculated that the thickness of a creatine kinase subunit was of the order of 5 nm, and considerations of the space group suggested that the enzyme molecule possesses a two-fold axis relating two identical subunits. To date, no further X-ray diffraction results have been reported.

MECHANISM OF ACTION

Arrangement of substrates at the catalytic site

The reaction catalysed is a transfer of a phosphoryl group from $MgATP^{2-}$ to creatine. There is no evidence for any phosphorylated enzyme intermediate. Detailed kinetic studies of the reaction, including the use of product inhibition have shown that the mechanism is of the rapid-equilibrium, random order type with synergism in substrate binding, i.e. the binding of metal–nucleotide to the enzyme facilitates the subsequent binding of creatine and vice versa (Morrison & James, 1965).

An important finding was that certain small, planar, anions (notably nitrate and formate) led to a remarkable decrease in the reactivity of a thiol group when added to a mixture of the enzyme with

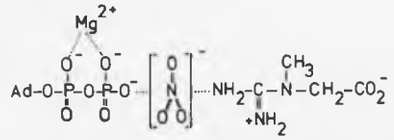


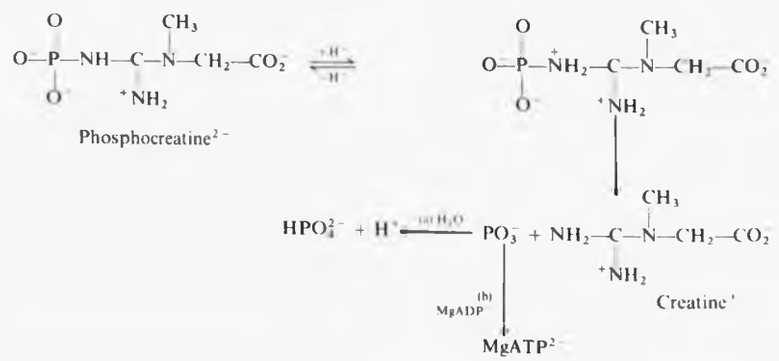
Fig. 1. The "transition state analogue" complex for the reaction catalysed by creatine kinase (adapted from Milner-White & Watts, 1971). Various other anions can replace nitrate in this complex, in which the thiol groups have only a very low reactivity towards iodoacetamide. It is postulated that in the transition state of the enzyme catalysed reaction, the planar metaphosphate ion (PO_3^-) would replace nitrate.

$MgADP^-$ and creatine. (Milner-White & Watts, 1971). On the basis of this work and other kinetic and magnetic resonance studies (Reed & Cohn, 1972; James & Cohn, 1974; McLaughlin *et al.*, 1976), it has been proposed that the anions can occupy the site normally occupied by the γ-phosphoryl group of $MgATP^{2-}$, so that a complex is formed which resembles the "transition state" for the enzyme catalysed transphosphorylation reaction (Fig. 1).

In a recent paper, Milner-White & Kelly have used the reactivity of this thiol group on each subunit towards iodoacetamide as a measure of the conformational state of the "working" enzyme, i.e. while it is catalysing the reaction between $MgATP^{2-}$ and creatine (Milner-White & Kelly, 1976). From their results, it appears that about 80% of the enzyme molecules are complexed in the $MgADP^- - PO_3^- -$creatine form (in which the thiol groups are assumed to be virtually unreactive by analogy with their behaviour in the "transition state analogue" complex), with the remainder present as a Michaelis complex (in which the thiol groups are fully reactive). This finding lends support to the idea that the "transition state analogue" complex is relevant to an important state in the enzyme catalysed reaction.

Structural studies of the arrangement of substrates at the active site of the enzyme have relied heavily on magnetic resonance methods, as X-ray diffraction work is still at a preliminary stage. For the magnetic resonance mapping experiments it is necessary to introduce paramagnetic centres into the enzyme molecule and this can be done either by using Mn^{2+} as the divalent metal ion [the V_{max} is approx 80% of that of the Mg^{2+} activated reaction in the direction of phosphocreatine synthesis (Watts, 1973)] or by attaching a stable, paramagnetic, "spin label" moiety to the reactive thiol group on each subunit (McLaughlin *et al.*, 1976). The conclusion from the magnetic resonance work is that the nucleotide and guanidino substrates are so aligned on the enzyme that the transferable phosphoryl group on one substrate is in apposition to the acceptor moiety on the second substrate. The divalent metal ion is probably liganded to the α- and β-phosphates of the nucleotide substrate and not directly liganded to the guanidino substrate. In addition, the metal ion—formate distance (0.5 nm) in the enzyme–MnADP$^-$–formate–creatine "transition state analogue" complex is fully consistent with the suggestion that the monovalent ion binds at the site normally occupied by the transferable phosphoryl group.

The actual mechanism of phosphoryl transfer in the catalytically active complex is still unresolved. Transfer could proceed via an "S_n2-type" mechanism in which the guanidino nitrogen would attack the phosphorus of the γ-phosphoryl group of ATP, simultaneously weakening the P–O bond. This would involve the γ phosphorus assuming a pentacoordinate trigonal bipyramid geometry in the transition state of the reaction; a mechanism which bears some analogies to the proposed mechanism of action of ribonuclease (Milner-White & Watts, 1971). The alternative "S_n1-type" mechanism of transfer would involve the participation of the planar, highly reactive metaphosphate ion (PO_3^-). The effect of nitrate and other planar anions in forming a "transition state analogue" complex would be consistent with either type of transfer mechanism. From detailed studies of the mechanism of hydrolysis of phosphocreatine, it has been concluded that the reaction does involve production of metaphosphate which then reacts with water to produce phosphate (Allen & Haake, 1973, 1976). This is depicted in Scheme 1, which also shows the proposed alternative fate of metaphosphate in the presence of $MgADP^-$ and creatine kinase (i.e. to yield $MgATP^{2-}$).



Scheme 1. Proposed mechanism for hydrolysis of phosphocreatine (a) and its relevance to the enzyme catalysed transphosphorylation reaction (b) (Allen & Haake, 1976)

This mechanism has several important implications for the enzyme catalysed transphosphorylation reaction, among which we might mention the following: (i) the phosphocreatine must be protonated before it can break down to yield PO_3^-. Presumably an amino acid side chain which can function as an acid catalyst must be close to the phosphocreatine site on the enzyme. (ii) The metaphosphate produced must be in very close association with the MgADP on the enzyme so that reaction occurs to form only $MgATP^{2-}$ and not phosphate (by attack of water on metaphosphate). The active site of the enzyme must be such as to exclude water, since no enzyme catalysed breakdown of phosphocreatine occurs in the absence of MgADP. It is worth noting in this connection that a number of studies using fluorescent probes which are thought to bind at the nucleotide site on the enzyme indicate that the active site provides a relatively hydrophobic environment (Roustan *et al.*, 1973; Somerville & Quiocho, 1977). (iii) Since water is to be excluded from the active site, it is proposed that there must be specific binding sites for the phosphate dianion of phosphocreatine (possibly H-bonded to a lysine or arginine group), for the guanidinium cation (possibly H-bonded to a carboxylate group) and for the carboxylate anion (possibly H-bonded to an arginine group). As yet none of these binding sites has been identified with certainty.

A good deal of effort has been expended in attempts to identify particular groups on the enzyme which are involved in the catalytic process. Most of this work has involved the technique of chemical modification of amino acid side chains and will be dealt with below.

Role of various groups on the enzyme

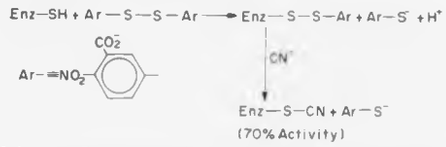
(a) *Thiol groups.* It has long been known that creatine kinase possesses one reactive thiol group per subunit, the modification of which by a variety of reagents (iodoacetamide, iodoacetate, 1-fluoro-2,4-dinitrobenzene etc.) leads to complete, or very nearly complete, inactivation of the enzyme (Watts, 1973). The importance of this thiol group in the mechanism of action of the enzyme has been the subject of considerable debate; one suggestion was that the thiol group withdrew a proton from the creatine guanidino group, facilitating nucleophilic attack by the nitrogen on the γ-phosphoryl group of $MgATP^{2-}$ (Watts, 1973).

Two groups of workers have recently shown that this thiol group cannot be directly involved in the catalytic mechanism, and is therefore to be regarded as a "non-essential" group. Enzyme derivatives, in which only small perturbations are made to the thiol group, retain a significant amount of activity. Kenyon and coworkers have described the conversion of —SH to —S—S—CH_3 (Scheme 2) to yield a derivative which retains 20% of the activity of unmodified enzyme (Smith & Kenyon, 1974; Smith *et al.*, 1975).

$$\text{Enz—SH} + CH_3\text{—S—}SO_3^-\text{—}CH_3 \rightarrow \underset{(20\%\ \text{Activity})}{\text{Enz—S—S—}CH_3} + CH_3SO_3^- + H^+$$

Scheme 2. Modification of the reactive thiol group of creatine kinase by reaction with methanethiolsulphonate to yield an active derivative.

More recently, it has been reported that the S-*cyano* derivative of creatine kinase retains about 70% of the activity of unmodified enzyme (del Terrossian & Kassab, 1976). The preparation of this derivative was achieved via a mixed disulphide as illustrated in Scheme 3.



Scheme 3. Preparation of the S-*cyano* derivative of creatine kinase.

Although these elegant studies clearly indicate that the thiol group can no longer be regarded as essential for the catalytic activity of the enzyme, its exact role remains something of an enigma. Binding studies have shown that the inactive derivative in which the thiol group has been reacted with iodoacetamide is capable of binding metal nucleotide substrate although no evidence could be found for the formation of a ternary complex on addition of creatine (O'Sullivan & Cohn, 1968) or for the formation of a "transition state analogue" complex on addition of creatine and nitrate (McLaughlin, 1974) to the enzyme metal nucleotide complex. The integrity of the thiol group may be viewed as important in the conformational transitions which the enzyme undoubtedly undergoes on formation of the catalytically active complexes (Maggio *et al.*, 1977). Some recent detailed investigations of the kinetic and binding properties of the CH_3—S-blocked enzyme derivative have shown how subtle some of the perturbations caused by chemical modification of amino acid side chains can be (Markham *et al.*, 1977; Maggio *et al.*, 1977). It appears for instance, that the derivative shows negative cooperativity in binding of metal nucleotide in contrast to the native enzyme which shows no such interactions, and that there is a loss of synergism in substrate binding.

These studies on the thiol group of creatine kinase serve to emphasise that considerable caution needs to be exercised when interpreting the results of chemical modification studies, since different effects can be observed depending on properties such as the size, charge, H-bonding ability etc., of the perturbing group introduced into the enzyme.

(b) *Lysine groups.* Previous work had indicated that a lysine group was present at or near the active site of the enzyme, on the basis of modification by reaction with dansyl chloride or *p*-nitrophenyl acetate (Watts, 1973). The role of this lysine group has been put on a firmer basis by some recent detailed magnetic resonance investigations (James & Cohn, 1974). From the results of NMR double-resonance studies, it was concluded that the ϵ-CH_2 group of the lysine is in close proximity to the proton of the formate anion in the "transition state analogue" complex (enzyme·MgADP·-formate·creatine) and hence by implication to the transferred phosphoryl group in the catalytically active complex.

(c) *Arginine groups.* Work with the arginine-specific reagents butanedione and phenylglyoxal has shown

that one arginine group per enzyme subunit can be modified with complete loss of activity (Borders & Riordan, 1975). The modified enzyme is incapable of binding nucleotides and inclusion of $MgATP^{2-}$ or $MgADP^{-}$ afforded protection against inactivation. On the basis of these results it was proposed that an arginine group is involved in the binding of nucleotides, presumably by an electrostatic interaction with the negatively charged oligophosphate moiety. This conclusion is supported by studies of the ADP binding site using NMR double-resonance techniques (James, 1976) which also indicate that an arginine group is in close proximity to the nucleotide substrate on the enzyme. It is reasonable to suppose that the arginine (and or lysine) groups referred to may fulfil certain of the binding roles suggested by the model studies on phosphocreatine breakdown mentioned earlier.

(d) *Other groups.* Chemical modification work has been performed on histidine (Pradel & Kassab, 1968) and tyrosine (Fattoum *et al.*, 1975) groups of the enzyme. Under appropriate conditions, modification leads to inactivation of the enzyme, but these experiments have not been interpreted in as much detail as those described earlier. It is possible that a histidine group could act as the acid (proton donating) group towards phosphocreatine postulated from the model studies of Allen & Haake (1976).

SUBUNIT BEHAVIOUR

Until recently, there has been little evidence for any subunit interactions in rabbit muscle creatine kinase, although some unusual features of the reaction of the enzyme from other sources with thiol-modifying reagents had been interpreted in terms of subunit interactions (Watts, 1973). However, using a fluorescent probe technique, McLaughlin (1974) reported that the binding of ADP to the rabbit muscle enzyme in the presence of creatine and nitrate (with or without Mg^{2+}) displayed features similar to negative cooperativity.

From the results of fluorescence energy transfer experiments (Haugland, 1975) and from our failure to observe cross-linking with dichloroketone reagents ($ClCH_2CO(CH_2)_nCOCH_2Cl$: $n = 3$, 4, 6 or 8) it can be concluded that the two active sites, or rather the reactive thiol groups near the active sites, are well separated on the dimeric molecule.

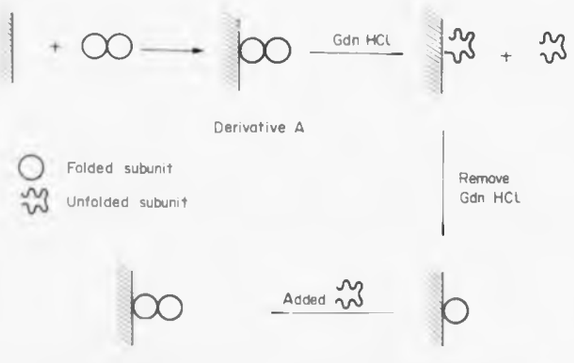
Our studies have been concerned with two particular aspects of subunit behaviour in the enzyme; namely (i) a study of the properties of isolated subunits of the enzyme, and a comparison with the properties of the dimeric form; and (ii) a study of the conditions under which the subunits act in a non-identical fashion. The aim of these studies is to understand the importance of the dimeric structure of the enzyme which may be reflected in various catalytic or regulatory aspects of the transphosphorylation process.

Study of isolated subunits

As previously mentioned, creatine kinase can be readily dissociated into subunits by agents such as urea or guanidinium chloride (Yue *et al.*, 1967). Removal of the denaturing agent by dilution or dialysis leads to reassociation of the subunits and regain of a substantial amount of activity (Dawson *et al.*, 1967). We have improved the conditions for renaturation of the denatured enzyme and have been able to obtain a product which is essentially identical with the native enzyme in terms of activity, kinetic parameters, circular dichroism spectra etc. (Bickerstaff & Price, 1977).

In order to study the properties of isolated subunits of the enzyme it is clearly necessary to separate the processes of subunit refolding and subunit reassociation when the denaturing agent is removed. We have used the matrix-binding method of Chan (1970) to achieve this. The principle of the method is illustrated in Scheme 4.

Enzyme is linked to a suitable matrix (such as CNBr-activated Sepharose) via one subunit only, to yield derivative A (Scheme 4). (Linkage via one subunit can be generally achieved by using low levels of CNBr activation, and can be tested for by performing appropriate protein determinations on the matrix-bound derivatives). Derivative A is then treated with denaturing agent and the non-matrix-bound subunit is washed away. On subsequent removal of the dena-



Scheme 4. Interconversions of matrix-bound derivatives of creatine kinase (Gdn HCl = guanidinium chloride).

Table 1. Protein content and activity of matrix-bound derivatives of creatine kinase

Derivative	Protein content (μg ml gel)	(%)	Specific activity (Units mg)
Derivative A	400	100.0	28.0
Derivative B	210	52.5	25.7
Derivative C	380	95.0	27.4

turing agent, the matrix-bound subunit can refold but is prevented from reassociation with other matrix-bound subunits by the rigidity of the matrix. This yields derivative B, with half the protein content of derivative A. Derivative B can reassociate with added subunits of the soluble enzyme to yield matrix-bound reassociated dimer (derivative C). If the enzyme were initially linked to the matrix via both subunits there would be no loss of protein on treatment with the denaturing agent.

The results obtained with creatine kinase showed that it was possible to prepare matrix-bound subunits of the enzyme, and that these subunits were indeed catalytically active (Bickerstaff & Price, 1976*a*). Typical results are shown in Table 1.

The specific activity of derivative A was about 50% of the value for soluble enzyme and further investigation showed that this decrease could be attributed to two factors. (i) The original assay conditions employed were not saturating with respect to $MgATP^{2-}$. The K_m for $MgATP^{2-}$ is raised approx 2.5-fold in derivative A compared with soluble enzyme. In subsequent assays more nearly saturating concentrations of $MgATP^{2-}$ have been employed. (ii) Acetate, which is included at concentrations of 0.1 M in enzyme assays to maintain the ionic strength (Milner-White & Watts, 1971) has a small (20%) activating effect on the soluble enzyme, but a much larger (70–90%) activating effect on matrix-bound derivatives A and C. By contrast the effect on matrix-bound subunit enzyme (derivative B) is small (5% activation) (Bickerstaff & Price, 1976).

When these factors were taken into account the V_{max} for the matrix-bound derivatives A and C in the presence of acetate (145 units/mg) is very similar to that of the soluble enzyme (150 units/mg) in the presence of acetate, whereas that of derivative B is somewhat lower (115 units/mg).

From these studies we can draw a number of conclusions (i) The general similarity of the V_{max} values for the various forms of the enzyme indicates that the general features of the catalytic site are not grossly disturbed when the enzyme is linked to the matrix. (ii) The changes in the K_m values for $MgATP^{2-}$ and for creatine (up to 2.5-fold) upon matrix-binding indicate that some small perturbation to the active site or its microenvironment does occur, although it is clear that the matrix-bound derivatives do still provide information relevant to the study of the soluble enzyme. (iii) The matrix-bound subunit form of the enzyme (derivative B) is catalytically active. This result is of considerable interest, as it shows that the quaternary structure of the enzyme is not a major controlling factor over the expression of catalytic activity. In this connection it should be noted that the enzyme arginine kinase which catalyses a closely related reaction in invertebrates occurs in a monomeric form in lobster (Morrison, 1973). Previous work has shown that lobster arginine kinase possesses notable similarities to the creatine kinase subunit, in terms of molecular weight and of sequence around the rapidly reacting thiol group (Morrison, 1973). (iv) The activator acetate would appear to operate via the dimeric stureture of the enzyme rather than via an effect on each subunit alone.

We are currently investigating various properties of the different matrix-bound forms of the enzyme, such as thiol group reactivity, conformational response to various ligands including the "transition state analogue" complex and the stability towards denaturing agents. The results of these studies should help in understanding the significance of the dimeric structure of the enzyme.

Non-identical behaviour of the subunits

Since the studies with matrix-bound derivatives of creatine kinase indicated that the dimeric structure of the enzyme was not required for catalytic activity, we have sought evidence that the two subunits might behave non-identically under certain conditions. Such behaviour might have possible regulatory significance.

An indication of non-identical behaviour came from a study of the kinetics of modification of the reactive thiol group on each subunit with a variety of reagents: iodoacetate, 7-chloro-4-nitrobenzofurazan and 5,5-dithiobis-(2-nitrobenzoic acid) (Price & Hunter, 1976). With each of these reagents the thiol groups on the two subunits reacted at the same rate as each other, both in the absence of ligands and in the presence of combinations of Mg^{2+}, ADP and creatine. However, in the presence of $MgADP^-$, creatine and nitrate (the "transition state analogue" complex) the reactions deviated markedly from normal second order kinetics, showing that the thiol groups are now no longer reacting at the same rate as each other. A similar effect was observed if Mg^{2+} was omitted from the components of the "transition state analogue" complex. Figure 2 shows the kinetic analyses for the reactions of iodoacetate with the enzyme in the absence of ligands and in the presence of the "transition state analogue" comples.

A study of ADP binding, using the equilibrium dialysis technique, also showed that the subunits of the enzyme behaved non-identically in the "transition state analogue" complex. In the absence of other ligands, or in the presence of Mg^{2+}, or Mg^{2+} and creatine, the binding of ADP shows a normal hyperbolic saturation curve with 1.8 binding sites per dimer. In the presence of Mg^{2+} and creatine and nitrate, the binding of ADP shows features characteristic of either negative cooperativity or non-identical sites (Price & Hunter, 1976). Figure 3 shows this binding data in the form of Scatchard plots.

The non-identical behaviour could arise either from an inherent asymmetry of the enzyme in the "transition state analogue" complex or from an asymmetry induced by dissociation of nucleotide from one subunit or by modification of the thiol group on one subunit. These possibilities might be distinguished if data on the symmetry of the enzyme in the "transition state analogue" complex were available, e.g. from

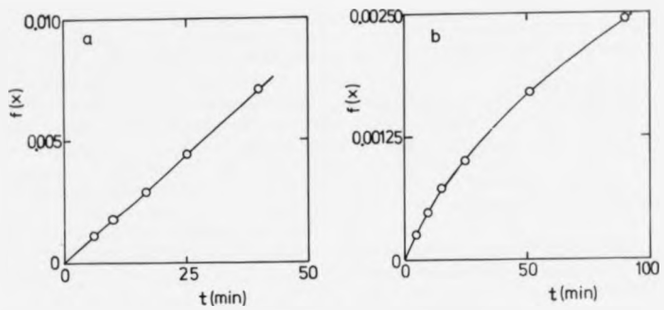


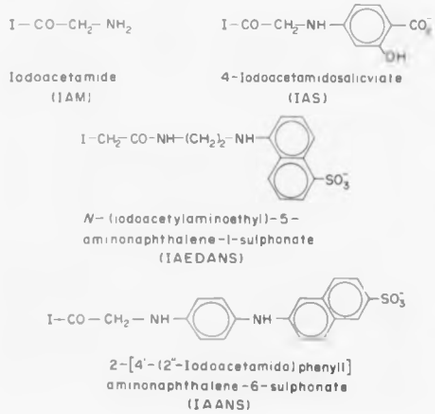
Fig. 2. Reactions of iodoacetate with creatine kinase in 50 mM sodium tricine buffer at pH 7.5 (T = 25 C) analysed as second order processes. (a) 275 μM iodoacetate reacted with 105 μM enzyme subunits; (b) 760 μM iodoacetate reacted with 120 μM enzyme subunits in the presence of 2 mM magnesium acetate, 1 mM ADP, 30 mM creatine and 10 mM sodium nitrate. The data shown cover 85% of the total reactions. On the ordinates, $f(x)$ is given by

$$f(x) = \left(\frac{1}{[A] - [B]}\right) \ln_e \left\{\frac{[B]([A] - [x])}{[A]([B] - [x])}\right\}$$

where [A] and [B] are the initial concentrations of iodoacetate and thiol groups reacting, respectively, and [x] is the concentration of carboxymethylated thiol groups formed at time t.

X-ray diffraction studies. The significance of the non-identical behaviour in this complex (which is thought to resemble the structure of the transition state of the enzyme catalysed reaction) is open to question. A rapid reaction study of the enzyme catalysed reaction has indicated that there is no large transient or lag phase in either direction, i.e. that the chemical reaction is rate limiting and that isomerisation of the enzyme must be rapid (Engelborghs *et al.*, 1975). This would argue against a "flip-flop" mechanism of the type described by Lazdunski (1972) for the dimeric alkaline phosphatase of *E. coli* (in which the events at one active site are linked to those at the other via conformational changes in the enzyme) being of importance in the case of creatine kinase.

Another indication of non-identical behaviour of the subunits of creatine kinase has come from a study of the reactions of the reactive thiol group on each subunit with iodoacetamide and various of its derivatives:



The kinetics of the modification reactions were studied at pH 8.0 and at 25 C and 0 C in the absence of added ligands. The pattern of the results is shown in Table 2 (Price, 1977).

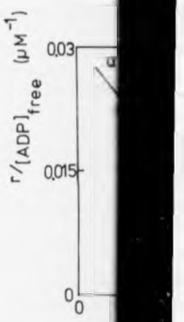
The results in Table 2 show that with the progressively larger reagents there is an increasing tendency towards biphasic reactions, i.e. incorporation of the bulky reagent on one subunit leads to a structural change transmitted to the second subunit, modifying the reactivity of its thiol group. The effect is particularly pronounced with the largest reagent studied (IAANS) and a preliminary estimate indicates that the difference in reactivity at 25 C in this case is of the order of 200-fold. Haugland (1975) has also shown from fluorescence studies that IAANS reacts with creatine kinase in a biphasic fashion.

The importance of this difference in thiol group reactivity is that "hybrid" enzyme molecules, in which the thiol group of only one subunit is reacted can be prepared by addition of one mole of IAANS per mole of enzyme dimer. We are currently studying the properties of these "hybrid" molecules as another means of assessing the importance of the dimeric structure for various catalytic and conformational properties of the enzyme.

Table 2. Characteristics of reactions of creatine kinase with various iodoacetamide derivatives

Reagent	Reaction characteristics	
	(25 C)	(0 C)
IAM	normal	normal
IAS	normal	biphasic
IAEDANS	normal	biphasic
IAANS	markedly biphasic	markedly biphasic

"Normal" refers to normal second-order kinetics being observed for at least 85% of the total reaction.



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SUM

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Acknowledgements—The
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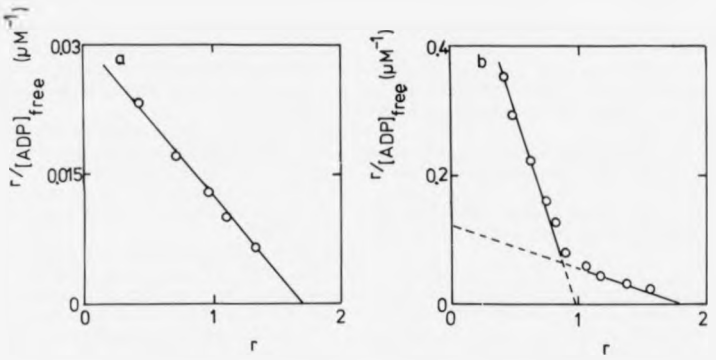

Fig. 3. Scatchard plots for the binding of ADP to creatine kinase in 50 mM sodium tricine buffer at pH 7.5 (T = 18 C). (a) Binding of ADP to enzyme (2 mg/ml): (b) binding of ADP to enzyme (0.4 mg/ml) in the presence of 5 mM magnesium acetate, 30 mM creatine and 10 mM sodium nitrate. On the axes, r represents the mole ADP bound per mole creatine kinase dimer.

The effect of temperature on the reactions of the enzyme with IAS and IAEDANS (Table 2) indicates that a difference exists in the strength and/or type of subunit interactions at the two temperatures. More detailed studies would be necessary to show whether creatine kinase exhibited a temperature transition of the type noted, e.g. for glycogen phosphorylase (Birkett *et al.*, 1971).

SUMMARY

This brief review has outlined some of the recent studies which have helped to show the organisation of the catalytic site of creatine kinase and the possible rôle of various amino acid groups in the mechanism of the transphosphorylation reaction. In addition, it has been possible to show that the dimeric structure of the enzyme is not essential for the expression of catalytic activity, and that under certain circumstances the subunits of the enzyme behave in a non-identical fashion.

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Properties of Matrix-Bound Dimer and Monomer Derivatives of Immobilized Creatine Kinase from Rabbit Skeletal Muscle

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Dimeric creatine kinase (EC 2.7.3.2) from rabbit skeletal muscle can be immobilized via a single subunit to CNBr-activated Sepharose 4B and subsequently treated with guanidine hydrochloride followed by renaturation to yield a catalytically active matrix-bound subunit derivative. The importance of the intact dimeric structure in the activation of the enzyme by acetate was demonstrated. Immobilization did not appear to alter the pH optimum of the enzyme, and the kinetic parameters for the matrix-bound derivatives were generally similar to those for the soluble enzyme, but the matrix-bound derivatives showed higher thermal stability and greater resistance to denaturation than did the soluble enzyme. The rates of reaction of thiol groups of the matrix-bound derivatives with iodoacetamide in the absence and in the presence of combinations of substrates were similar to those of the soluble enzyme. Studies with 5,5′-dithiobis-(2-nitrobenzoic acid) and with iodoacetamide revealed the presence of an additional reactive thiol group in the matrix-bound subunit derivative, which is presumably masked in the dimeric derivatives.

The technique of matrix immobilization was first used by Chan and co-workers to study the properties of isolated subunits of aldolase (Chan, 1970) and transaldolase (Chan *et al.*, 1973*a*). Subsequently, the technique has been applied to various enzymes and the results indicate that certain multisubunit enzymes, e.g. fructose bisphosphatase (Grazi *et al.*, 1973) and arginase (Carjaval *et al.*, 1977), do not require the intact oligomeric structure for the expression of catalytic activity. However, phosphoglucose isomerase (Bruch *et al.*, 1976) and lactate dehydrogenase (Chan & Mosbach, 1976) were essentially inactive in the subunit form, indicating that the oligomeric structure and the subunit interactions are essential for enzyme activity.

In previous papers we reported briefly on the preparation and some catalytic properties of matrix-bound derivatives of rabbit muscle creatine kinase (ATP–creatine phosphotransferase, EC 2.7.3.2). It was shown that the dimeric structure of the enzyme was not required for the expression of enzyme activity, since the matrix-bound subunit form was catalytically active (Bickerstaff & Price, 1976*a*,*b*). A detailed study of the denaturation and renaturation of soluble creatine kinase (Bickerstaff & Price, 1977) has demonstrated that denaturation by guanidine hydrochloride is essentially completely reversible; this finding is an important prerequisite for the detailed interpretation of experiments involving matrix-bound subunit forms of enzymes (Chan, 1976). In the present paper we describe more detailed studies of the properties of the matrix-bound dimer and subunit forms of creatine kinase, which were undertaken to assess the importance of the dimeric structure of the enzyme. The properties examined include kinetics of the enzyme-catalysed reaction, stability towards denaturation, inhibition by reaction of thiol groups with iodoacetamide (Watts, 1973), reactivity of thiol groups and ligand-induced conformational changes (monitored by the reactivity of thiol groups towards iodoacetamide; Watts, 1973).

Abbreviations used: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid); Nbs^{2-}, 2-nitro-5-thiophenolate anion.

Materials and Methods

Materials

Creatine kinase was isolated from rabbit skeletal muscle as described by Milner-White & Watts (1971). Enzyme preparations were judged to be more than 90% homogeneous by polyacrylamide-gel electrophoresis at pH 7.5 (Davis, 1964) and had a specific activity of 130–150 μmol of ATP consumed/min per mg of protein, when assayed in the forward direction (phosphocreatine synthesis) under the conditions described below.

Pyruvate kinase (specific activity 200 units/mg of protein) and lactate dehydrogenase (specific activity

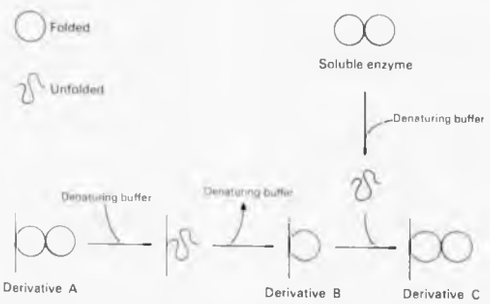
550 units/mg of protein) were obtained as $(NH_4)_2SO_4$ suspensions from Boehringer, Lewes, Sussex, U.K. (1 unit of enzyme activity refers to the consumption of 1 μmol of substrate/min.). Before use, the suspensions were centrifuged at 1000*g* for 5min and the resulting pellets dissolved in 0.1M-glycine/NaOH buffer at pH9.0. ATP, ADP, NADH, dithiothreitol, Nbs_2 and trypsin (specific activity 11000 units/mg of protein in the *N*-benzoyl-L-arginine ethyl ester assay) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Creatine, guanidine hydrochloride, glycine, Tris and iodoacetamide were obtained from BDH Chemicals, Poole, Dorset, U.K. The guanidine hydrochloride was recrystallized four times from ethanol and the iodoacetamide recrystallized three times from aq. 50% (v/v) ethanol. Sepharose 4B was purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K., and CNBr from Koch-Light, Colnbrook, Bucks., U.K. Iodo[1-^{14}C]-acetamide (specific radioactivity 58Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. A stock solution of radioactive iodoacetamide was prepared by addition of 50μCi of iodo[1-^{14}C]acetamide to 3ml of 10mM-iodoacetamide in 0.1M-glycine/NaOH at pH9.0. Other reagents were of analytical-reagent grade.

Preparation of matrix-bound creatine kinase

CNBr-activated Sepharose 4B was prepared by the procedure of March *et al.* (1974) but by using a lower degree of activation (5mg of CNBr/ml of packed gel). Coupling of creatine kinase to the activated gel and subsequent blocking of remaining activated groups on the Sepharose were carried out as described previously (Bickerstaff & Price, 1976*a*). Packed-gel volumes were determined after centrifugation at 200*g* for 2min in graduated centrifuge tubes. A stock suspension of the matrix-bound derivative was prepared by mixing equal volumes of gel and standard phosphate buffer (10mM-sodium phosphate buffer adjusted to pH8.0 with NaOH containing 1mM-EDTA). Accurate samples could then be removed from the stirred suspensions by using an automatic plunger-type pipette with the plastic tips cut to increase the aperture.

Preparation of matrix-bound subunit and reassociated derivatives

The relationships between the various matrix-bound derivatives of creatine kinase are shown in Scheme 1, and the principles involved in the preparation of such derivatives are discussed in detail by Chan (1976). Matrix-bound enzyme (derivative A) was incubated with denaturing buffer (0.1M-Tris/HCl, pH7.5, containing 5mM-dithiothreitol and 6M-guanidine hydrochloride) for 1h at 20°C and then washed in a sintered-glass column (1cm × 10cm) with denaturing buffer to remove non-covalently bound subunits. The denaturing buffer was then replaced by renaturing buffer (0.1M-Tris/HCl, pH7.5, containing 5mM-dithiothreitol) and renaturation was allowed to proceed for 3h at 20°C, followed by 18h at 4°C. The gel was then washed extensively with standard phosphate buffer (200ml) to displace the renaturation buffer and was finally suspended in standard phosphate buffer in a 1:1 (v/v) suspension. This procedure produced the matrix-bound subunit form (derivative B). In the preparation of matrix-bound reassociated enzyme (derivative C), small samples of a solution of dissociated soluble enzyme



Scheme 1. *Scheme depicting the relationships between the matrix-bound derivatives of creatine kinase*
The soluble enzyme was coupled to CNBr-activated Sepharose 4B to yield the matrix-bound enzyme (derivative A). Successive treatment of derivative A with denaturing and renaturing buffer produced the matrix-bound subunit (derivative B). The matrix-bound reassociated enzyme (derivative C) was prepared from derivative B by reassociation with added subunits of denatured soluble enzyme.

(previously incubated with d
added to a stirred suspens
renaturing buffer (gel/buffer
of a 4-fold excess of the diss
(expressed relative to the
derivative B), the mixture wa
3h at 20°C and then for a
gel was then washed exte
phosphate buffer, and a sta
(1:1, v/v) was prepared as o

Protein measurement and enz

The concentration of th
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concentrations of matrix-b
determined by using a sligh
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staff & Price, 1976*a*). Creat
determined in the forward di
synthesis) by using a coupled
pyruvate kinase and lact
auxiliary coupling enzymes.
formed at 30°C in 0.1M-glyc
the standard assay mixtur
creatine, 4mM-ATP, 5mM-m
phosphoenolpyruvate, 133μ
acetate and 24 units each
lactate dehydrogenase in a
When assays were perfor
acetate, the sodium acetat
magnesium acetate replace
enzyme activity of the matri
determined after addition
suitably diluted suspension
which was maintained at
stirred with an apparatus d
(1973).

Kinetic parameters for t
and creatine) were obtain
assays in which in turn th
substrate was varied in t
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concentration was varied.
concentration was adjuste
Mg^{2+} concentration at 1 m

Determination of pH optim

Enzyme activity was m
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except that the following b
Tris/acetate at pH7.5 and
at pH9.0, 9.5 and 10.5. The
was checked before and aft
case did the pH alter by mor
assay mixtures at pH9.5 an
coupling enzymes (90 unit

(previously incubated with denaturing buffer) were added to a stirred suspension of derivative B in renaturing buffer (gel/buffer 1:5, v/v). After addition of a 4-fold excess of the dissociated soluble enzyme (expressed relative to the quantity of protein in derivative B), the mixture was allowed to renature for 3h at 20°C and then for a further 18h at 4°C. The gel was then washed extensively with standard phosphate buffer, and a standard stock suspension (1:1, v/v) was prepared as outlined above.

Protein measurement and enzyme assays

The concentration of the soluble enzyme was determined from the A_{280} by using $A^{1\%}_{1cm} = 8.96$ and a mol.wt. of 82000 (Noda *et al.*, 1960). Protein concentrations of matrix-bound derivatives were determined by using a slightly modified version of the Lowry method as described previously (Bickerstaff & Price, 1976*a*). Creatine kinase activity was determined in the forward direction (phosphocreatine synthesis) by using a coupled assay system involving pyruvate kinase and lactate dehydrogenase as auxiliary coupling enzymes. All assays were performed at 30°C in 0.1M-glycine/NaOH, pH9.0, and the standard assay mixture consisted of 40mM-creatine, 4mM-ATP, 5mM-magnesium acetate, 1mM-phosphoenolpyruvate, 133μM-NADH, 0.1M-sodium acetate and 24 units each of pyruvate kinase and lactate dehydrogenase in a final volume of 3ml. When assays were performed in the absence of acetate, the sodium acetate was omitted and the magnesium acetate replaced by 5mM-$MgSO_4$. The enzyme activity of the matrix-bound derivatives was determined after addition of small samples of a suitably diluted suspension to the assay mixture, which was maintained at 30°C and continuously stirred with an apparatus described by Mort *et al.* (1973).

Kinetic parameters for the substrates (MgATP and creatine) were obtained from the results of assays in which in turn the concentration of one substrate was varied in the presence of several fixed concentrations of the other. When the ATP concentration was varied, the magnesium acetate concentration was adjusted to maintain the free Mg^{2+} concentration at 1mM (Watts, 1973).

Determination of pH optimum

Enzyme activity was monitored over the pH range 7.5–10.5 under standard assay conditions, except that the following buffers were used: 0.1M-Tris/acetate at pH7.5 and 8.5; 0.1M-glycine/NaOH at pH9.0, 9.5 and 10.5. The pH of the assay mixture was checked before and after each assay and in no case did the pH alter by more than 0.1 pH unit. In the assay mixtures at pH9.5 and 10.5 larger amounts of coupling enzymes (90 units of each enzyme) were needed to ensure that the coupling reactions were not rate-limiting at these pH values.

Stability towards thermal inactivation

The rates of thermal inactivation of the enzyme and its matrix-bound derivatives were studied at 45°C in 0.1M-glycine/NaOH buffer at pH8.5 (45°C). Buffer (2ml) was equilibrated in a thermostatically controlled cuvette for 15min before a small sample (0.1ml) of enzyme was added, to give a final concentration of about 30μg of protein/ml. All mixtures were stirred continuously with a small magnetic stirring bar (0.3cm long). Samples were removed at set times and assayed directly for residual enzyme activity by using the standard assay conditions.

Stability towards inactivation by guanidine hydrochloride

The stability of the enzyme and its matrix-bound derivatives towards inactivation by guanidine hydrochloride was studied by incubating samples (in which the enzyme concentration was about 30μg/ml) at 30°C in denaturing buffer in which the concentrations of Tris/HCl and dithiothrietol were kept constant at 0.1M and 5mM respectively and the guanidine hydrochloride concentration was varied over the range 0–1.0M. After a 20min incubation period, during which the mixtures were kept continuously stirred, a sample was removed for direct assay of enzyme activity. The standard assay system was supplemented with 15μg of trypsin to prevent renaturation within the assay mixture. Control experiments showed that neither the residual denaturant nor the trypsin in the assay mixture affected the activity of creatine kinase or the coupling enzymes. It was also shown that trypsin effectively prevented renaturation within the assay mixture, as was found in a study of aldolase subunits (Chan *et al.*, 1973*b*).

Inhibition by iodoacetamide

Inhibition of the enzyme and its matrix-bound derivatives by iodoacetamide was studied at 30°C in 0.1M-glycine/NaOH at pH9.0. Enzyme was allowed to equilibrate with any added substrates or ligands for 20min in a stirred solution of total volume 2ml. A control sample (0.1ml) was transferred to a stirred solution (1.9ml) of 1mM-dithiothreitol in standard phosphate buffer, and the reaction was then started by addition of iodoacetamide to the remainder of the enzyme. The progress of the inhibition reaction was monitored by removing samples at set times and diluting them into stirred solutions of 1mM-dithiothreitol. Samples were then assayed for residual activity by the standard assay procedure. The enzyme activity of these diluted samples did not change over a period of at least 3h at 20°C.

Determination of reactive thiol groups

The numbers of reactive thiol groups present in the enzyme and its matrix-bound derivatives were measured by using Nbs_2, by assuming an absorption coefficient of 13.6 litre·mmol^{-1}·cm^{-1} for the Nbs^2 anion at 412nm (Ellman, 1959). Nbs_2 (final concn. 250μM) was added to a gently stirred suspension of 1ml of gel in 1ml of standard phosphate buffer, and the stirring was continued for 15min at 20°C. The suspension was then centrifuged at 1000*g* for 5min and the A_{412} of the supernatant measured. Control experiments showed that small corrections were necessary with the matrix-bound subunit and the matrix-bound reassociated enzyme to take account of the reaction of Nbs_2 with residual dithiothreitol from the renaturing buffer. The correction was always less than 10% of the total observed absorbance. In every case there was no further reaction with Nbs_2 over an additional 15min period.

The numbers of reactive thiol groups were also determined by using iodo[1-^{14}C]acetamide. A small sample of the stock radioactive iodoacetamide solution was added to a gently stirred suspension of 1ml of matrix-bound derivative in 1ml of 0.1M-glycine/NaOH at pH9.0, to give a final iodoacetamide concentration of 0.476mM. The mixture was stirred at 30°C for 15min before the reaction was stopped by the addition of 0.5ml of standard phosphate buffer containing 10mM-dithiothreitol. The gel was then transferred to a sintered-glass column (1cm × 10cm) and washed extensively with standard phosphate buffer (200ml) to remove unchanged iodo-[1-^{14}C]acetamide. Radioactivity of matrix-bound derivatives was determined after hydrolysis of a 0.2ml sample of a 1:1 (v/v) gel suspension in standard phosphate buffer in 0.5ml of 12M-HCl, followed by addition of 0.5ml of water and 15ml of scintillation fluid (Bruch *et al.*, 1976). Control experiments showed that the presence of neither Sepharose nor enzyme affected the observed radioactivity (c.p.m.) of a standard solution of iodo-[1-^{14}C]acetamide and that more than 99.9% of the unchanged iodo[1-^{14}C]acetamide was removed from the gel by washing with standard phosphate buffer as described. Small corrections were necessary, however, with the matrix-bound subunit and matrix-bound reassociated enzyme to account for the reaction of iodo[1-^{14}C]acetamide with residual dithiothreitol from the renaturing buffer. The correction was always less than 15% of the total observed radioactivity. The incorporation of iodo-[1-^{14}C]acetamide into soluble enzyme was monitored by the general method previously described (Griffiths *et al.*, 1975).

In all cases there was no further incorporation of radioactivity after an additional 45min period of incubation with the iodo[1-^{14}C]acetamide solution.

Results

Protein content and enzyme activity of derivatives

Typical protein contents and enzyme activity values for the matrix-bound derivatives are shown in Table 1. The previously reported lower specific activities for these derivatives (Bickerstaff & Price, 1976*a*) were obtained under conditions that differed with respect to both substrate concentration and temperature from those used in the present work. In addition, later work (Bickerstaff & Price, 1976*b*) revealed the extent of the acetate-induced activation of matrix-bound enzyme derivatives. The results shown in Table 1 indicate that the specific activity of matrix-bound subunit (derivative B) is not increased significantly (by less than 5%) by the inclusion of 0.1M-acetate in the assay system, whereas the activities of both matrix-bound enzyme (derivative A) and matrix-bound reassociated enzyme (derivative C) are increased considerably (by 95 and 70% respectively). These results suggest that acetate exerts its activating effect via a subunit interaction and not by a direct influence on the catalytic site of each subunit. The activity of soluble enzyme is enhanced by 17% on inclusion of 0.1M-acetate in the assay system (Table 1). If acetate exerts its effect via a conformational change in the enzyme (Watts, 1973), it would appear that immobilization of the enzyme has affected the conformation in a way that is opposite to the change caused by acetate.

pH optimum and kinetic parameters of derivatives

The effects of pH on the enzyme activities of soluble enzyme and the various matrix-bound derivatives are shown in Table 2. In all cases there was a broad peak in the pH–activity profile, with maximum activity being observed at pH9.0. The

Table 1. *Protein content and enzyme activity values of matrix-bound derivatives*

The preparation of the matrix-bound derivatives is outlined in Fig. 1 and is described in the Materials and Methods section. Enzyme assays were performed in the absence and in the presence of acetate (0.1M) as described in the Materials and Methods section. Soluble creatine kinase had a specific activity of 115units/mg in the absence of acetate and 135units/mg in the presence of acetate. Protein contents are expressed as μg/ml of packed gel.

Matrix-bound derivative	Protein content (μg/ml)	Protein content (%)	Specific activity (μmol/min per mg) Acetate absent	Specific activity (μmol/min per mg) Acetate present
Derivative A	400	100	50	98
Derivative B	200	50	48	50
Derivative C	380	95	50	80

results obtained with the soluble
pH range 7.5–9.0 are comparable w
under slightly different condition
1960).

A double-reciprocal plot of th
observed for the matrix-bound e
concentrations of MgATP in the
concentrations of creatine is sh
Corresponding plots were obtain
enzyme and the other matrix-bou
all showed the same intersectin
teristic of synergism, in the bindi
strates to the enzyme (Watts, 19
meters were estimated from secon
intercepts and slopes of the
reciprocal plots, as described by
(1957) and shown in Fig. 1(*b*). The
for the soluble enzyme and
derivatives are collected in Table

The results show that in the
acetate the values of $V_{max.}$ for m
and matrix-bound reassociated
similar to that of the soluble e
bound subunit form has a lower

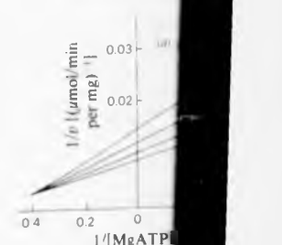

Fig. 1. *Double-reciprocal pl*
(*a*) Effect of varying the [MgAT
, 20mM; □, 10mM; ●, 5mM.
velocity (○) and gradients (
The assays were performed in

Table 3. *Kinetic*
The kinetic constants were e
[magnesium acetate] was adju

Matrix-bound derivative

Derivative A
Derivative B
Derivative C
Soluble enzyme

results obtained with the soluble enzyme over the pH range 7.5–9.0 are comparable with those reported under slightly different conditions (Noda *et al.*, 1960).

A double-reciprocal plot of the initial velocities observed for the matrix-bound enzyme at various concentrations of MgATP in the presence of fixed concentrations of creatine is shown in Fig. 1(*a*). Corresponding plots were obtained for the soluble enzyme and the other matrix-bound derivatives, and all showed the same intersecting pattern, characteristic of synergism, in the binding of the two substrates to the enzyme (Watts, 1973). Kinetic parameters were estimated from secondary replots of the intercepts and slopes of the primary double-reciprocal plots, as described by Florini & Vestling (1957) and shown in Fig. 1(*b*). The kinetic parameters for the soluble enzyme and the matrix-bound derivatives are collected in Table 3.

The results show that in the presence of 0.1M-acetate the values of $V_{max.}$ for matrix-bound enzyme and matrix-bound reassociated enzyme are very similar to that of the soluble enzyme. The matrix-bound subunit form has a lower $V_{max.}$ (75% of the value for soluble enzyme). There are some small variations in the values of K_m for MgATP, with those for the matrix-bound enzyme being raised by approx. 2.5-fold compared with soluble enzyme

Table 2. *Effect of pH on the activity of soluble and matrix-bound derivatives of creatine kinase*

The activity of the soluble and matrix-bound derivatives (see Scheme 1) was determined in the following buffers: 0.1M-Tris/acetate at pH7.5 and 8.5; 0.1M-glycine/NaOH at pH9.0, 9.5 and 10.5. The results in each case are expressed as a percentage of the maximum activity, which in all cases was observed at pH9.0.

	Relative enzyme activity			
		Matrix-bound derivative		
Buffer pH	Soluble enzyme	A	B	C
7.5	78	74	72	72
8.5	98	96	94	92
9.0	100	100	100	100
9.5	88	92	80	88
10.5	67	52	60	68

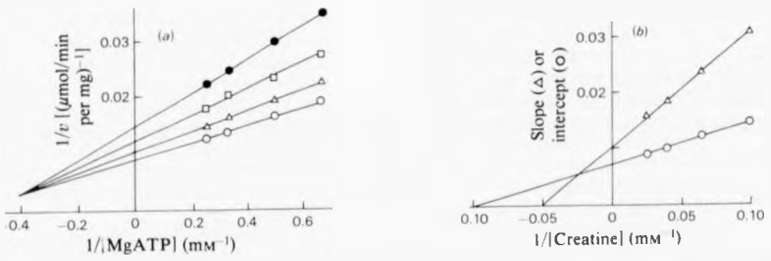

Fig. 1. *Double-reciprocal plot and secondary-replot data for the reaction catalysed by the matrix-bound enzyme*

(*a*) Effect of varying the [MgATP] in the presence of several fixed concentrations of creatine, which were: ▽, 40mM; △, 20mM; □, 10mM; ●, 5mM. (*b*) Secondary replot of the data in (*a*) showing the variation of maximum initial velocity (○) and gradients (△) of the primary plot as a function of the reciprocal of the creatine concentration. The assays were performed in 0.1M-glycine/NaOH at pH9.0 and 30°C in the presence of 0.1M-sodium acetate.

Table 3. *Kinetic parameters for soluble and matrix-bound derivatives of creatine kinase*

The kinetic constants were estimated from data of the type presented in Fig. 1. When [MgATP] was varied, [magnesium acetate] was adjusted to maintain the free $[Mg^{2+}]$ at 1mM.

		Apparent kinetic constants (mM)			
		MgATP		Creatine	
Matrix-bound derivative	$V_{max.}$ (μmol/min per mg)	K_m	K_s	K_m	K_s
Derivative A	145	0.9	2.5	5.0	13.9
Derivative B	115	1.2	2.4	17.0	32.0
Derivative C	142	1.0	2.5	8.5	25.0
Soluble enzyme	150	0.4	1.1	10.0	28.0

(Table 3). It is also apparent that the ratio K_i/K_m for each substrate (which gives a measure of the extent of synergism in substrate binding) is decreased in all the matrix-bound derivatives compared with soluble enzyme. The difference in the ratio K_i/K_m for the monomeric and dimeric forms of the enzyme is small and probably within experimental error. These studies show that immobilization of the enzyme has not drastically affected the integrity of the active site, although small changes in its three-dimensional structure cannot be excluded. The phenomenon of synergism in substrate binding does not appear to depend on the presence of a dimeric structure in the enzyme.

Stability of derivatives

In common with the findings of studies on other enzymes (Fukui *et al.*, 1975; Goheer *et al.*, 1976), it was found that immobilization of creatine kinase led to an enhancement of stability towards either thermal inactivation (Fig. 2*a*) or unfolding by guanidine hydrochloride (Fig. 2*b*). In both types of study the order of stabilities observed was: soluble enzyme <derivative B <derivatives A and C. Fig. 2(*a*) shows that the first-order rate constants for inactivation at 45°C are 0.028, 0.020 and 0.011 min^{-1} for soluble enzyme, derivative B and derivatives A and C respectively. The concentrations of guanidine hydrochloride that cause a 50% loss of activity for these samples are 0.52M, 0.57M and 0.64M respectively (Fig. 2*b*). It should be noted, however, that with both soluble enzyme and the matrix-bound derivatives it proved impossible to achieve an equilibrium limiting value of the remaining enzyme activity at a given concentration of guanidine hydrochloride; a progressive loss of activity was observed over a period of several hours. For this reason experiments were conducted with a fixed incubation time (20min) at a given concentration of guanidine hydrochloride, so the results obtained presumably represent a combination of kinetic and thermodynamic aspects of the unfolding of the protein structure. Any detailed analysis of the relative thermodynamic stabilities of the soluble enzyme and the various matrix-bound derivatives by using an approach such as that suggested, for example, by Tanford (1968) would be inappropriate.

Inhibition by iodoacetamide

The reactivity of a thiol group in each subunit of creatine kinase towards iodoacetamide has been widely used as an index of the conformational state of the enzyme, and in particular as a means of detecting ligand-induced conformational changes in the enzyme (Watts, 1973; Milner-White & Kelly, 1976). The results of studies on the reactivity of the thiol group of soluble creatine kinase and its matrix-bound derivatives are shown in Table 4. These results were obtained by measuring the rate of inactivation of the enzyme by iodoacetamide, since it is known that the modified enzyme is completely inactive or very nearly so (Watts, 1973).

The data in Table 4 show that the reactivity of the thiol group in the various forms of the enzyme is quite similar, suggesting that immobilization has not significantly affected the conformation of the enzyme

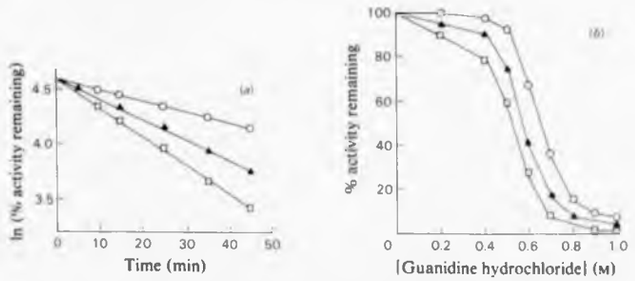

Fig. 2. *Stability of creatine kinase derivatives*

(*a*) Semi-logarithmic plot comparing the rate of thermal inactivation of soluble and matrix-bound derivatives of creatine kinase. ○, Soluble enzyme; ▲, matrix-bound subunit; □, matrix-bound dimer. The studies were made in 0.1M-glycine/NaOH at pH8.5 at 45°C in a final volume of 2ml. The enzyme concentrations was approx. 30μg/ml in all cases and samples were transferred from the inactivation mixture at the times shown and assayed directly for residual enzyme activity during the standard assay procedure. (*b*) Effect of guanidine hydrochloride on the activity of soluble and matrix-bound derivatives of creatine kinase at pH7.5 and 30°C. ○, Soluble enzyme; ▲, matrix-bound subunit; □, matrix-bound dimer. The concentration of guanidine hydrochloride-inactivated creatine kinase was approx. 30μg/ml in each case and the period of incubation was 20min. The standard assay system was used and supplemented with trypsin (15μg) as described in the Materials and Methods section.

Table 4. *Second-order rate constant*

Inhibition of the soluble and the
substrates or substrate complexes
centration was 0.476mM and the c
tions used were: ADP, 1mM; ma
are percentage changes in *k*. Each

Reaction mixture
E (enzyme only)
E + MgADP
E + MgADP + creatine
E + MgADP + creatine + NO_3^-

in the region of the thiol group.
constant for inhibition of the
iodoacetamide is comparable w
Milner-White & Kelly (1976) unde

The effects of the ligand co
ADP and Mg^{2+} + ADP + creat
of the thiol group were also ver
derivatives (Table 4). Of partic
results obtained on addition
creatine + nitrate, a combinatio
produce a transition-state analog
the nitrate occupies the positio
phosphoryl group in the catalyt
(Watts, 1973; James & Cohn, 19
Kelly, 1976). In each case a de
90% in the rate constant for the
was observed (Table 4).

These results indicate that
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ligands do not require a dime
connection, it might be noted
arginine kinase isolated from
americanus is also capable of
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+ L-arginine + nitrate to the
Cohn, 1974).

Determination of reactive thiol

The numbers of reactive thio
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determined by reaction with
easily applicable to such deriv
Nbs^{2-} anion product is relea
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centrifuged (500*g* for 5min).
with the various derivatives
and indicate that the soluble
enzyme and matrix-bound re

Table 4. *Second-order rate constants (k) for inactivation of soluble and matrix-bound derivatives of creatine kinase by iodoacetamide*

Inhibition of the soluble and the matrix-bound derivatives by iodoacetamide in the absence and in the presence of substrates or substrate complexes was studied at 30°C in 0.1M-glycine/NaOH at pH9.0. The iodoacetamide concentration was 0.476mM and the enzyme concentration was approx. 1.5μM-subunits in all cases. Substrate concentrations used were: ADP, 1mM; magnesium acetate, 10mM; creatine, 40mM; $NaNO_3$, 0.1M. Values in parentheses are percentage changes in *k*. Each value of *k* was determined at least three times and was reproducible to within 5%.

	k (M^{-1} min^{-1})					
		Matrix-bound derivative				
Reaction mixture	Soluble enzyme	A		B	C	
E (enzyme only)	1100 —	800 —		1000 —	850 —	
E + MgADP	1200 (+9)	900 (+12)		1000 (0)	1000 (+17)	
E + MgADP + creatine	850 (−23)	600 (−25)		700 (−30)	600 (−30)	
E + MgADP + creatine + NO_3^-	80 (−93)	50 (−94)		90 (−91)	70 (−92)	

in the region of the thiol group. The value of the rate constant for inhibition of the soluble enzyme by iodoacetamide is comparable with that reported by Milner-White & Kelly (1976) under similar conditions.

The effects of the ligand combinations Mg^{2+} + ADP and Mg^{2+} + ADP + creatine on the reactivity of the thiol group were also very similar for all the derivatives (Table 4). Of particular interest are the results obtained on addition of Mg^{2+} + ADP + creatine + nitrate, a combination that is thought to produce a transition-state analogue complex in which the nitrate occupies the position of the transferable phosphoryl group in the catalytically active complex (Watts, 1973; James & Cohn, 1974; Milner-White & Kelly, 1976). In each case a decrease of more than 90% in the rate constant for the inactivation reaction was observed (Table 4).

These results indicate that the conformational responses of the various forms of the enzyme of the ligands do not require a dimeric structure. In this connection, it might be noted that the monomeric arginine kinase isolated from the lobster *Homarus americanus* is also capable of forming a transition-state analogue complex on addition of Mg^{2+} + ADP + L-arginine + nitrate to the enzyme (Buttlaire & Cohn, 1974).

Determination of reactive thiol groups

The numbers of reactive thiol groups in the matrix-bound derivatives of creatine kinase were initially determined by reaction with Nbs_2. This reaction is easily applicable to such derivatives, since the yellow Nbs^{2-} anion product is released into solution and can be readily measured spectrophotometrically in the supernatant after the gel suspension has been centrifuged (500*g* for 5min). The results obtained with the various derivatives are shown in Table 5 and indicate that the soluble enzyme, matrix-bound enzyme and matrix-bound reassociated enzyme all possess one reactive thiol group per subunit, whereas there are two reactive thiol groups in the matrix-bound subunit form.

Because the absorbance changes in the reaction with Nbs_2 were relatively small at the protein concentrations obtainable with the matrix-bound derivatives, a check on these results was performed by using iodo[1-^{14}C]acetamide as a thiol-modifying reagent. The results (summarized in Table 5) confirm the pattern observed with Nbs_2, i.e. one reactive thiol group per subunit for soluble enzyme, matrix-bound enzyme and matrix-bound reassociated enzyme and two reactive thiol groups per subunit for the matrix-bound subunit form.

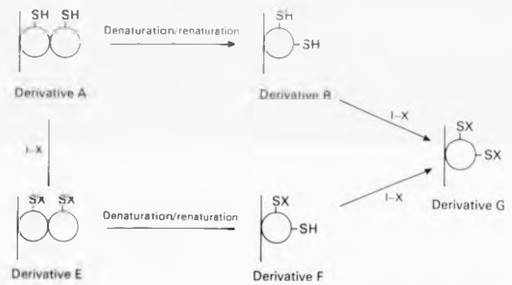
It should be noted that the derivatives that had been treated with iodo[1-^{14}C]acetamide to the limiting extents shown in Table 5 did not react further with added Nbs_2, indicating that the two reagents are reacting with the same thiol group(s) on the enzyme.

Further experiments with carbamoylated enzyme derivatives

The interconversions of the various carbamoylated enzyme derivatives are illustrated in Scheme 2. Derivatives A and B represent the matrix-bound enzyme and matrix-bound subunit respectively (cf. Scheme 1); derivative E is the carbamoylated matrix-bound dimer (reacted to the limiting extent of one thiol group per subunit); derivative F is the monocarbamoylated matrix-bound subunit form obtained from derivative E by a denaturation–renaturation sequence analogous to that used in the preparation of derivative B from derivative A (Scheme 1); derivative G is the dicarbamoylated matrix-bound subunit form obtained either from derivative F or directly from derivative B by reaction with iodoacetamide in a fashion analogous to that described in the Materials and Methods section.

Table 5. *Measurement of reactive thiol groups in soluble and matrix-bound derivatives of creatine kinase*
Reactive thiol groups were measured by two methods: release of the anion Nbs^{2-} and incorporation of [1-^{14}C]-acetamide. The protein concentration refers to the final concentration of protein in the reaction mixture and not to the protein content of the packed gel. Experimental conditions are described in the Materials and Methods section. Results are means of six measurements.

Matrix-bound derivative	Protein concentration (μM-subunits)	Nbs^{2-} released (μM)	[1-^{14}C]Acetamide incorporated (μM)	No. of reactive thiol groups/subunit	
				Nbs_2	Iodoacetamide
Derivative A	5.8	6.1	6.2	1.05	1.07
Derivative B	2.9	6.2	6.4	2.14	2.20
Derivative C	5.6	6.2	6.3	1.10	1.12
Soluble enzyme	5.8	5.5	5.7	0.95	0.98



Scheme 2. *Scheme depicting the relationship between the various carbamoylated matrix-bound derivatives of creatine kinase*
Derivatives A and B were obtained as described in Scheme 1. Derivative E was prepared by treating derivative A with a large excess of iodoacetamide (I–X). Derivative F was then derived from derivative E by the denaturation–renaturation procedure outlined in the Materials and Methods section. Derivative G was obtained either from derivative F or directly from derivative B by reaction with a large excess of iodoacetamide.

Conversion of derivative E into derivative F by the denaturation–renaturation sequence was accompanied by the loss of 52% of the protein content and 54% of the radioactivity, confirming that in derivative E the iodo[1-^{14}C]acetamide had reacted uniformly with the two subunits of the matrix-bound dimer.

The inactivation of soluble enzyme, matrix-bound enzyme and matrix-bound reassociated enzyme is accompanied by a proportional incorporation of one acetamide moiety per subunit, whereas with the matrix-bound subunit form complete inactivation is accompanied by reaction of two thiol groups. From studies of the rate of incorporation of radioactivity from iodo[1-^{14}C]acetamide into the matrix-bound subunit form it appears that these two thiol groups are of comparable reactivity, i.e. there is no rapid reaction of one thiol group followed by a slower reaction of the second. Thus it is possible that reaction of only one thiol group (the same one as in the dimeric forms) is required for inactivation of the matrix-bound subunit form and reaction of the second thiol group does not affect the activity. This possibility is supported by the observation that the monocarbamoylated matrix-bound subunit form (derivative F in Scheme 2) is inactive ($\leq$2% of the activity of derivative B).

Discussion

The results obtained in these experiments show that there are considerable similarities between soluble and matrix-bound creatine kinase in terms of (i) the parameters of the enzyme-catalysed reaction, (ii) the reactivity of a single thiol group per subunit towards iodoacetamide and (iii) changes in the reactivity of this thiol group in the presence of various combinations of substrates and ligands. These data indicate that neither the conformation of the enzyme (at the active site and at the reactive thiol group) nor the substrate-induced conformational changes (monitored by changes in the reactivity of

the thiol group) are grossly altere
of soluble enzyme. Extrapol
obtained with the matrix-bound
to the behaviour of soluble e
made with reasonable confid
observed changes in the degr
substrate binding and in the
induced activation indicate th
turbations in structure do occu
The similarities between matri
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structure of the enzyme is n
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studies on arginine kinases iso
cucumber *Holothuria forskal*
Homarus vulgaris, which occur
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the properties of the enzyme in
is open to question.

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We thank the Science Research
ship (to G. F. B.) and for general

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and incorporation of [1-^{14}C]-
a reaction mixture and not to
aterials and Methods section.

f reactive thiol groups/subunit

Nbs_2	Iodoacetamide
1.05	1.07
2.14	2.20
1.10	1.12
0.95	0.98

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-SX
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the thiol group) are grossly altered by immobilization of soluble enzyme. Extrapolation from results obtained with the matrix-bound enzyme derivatives to the behaviour of soluble enzyme can thus be made with reasonable confidence, although the observed changes in the degree of synergism of substrate binding and in the extent of acetate-induced activation indicate that some small perturbations in structure do occur on immobilization. The similarities between matrix-bound dimer and subunit forms of the enzyme, as far as kinetic parameters and substrate-induced changes in thiol-group reactivity are concerned, show that the dimeric structure of the enzyme is not required for the expression of catalytic activity or of substrate-induced conformational changes. The greater stability of the dimer form with regard to thermal denaturation or to denaturation by guanidine hydrochloride can be explained by the favourable subunit interactions in the dimer form and is comparable with the pattern observed by Anosike *et al.* (1975) in their studies on arginine kinases isolated from the seacucumber *Holothuria forskali* and the lobster *Homarus vulgaris*, which occur in dimer and monomer forms respectively. As with these arginine kinases, the differences in stability between the matrix-bound dimer and subunit forms of creatine kinase are only observed under non-physiological conditions, and the relevance of these findings to the properties of the enzyme in a physiological state is open to question.

Perhaps the most noteworthy difference that we have observed between the matrix-bound dimer and monomer forms of creatine kinase is the presence of an extra reactive thiol group in the monomer form. It is tempting to suggest that this extra thiol group may be masked in the dimer form, possibly by being in close proximity to the subunit interface. This possibility could be further investigated by determining the extent to which the unmodified, mono- and di-carbamoylated forms of matrix-bound subunit creatine kinase (derivatives B, F and G respectively in Scheme 2) can reassociate with added subunits of soluble enzyme.

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