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.*, 1973a). Subsequently, the technique has been applied to various enzymes and the results indicate that certain multisubunit enzymes, e.g. fructose biphosphatase (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, 1976a,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;

Abbreviations used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); Nbs²⁻, 2-nitro-5-thiophenolate anion.

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).

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 μ mol 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 $(\text{NH}_4)_2\text{SO}_4$ suspensions from Boehringer, Lewes, Sussex, U.K. (1 unit of enzyme activity refers to the consumption of $1\text{ }\mu\text{mol}$ of substrate/min.). Before use, the suspensions were centrifuged at $1000g$ for 5 min and the resulting pellets dissolved in 0.1 M -glycine/NaOH buffer at pH 9.0. ATP, ADP, NADH, dithiothreitol, Nbs₂ 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[1-¹⁴C]-acetamide (specific radioactivity 58 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. A stock solution of radioactive iodoacetamide was prepared by addition of $50\text{ }\mu\text{Ci}$ of iodo[1-¹⁴C]acetamide to 3 ml of 10 mM -iodoacetamide in 0.1 M -glycine/NaOH at pH 9.0. 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 (5 mg 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, 1976a).

Packed-gel volumes were determined after centrifugation at $200g$ for 2 min in graduated centrifuge tubes. A stock suspension of the matrix-bound derivative was prepared by mixing equal volumes of gel and standard phosphate buffer (10 mM -sodium phosphate buffer adjusted to pH 8.0 with NaOH containing 1 mM -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 (0.1 M -Tris/HCl, pH 7.5, containing 5 mM -dithiothreitol and 6 M -guanidine hydrochloride) for 1 h at 20°C and then washed in a sintered-glass column ($1\text{ cm} \times 10\text{ cm}$) with denaturing buffer to remove non-covalently bound subunits. The denaturing buffer was then replaced by renaturing buffer (0.1 M -Tris/HCl, pH 7.5, containing 5 mM -dithiothreitol) and 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 standard phosphate buffer (200 ml) 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 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 $A_{1\text{cm}}^{1\%} = 8.96$ 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, 1976a). 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, 133 μ M-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-MgSO₄. 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 Mg²⁺ 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 30 μ g 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 30 μ g/ml) at 30°C in denaturing buffer in which the concentrations of Tris/HCl and dithiothreitol 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 15 μ g 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.*, 1973b).

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 $13.6 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ for the Nbs_2^- anion at 412 nm (Ellman, 1959). Nbs_2 (final concn. $250 \mu\text{M}$) was added to a gently stirred suspension of 1 ml of gel in 1 ml of standard phosphate buffer, and the stirring was continued for 15 min at 20°C . The suspension was then centrifuged at $1000g$ for 5 min 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 15 min 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 1 ml of matrix-bound derivative in 1 ml of 0.1 M-glycine/NaOH at pH 9.0, to give a final iodoacetamide concentration of 0.476 mM. The mixture was stirred at 30°C for 15 min before the reaction was stopped by the addition of 0.5 ml of standard phosphate buffer containing 10 mM-dithiothreitol. The gel was then transferred to a sintered-glass column ($1 \text{ cm} \times 10 \text{ cm}$) and washed extensively with standard phosphate buffer (200 ml) to remove unchanged iodo[1- ^{14}C]acetamide. 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, followed by addition of 0.5 ml of water and 15 ml 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 45 min 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, 1976a) 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, 1976b) 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 0.1 M-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 0.1 M-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 pH 9.0. 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 (0.1 M) as described in the Materials and Methods section. 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 $\mu\text{g/ml}$ of packed gel.

Matrix-bound derivative	Protein content		Specific activity ($\mu\text{mol/min per mg}$)	
	($\mu\text{g/ml}$)	(%)	Acetate absent	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 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.

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

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 $[\text{Mg}^{2+}]$ at 1 mM.

Matrix-bound derivative	$V_{\max}$ ($\mu\text{mol/min per mg}$)	Apparent kinetic constants (mM)			
		MgATP		Creatine	
		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_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 soluble enzyme. The difference in the ratio K_s/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. 2a) or unfolding by guanidine hydrochloride (Fig. 2b). 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. 2b). 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 (20 min) 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 0.1 M-glycine/NaOH at pH 8.5 at 45°C in a final volume of 2 ml. The enzyme concentrations was approx. 30 µg/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 pH 7.5 and 30°C. □, Soluble enzyme; ▲, matrix-bound subunit; ○, matrix-bound dimer. The concentration of guanidine hydrochloride-inactivated creatine kinase was approx. 30 µg/ml in each case and the period of incubation was 20 min. The standard assay system was used and supplemented with trypsin (15 µg) as described in the Materials and Methods section.

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. 1.5 μ M-subunits in all cases. Substrate concentrations used were: ADP, 1mM; magnesium acetate, 10mM; creatine, 40mM; NaNO₃, 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%.

Reaction mixture	k (M ⁻¹ ·min ⁻¹)				
	Soluble enzyme	Matrix-bound derivative			
		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 Mg²⁺ + ADP and Mg²⁺ + 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 Mg²⁺ + 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 Mg²⁺ + 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 Nbs₂. This reaction is easily applicable to such derivatives, since the yellow Nbs₂⁻ anion product is released into solution and can be readily measured spectrophotometrically in the supernatant after the gel suspension has been centrifuged (500g 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 Nbs₂ were relatively small at the protein concentrations obtainable with the matrix-bound derivatives, a check on these results was performed by using iodo[1-¹⁴C]acetamide as a thiol-modifying reagent. The results (summarized in Table 5) confirm the pattern observed with Nbs₂, 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[1-¹⁴C]acetamide to the limiting extents shown in Table 5 did not react further with added Nbs₂, 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 $[1-^{14}\text{C}]$ -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)	$[1-^{14}\text{C}]$ 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 $[1-^{14}\text{C}]$ 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 $[1-^{14}\text{C}]$ 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 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 sea-cucumber *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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