A Study of the Role of the Reactive Thiol Group of Rabbit Muscle Creatine Kinase with a Chromophoric Reporter Group

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Substrate- and ligand-induced conformational changes were studied in a series of thiol-modified derivatives of rabbit muscle creatine kinase that retained different amounts of enzymic activity. The results indicate that the 'reactive' thiol group of the enzyme is required for the conformational changes associated with formation of a 'transition-state analogue' complex.

Creatine kinase (ATP-creatine *N*-phosphotransferase, EC 2.7.3.2) from rabbit muscle possesses one reactive thiol group per subunit, modification of which by a variety of reagents (e.g. iodoacetamide, iodoacetate and 1-fluoro-2,4-dinitrobenzene) leads to inactivation of the enzyme (Watts, 1973). Modification procedures that introduce only small perturbations have shown that this thiol group is not essential for retention of enzymic activity; thus the E-S-CN and E-S-S-CH₃ derivatives retain 70 and 30% respectively of the activity of unmodified enzyme (der Terrossian & Kassab, 1976; Maggio *et al.*, 1977).

Quiocho and his co-workers (Quiocho & Thomson, 1973; Laue & Quiocho, 1977) have shown that the 2-mercuri-4-nitrophenol group can be introduced into the enzyme from chicken breast muscle at a thiol group distinct from that which is reactive towards iodoacetamide. They also showed that the spectrum of the 2-mercuri-4-nitrophenol group changes on addition of various substrate and ligand combinations to the MNP-enzyme. We have used this reporter group to examine the ligand-induced conformational changes of various MNP derivatives of the rabbit muscle enzyme, and in particular to study those conformational changes that are associated with formation of the complex (enzyme + MgADP + creatine + nitrate) that has been postulated to resemble the transition state of the enzyme-catalysed reaction (Milner-White & Watts, 1971; James & Cohn, 1974; Milner-White & Kelly, 1976). The present results lend support to the

hypothesis 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.

Experimental

Tricine and iodoacetamide were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. The iodoacetamide was recrystallized from hot water before use. 2-Chloromercuri-4-nitrophenol was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. 8-Anilino-naphthalene-1-sulphonic acid was obtained as the magnesium salt from BDH Chemicals, Poole, Dorset, U.K. All other reagents were of A.R. grade.

Creatine kinase was isolated from rabbit skeletal muscle and assayed as described previously (Price & Hunter, 1976). *K_m* values were determined at 30°C in 0.1 M-glycine/NaOH buffer, pH 9.0. The free Mg²⁺ concentration was maintained at 1 mM. The E-S-CN and E-S-S-CH₃ derivatives were prepared as described by der Terrossian & Kassab (1976) and Smith *et al.* (1975). In our hands the E-S-S-CH₃ derivative retained 40% of the activity of unmodified enzyme, compared with the published value of 30% under identical conditions (Maggio *et al.*, 1977). The E-S-CH₂CONH₂ derivative was prepared by reacting enzyme with a 10% excess of iodoacetamide (with respect to the subunit concentration) in 50 mM-Tricine (sodium salt) buffer, pH 8.0, for 3 h at 25°C. After dialysis, the product was assayed and found to possess $\leq 2\%$ of the activity of unmodified enzyme. Reaction of the enzyme derivatives with 2-chloromercuri-4-nitrophenol was carried out as described by Quiocho & Thomson (1973), except that the reactions were performed in 50 mM-Tricine (sodium salt) buffer, pH 8.0, at 0°C. After dialysis, the extents of incorporation of the 2-mercuri-4-nitrophenol group were determined by using the published absorption coefficient (Quiocho & Thomson, 1973).

Abbreviations used: E-S-CN, E-S-S-CH₃ and E-S-CH₂CONH₂ represent derivatives of unmodified enzyme (E-S-H) in which the thiol group reactive towards iodoacetamide has been converted into the -S-CN, -S-S-CH₃ and -S-CH₂CONH₂ derivatives respectively. These forms of the enzyme are then reacted with 2-chloromercuri-4-nitrophenol to yield the 2-mercuri-4-nitrophenol derivatives (MNP-E-S-H, MNP-E-S-CN, MNP-E-S-S-CH₃ and MNP-E-S-CH₂CONH₂); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

The binding of ADP to the enzyme and its various 2-mercuri-4-nitrophenol derivatives was monitored by the 8-anilino-naphthalene-1-sulphonic acid fluorescence-quenching method, and the data were analysed as described by McLaughlin (1974). Binding studies were performed in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C with enzyme concentrations of 0.25 mg/ml.

The difference-spectra measurements were made over the range 375–500 nm on a Varian series 634 spectrophotometer with cuvettes of 4.5 mm path length. The studies were performed in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C. Since the changes in absorbance were small, at least four determinations were made of each absorbance change reported. In no case did the values of (or differences in) absorbance differ from those in Table 1 by more than $10^{-3} \times 0.5$ unit.

Results and Discussion

Rabbit muscle creatine kinase was found to incorporate one 2-mercuri-4-nitrophenol group per subunit without loss of activity, a situation analogous to that described by Quiocho & Thomson (1973) for the enzyme from chicken breast muscle. A detailed analysis of the enzyme-catalysed reaction showed that the K_m values for substrates (0.5 mM and 10 mM for MgATP and creatine respectively) were essentially identical for MNP-enzyme and unmodified enzyme. In addition, the binding of ADP in the presence of other ligands was not significantly affected by incorporation of the 2-mercuri-4-nitrophenol group (Table 1).

The activity of MNP-enzyme was, however, sensitive to iodoacetamide, although the rate constant for this inactivation [$55 \text{ M}^{-1} \cdot \text{min}^{-1}$ at 25°C in 50mM-Tricine (sodium salt), pH 8.0] was substantially lower than that for unmodified enzyme ($500 \text{ M}^{-1} \cdot \text{min}^{-1}$) under the same conditions. The unmodified enzyme and MNP-enzyme exhibited similar conformational behaviour in the presence of Mg^{2+} (2 mM) plus ADP (1 mM) plus creatine (40 mM) plus nitrate (10 mM), as shown by the large decrease in the reactivity of the thiol group towards iodoacetamide (the rate constants were decreased by 82 and 68% for unmodified and MNP-enzyme respectively).

The present results show that the 2-mercuri-4-nitrophenol group is a useful probe for monitoring conformational changes in the enzyme since it causes minimal perturbation of the active site. Although the environment of the thiol group is changed to some extent in the MNP-enzyme (as shown by the lower rate constant for the iodoacetamide reaction), the conformational properties of the enzyme with regard to formation of the 'transition-state analogue' complex do not appear to be substantially altered.

Table 1. Nucleotide-binding and difference-spectra data for MNP-enzyme derivatives

Derivative	Relative activity	Dissociation constant for ADP (μM)			ΔA_{425}		Effect of nitrate (B)–(A)
		+Mg ²⁺	+Mg ²⁺ + creatine	Mg ²⁺ + creatine + nitrate	+Mg ²⁺ + ADP + creatine (A)	+Mg ²⁺ + ADP + creatine + nitrate (B)	
E-S-H	100	130	130	2, 12*	—	—	—
MNP-E-S-H	100	100	135	1.5, 10*	–0.0115	–0.0195	–0.008
MNP-E-S-CN	70	140	130	8, 60*	–0.0115	–0.0165	–0.005
MNP-E-S-S-CH ₃	40	110	140	15, 90*	–0.0110	–0.0145	–0.0035
MNP-E-S-CH ₂ CONH ₂	≤2	135	140	230	–0.0115	–0.0115	0.000

* Evidence for negative co-operativity in nucleotide binding.

Nucleotide-binding data were obtained in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C. Concentrations of ligands when added were: magnesium acetate, 5 mM; creatine, 40 mM; sodium nitrate, 10 mM. The values quoted represent those for K_1 and K_2 , respectively. The range of ADP concentrations used in the titrations was 2.5–700 μM . Difference-spectra measurements were made in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C, with additions of ligands as described in the legend to Fig. 1. The values have all been corrected (for the effects of dilution on addition of ligands) to the initial enzyme concentration of 0.7 mg/ml.

Fig. 1. Difference spectra obtained on addition of ligands to MNP-creatine kinase

Measurements were made in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C. I, Baseline scan (each cuvette containing 1.0ml of 0.7 mg of MNP-enzyme/ml and 2.4mM-magnesium acetate). II, 0.035 ml of 40mM-ADP was added to the sample cell in I. III, 0.455 ml of 100mM-creatine was added to the sample cell in II. IV, 0.01 ml of 1.5M-sodium nitrate was added to the sample cell in III. In all cases, a corresponding volume of buffer was added to the reference cell.

Introduction of the 2-mercuri-4-nitrophenol group also caused no significant change in the enzyme activities of the other derivatives studied (E-S-CN, E-S-S-CH₃ and E-S-CH₂CONH₂). However, as noted by Quijcho & Thomson (1973), it was found that at higher concentrations of 2-chloromercuri-4-nitrophenol more than one 2-mercuri-4-nitrophenol group could be introduced per enzyme subunit with a progressive loss of activity. Thus, at an extent of incorporation of 1.5 2-mercuri-4-nitrophenol groups into the enzyme, the activity had dropped to 80% of that of unmodified enzyme. In all experiments reported in the present paper, the degree of incorporation never exceeded 1.1 2-mercuri-4-nitrophenol groups per subunit.

The difference-spectra measurements on the MNP-enzyme derivatives showed the following. (i) Addition of Mg²⁺ at concentrations up to 5mM caused no difference spectrum. This is consistent with earlier studies that showed that the bivalent metal ion binds only very weakly to the enzyme in the absence of nucleotide (Watts, 1973). However, addition of ADP

(in the presence of Mg²⁺) caused a negative difference spectrum with a minimum at about 410nm (Fig. 1). Control experiments showed that there was no detectable change in the size of this difference spectrum when the ADP concentration was lowered from 1.35mM to 1mM (at a total Mg²⁺ concentration of 2.4mM) and when the total Mg²⁺-concentration was varied between 1.5mM and 3.5mM at an ADP concentration of 1mM. The magnitude of this difference spectrum induced by Mg²⁺ plus ADP was essentially identical for all the 2-mercuri-4-nitrophenol derivatives studied, irrespective of the degree of residual activity (Table 1). It would appear that the conformational change induced in the enzyme by binding of nucleotide is not affected by modification of the thiol group reactive towards iodoacetamide. The results of the binding studies (Table 1) also showed that the dissociation constants for ADP in the presence of Mg²⁺ were very similar for all the 2-mercuri-4-nitrophenol derivatives studied. (ii) Addition of creatine to the solution containing MNP-enzyme plus Mg²⁺ plus ADP led to a diminution in the difference spectrum accompanied by a small red shift (Fig. 1). The magnitude of this effect was identical for all the 2-mercuri-4-nitrophenol derivatives studied and was not due to a weakening of ADP binding, since the binding studies (Table 1) show that in all cases under the conditions employed the degree of saturation of the nucleotide site was at least 87%. The dilution of the solution on addition of creatine is corrected for in the results of the difference-spectra measurements summarized in Table 1. Control experiments showed that this procedure was valid since the size of the difference spectrum under these conditions was proportional to the concentration of enzyme present. It is apparent from these results that the ability of the enzyme to undergo the conformational changes associated with formation of the ternary complex (enzyme + MgADP + creatine) is not affected by modification of the thiol group reactive towards iodoacetamide. (iii) Addition of sodium nitrate (10mM) to the ternary complex (MNP-enzyme + MgADP + creatine) led to a marked increase in the size of the negative difference spectrum (Fig. 1). There was no detectable change in the difference spectrum when 10mM-sodium nitrate was added to the inactive MNP-E-S-CH₂CONH₂ derivative in the presence of MgADP plus creatine (Table 1). As shown in Table 1, the addition of 10mM-sodium nitrate led to intermediate increases in the difference spectra of the MNP-E-S-CN and MNP-E-S-S-CH₃ derivatives, which retained intermediate amounts of enzyme activity.

The addition of a higher concentration (200mM) of sodium nitrate to the ternary complexes led to larger changes in the difference spectra. These larger effects were not due solely to an increase in the ionic strength of the solutions since the addition of 200mM-sodium

acetate to ternary complexes caused no changes in the difference spectra. The sizes of the absorbance changes caused by addition of 200mM-sodium nitrate (-0.0205 , -0.0165 , -0.010 and -0.004 at 425 nm for the 2-mercuri-4-nitrophenol derivatives of E-S-H, E-S-CN, E-S-S-CH₃ and E-S-CH₂CONH₂ respectively at concentrations of 0.7mg/ml) could also be roughly correlated with the degree of activity of these derivatives (Table 1). However, a problem in the interpretation of the results obtained by addition of 200mM-sodium nitrate to the ternary complexes is that the inactive MNP-E-S-CH₂CONH₂ derivative shows a change in the difference spectrum on addition of nitrate. It is known that high concentrations of certain anions (e.g. nitrate) can affect the binding of nucleotides to inactivated forms of creatine kinase (Haugland, 1975), and it is possible that there may also be some other effects of high concentrations of nitrate, e.g. on the binding of creatine, which contains a carboxylate function. Nevertheless the results, taken together with those obtained at the lower (10mM) concentrations of nitrate, do strongly support the suggestion (Markham *et al.*, 1977) that the integrity of the thiol group reactive towards iodoacetamide is required for the conformational changes that the enzyme undergoes on formation of the 'transition-state analogue' complex (enzyme + MgADP + creatine + nitrate). Milner-White & Kelly (1976) have shown that the conformation of the enzyme in this complex is similar to that of the working enzyme, as judged by the reactivity of the thiol group towards iodoacetamide.

Addition of nitrate to the ternary complex (enzyme + MgADP + creatine) has been shown to tighten the binding of nucleotide (McLaughlin, 1974; Price & Hunter, 1976). As the data in Table 1 show, addition of 10mM-nitrate to the ternary complex of the inactive MNP-E-S-CH₂CONH₂ derivative weakened the binding of nucleotide, whereas in the cases of the partially active MNP-E-S-CN and MNP-E-S-S-CH₃ derivatives addition of nitrate caused a tightening of nucleotide binding, although

the degree of tightening was rather lower than that for the fully active MNP-E-S-H derivative. The present results can be correlated with the ability of the various derivatives to undergo the conformational changes associated with formation of the 'transition-state analogue' complex.

In summary, our results lend strong support to the hypothesis that the integrity of the thiol group in creatine kinase that is reactive towards iodoacetamide is required for the conformational changes that the enzyme undergoes on formation of the catalytically active complex. It is not apparently required for formation of the binary (enzyme + MgADP) or ternary (enzyme + MgADP + creatine) complexes.

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