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NON-IDENTICAL BEHAVIOUR OF THE SUBUNITS OF RABBIT MUSCLE CREATINE KINASE

NICHOLAS C. PRICE and MORAG G. HUNTER

Department of Biochemistry, University of Stirling, Stirling FK9 4LA, Scotland (U.K.)

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Summary

1. The dimeric enzyme creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) from rabbit muscle was reacted with three separate reagents, each of which specifically modifies one thiol group per subunit.

2. The reactions of the enzyme with these reagents (4-chloro-7-nitrobenzofurazan, 5,5′-dithiobis-(2-nitrobenzoic acid) and iodoacetate) all behave as normal second-order processes. This indicates that the thiol groups on the two subunits of the enzyme react at the same rate as each other in all three cases.

3. The effects of various ligands (Mg^{2+}, ADP and creatine, and combinations of these) on the kinetics of the reactions were studied. In all cases the reactions behave as normal second-order processes.

4. In the presence of the ligand combination Mg^{2+} plus ADP plus creatine plus nitrate, which has been postulated to form a "transition state analogue" complex with the enzyme, the reactions of the thiol group show considerable deviation from second-order kinetics. This indicates that the thiol groups on the two subunits react at different rates from each other. A similar effect is also noted in the presence of the combination ADP plus creatine plus nitrate.

5. The binding of ADP to the enzyme (studied by equilibrium dialysis) is hyperbolic in the absence of other ligands or in the presence of Mg^{2+} or Mg^{2+} plus creatine. The dissociation constant is similar in all three cases.

6. In the presence of creatine plus nitrate (with or without Mg^{2+}) the binding of ADP to the enzyme is tightened considerably and the binding plots indicate the presence of either negative interactions between the subunits or two distinct types of binding sites.

7. Possible causes for the observed non-identical behaviour of the two subunits of the enzyme are discussed.

Abbreviations: tricine, *N*-tris(hydroxymethyl)methyl glycine; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid); Nbf-Cl, 4-chloro-7-nitrobenzofurazan (this compound has been abbreviated as NBD-chloride elsewhere: the name and abbreviations here follow C.B.N. recommendations); ANS, 8-anilino-1-naphthalene sulphonate.

Introduction

Creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) from rabbit muscle occurs as a dimeric enzyme of molecular weight 82 000 [1]. The two subunits, which are very similar, if not identical, each possess a reactive thiol group, modification of which by a variety of reagents leads to inactivation of the enzyme [1]. As a result of a careful study of the effects of various anions on the properties of the enzyme in the presence of combinations of substrates, it has been postulated that a complex resembling the transition state of the reaction can be formed when various small planar anions (e.g. nitrate and formate) are added to a mixture of enzyme with Mg^{2+} plus ADP plus creatine [2]. This view has been supported by other studies involving magnetic resonance techniques [3,4] and fluorescent probes [5].

Until recently, little evidence had been presented to show that the two subunits of the rabbit muscle enzyme might not behave identically. However, using a fluorescent probe technique, McLaughlin [5] has reported that the binding of ADP to the enzyme in the presence of creatine plus nitrate (with or without Mg^{2+}) displayed features similar to negative cooperativity. The reactions of creatine kinase isolated from other sources (e.g. chicken heart, dogfish muscle and bovine brain) with thiol-modifying reagents display some unusual features [6—8] and an explanation has been proposed which involves interactions between the subunits of these enzymes [1].

In this paper we show that under conditions in which the "transition state analogue" complex is formed there is strong evidence for the non-identical behaviour of the two subunits of rabbit muscle creatine kinase. This shown in (i) studies of the reactivity of the thiol groups on the two subunits towards three reagents, and (ii) studies of ADP binding by equilibrium dialysis.

Materials and Methods

Creatine kinase was isolated from rabbit skeletal muscle as described previously [2]. Enzyme concentrations were determined spectrophotometrically using the published extinction coefficient [2]. The enzyme activity was assayed in the forward direction (phosphocreatine synthesis) by a coupled assay procedure involving pyruvate kinase and lactate dehydrogenase as coupling enzymes [9]. Under conditions chosen to provide more nearly saturating substrate concentrations (5 mM magnesium acetate, 4 mM ATP, 40 mM creatine in the presence of 100 mM sodium acetate at pH 9.0, $T = 30°C$) the specific activity of the enzyme was 130—150 μmol ATP consumed/min per mg protein, in agreement with previous determinations performed under these conditions using a pH-stat assay procedure [2]. The enzyme was tested for homogeneity by performing electrophoresis on polyacrylamide gels at pH 8.3 [10], and at pH 7.2 in the presence of sodium dodecyl sulphate [11]. In each case more than 90% of the total protein stained by Coomassie Blue migrated as a single band.

Tricine, Nbs_2, iodoacetic acid and the assay components were purchased from Sigma. The iodoacetic acid was recrystallised from light petroleum (b.p. 60—80°C) before use [12]. Iodo[2-^{14}C]acetic acid (specific activity 48 Ci/mol) was purchased from the Radiochemical Centre, Amersham, Bucks. Nbf-Cl was

purchased from Regis Chemical Co., Morton Grove, Ill., U.S.A. The reactions of Nbs_2 and Nbf-Cl with the enzyme were monitored spectrophotometrically by methods analogous to those previously described [13]. The reaction of iodoacetate with the enzyme was monitored in a number of ways: (i) by incorporation of radioactivity from iodo[2-^{14}C]acetate after aliquots of the reaction mixture had been quenched at known times in a 1% (v/v) solution of formic acid followed by dialysis [14], (ii) by observing the loss in enzyme activity [15], and (iii) by titrating the remaining thiol groups of aliquots withdrawn and diluted at known times, using excess (250 μM) Nbs_2. The third method was found to be the most convenient, but all three methods gave the same rate constant for the reaction of the enzyme with iodoacetate under standard conditions. The third type of method was also used to monitor the reaction of glutathione with iodoacetate.

Binding of ADP to creatine kinase was studied by equilibrium dialysis according to the previously published method [16]. ADP (sodium salt: Grade I) was purchased from Sigma. [8-^{14}C]ADP (specific activity 57 Ci/mol) was purchased as the ammonium salt from the Radiochemical Centre, Amersham, Bucks. During the period of equilibrium, there was a slight ($\leqslant$ 5%) breakdown of ADP, as measured by the pyruvate kinase-lactate dehydrogenase coupled assay system. No attempt was made to correct the results for this.

The results of these studies were analysed by Scatchard plots, according to the equation which characterises the binding of a ligand to equivalent and independent sites on a macromolecule [17]:

$$\frac{r}{[A]} = \frac{n}{K} - \frac{r}{K}$$

Where r equals the number of moles of ligand bound per mole of macromolecule, [A] equals the concentration of free ligand, n equals the number of ligand binding sites on the macromolecule and K equals the dissociation constant characterising the interaction of ligand and macromolecule.

If the plot of $\frac{r}{[A]}$ against r is linear, the sites are shown to be equivalent and independent. The intercept on the abscissa gives the value of n and the slope of the plot equals $-\frac{1}{K}$. Non-linearity of the plot of $\frac{r}{[A]}$ against r indicates that the sites are either not independent or are non-equivalent.

Results

The site of reaction of Nbf-Cl and Nbs_2 with creatine kinase

The reaction of iodoacetate with creatine kinase has been investigated in detail. Reaction of one thiol group in each subunit leads to inactivation [1,15]. It is not possible to check whether Nbf-Cl and Nbs_2 react at the same thiol group by standard peptide mapping techniques, because of the instability of the products of reaction especially towards disulphide interchange reactions [18]. However, a check on the site of reaction was performed by studying the effect of pre-treatment of the enzyme with iodoacetate on the subsequent reaction with Nbf-Cl and Nbs_2. The results of this type of experiment are shown in Fig. 1. Fig. 1a shows the data obtained when samples of enzyme which had been incubated with different amounts of iodoacetate were then assayed for

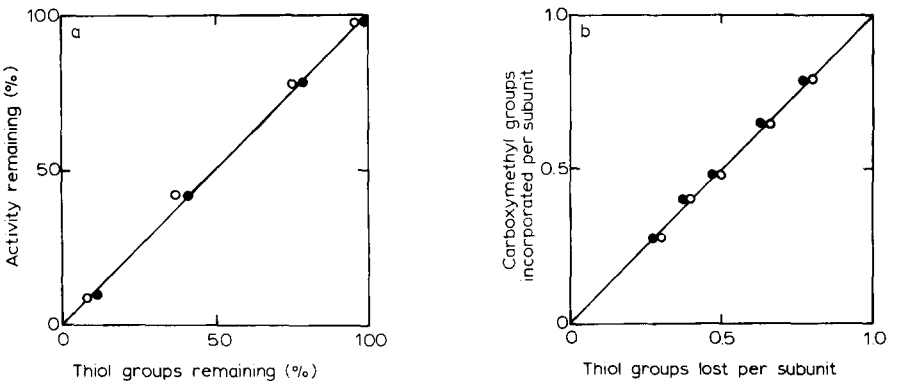

Fig. 1. The effect of pre-treatment of creatine kinase with iodoacetate on the subsequent reaction of the enzyme with Nbf-Cl and Nbs_2. (a) Enzyme incubated at 2 mg/ml in 50 mM sodium tricine buffer at pH 7.5 (T = 25° C) with various concentrations of iodoacetate. After dialysis to remove unreacted iodoacetate the samples were assayed for remaining enzyme activity and thiol group reaction using an excess of Nbf-Cl (○) or Nbs_2 (●). The values are expressed relative to a sample of enzyme which had been incubated under similar conditions with no iodoacetate. (b) Enzyme incubated under the conditions of Fig. 1a with iodo[2-^{14}C]acetate. After dialysis samples were assayed for incorporation of [^{14}C]carboxymethyl groups and for thiol group reaction with Nbf-Cl (○) and Nbs_2(●).

remaining activity and remaining reaction with Nbf-Cl and Nbs_2. Fig. 1b shows the result of an experiment in which the incorporation of [^{14}C]carboxymethyl groups from iodoacetate was measured and compared with the loss of subsequent reaction with Nbf-Cl and Nbs_2. The proportionality of the plots shown in Fig. 1 suggests very strongly that the two reagents react at the same thiol group on each subunit as does iodoacetate. It should be noted that in the one-well documented case in which a reagent (2-chloromercuri-4-nitrophenol) has been shown to modify a different thiol group in the native enzyme [19], prior reaction of the enzyme with iodoacetate has no effect on the subsequent modification reaction (Price, N.C., unpublished observation).

Reaction of Nbf-Cl with creatine kinase

As previously observed, Nbf-Cl reacts almost completely specifically with thiol groups at near neutral pH to give rise to a new absorption maximum near 420 nm [13]. Using the published value for the extinction coefficient of the product [13], a stoichiometry of 1.8 thiol groups reacted per creatine kinase dimer was calculated. This value was observed in all cases. There was no evidence from the absorption spectrum of the product for reaction of Nbf-Cl with any other amino acid side chains of the enzyme under the conditions used [13].

The reaction of Nbf-Cl with the enzyme was too rapid to analyse satisfactorily when carried out under pseudo first-order conditions (i.e. when Nbf-Cl was present in 15-fold excess over an enzyme concentration sufficient to yield a suitable increase in absorbance at 420 nm on reaction). Second-order conditions were thus routinely employed: the enzyme concentration in various experiments being in the range 10—25 μM (subunits) and the Nbf-Cl concentration being around 50 μM. The buffer in these experiments was 50 mM sodium tricine at pH 7.5 (T = 25° C).

Under these conditions the reaction of Nbf-Cl with the enzyme could be analysed as a second-order process (Fig. 2). The linearity of the plot (for at least 90% of the total reaction) shows that the two thiol groups react at the same rate with the reagent. From the slope of the second-order plot, the rate constant for the reaction is calculated to be $3.8 \cdot 10^4$ M^{-1}, compared with a value of $5.7 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ for the reaction of glutathione with Nbf-Cl under similar conditions.

The reaction of Nbf-Cl with the enzyme was also carried out in the presence of a variety of ligands, with the results shown in Table I. ADP (0.94 mM) causes a 20% increase in the rate constant; higher concentrations are without further effect. Mg^{2+} plus ADP cause an approximate doubling in the rate constant. When included separately in the reaction mixture magnesium acetate (2 mM), creatine (30 mM) and $NaNO_3$ (10 mM) have no significant effect on the rate constant. In all these cases, the reactions could be analysed as normal second-order processess for at least 90% of the total reaction. When the reaction is carried out in the presence of Mg^{2+} plus ADP plus creatine plus nitrate, however, two distinct effects are observed (Fig. 3). Firstly the reaction is slowed substantially compared with that observed in the presence of Mg^{2+} plus ADP plus creatine. Secondly, the second-order plot now shows distinct curva-

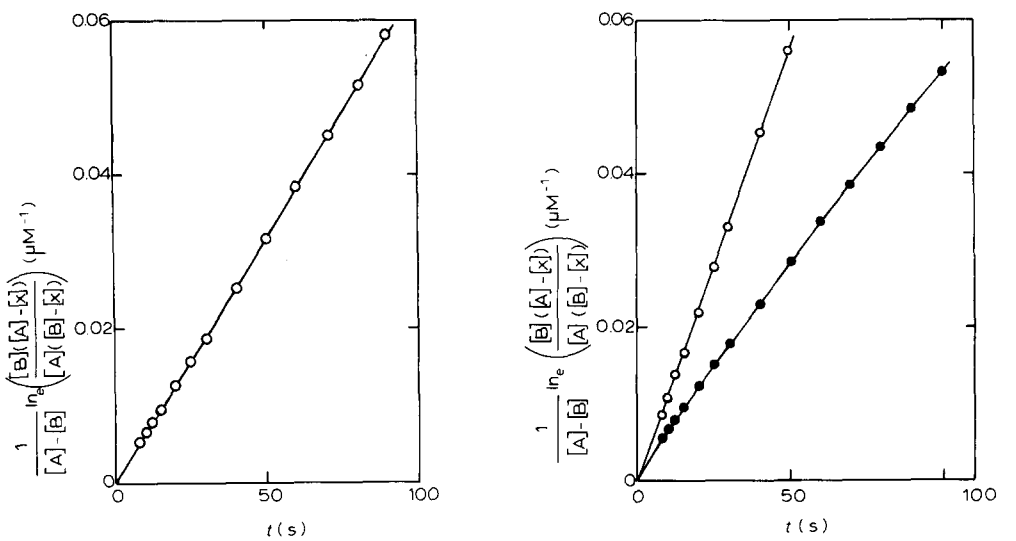

Fig. 2. The reaction of Nbf-Cl (54 μM) with creatine kinase (25 μM subunits) in 50 mM sodium tricine buffer at pH 7.5 (T = 25°C) analysed as a second-order process. The data cover 90% of the total reaction. On the ordinate. [A] refers to the initial concentration of Nbf-Cl, [B] to the initial concentration of thiol groups reacting (determined by the final absorbance change at 420 nm), and [x] to the concentration of product formed at time t.

Fig. 3. The effect of ligands on the reaction of Nbf-Cl with creatine kinase. ○, reaction performed in the presence of Mg^{2+} plus ADP plus creatine plus acetate; ●, reaction performed in the presence of Mg^{2+} plus ADP plus creatine plus nitrate (similar data were obtained when Mg^{2+} was omitted). The significance of the ordinate, and other conditions are as in Fig. 2. The concentrations of ligands, when added, are as in Table I.

TABLE I

THE REACTION OF RABBIT MUSCLE CREATINE KINASE WITH Nbf-Cl

The concentrations of Nbf-Cl and enzyme (subunits) were 54 and 25 μM, respectively, in 50 mM sodium tricine buffer at pH 7.5 (T = 25°C). The concentrations of ligands when added were: ADP, 0.94 mM; magnesium acetate, 2 mM; creatine, 30 mM; sodium nitrate, 10 mM; sodium acetate, 10 mM.

Additions	Second-order rate constant ($\times 10^{-4}$) ($M^{-1} \cdot min^{-1}$)
None	3.8
ADP	4.8
Mg^{2+} + ADP	7.8
Creatine	3.7
ADP + creatine	5.0
Mg^{2+} + ADP + creatine	7.0
Mg^{2+} + ADP + creatine + acetate	6.8

ture, which is especially apparent when the data from the later stages of the reaction are considered (Fig. 3). This shows that the two thiol groups are no longer reacting at the same rate with Nbf-Cl. These effects were also observed when the reaction was carried out in the presence of ADP plus creatine plus nitrate. In all other cases (i.e. with all other combinations of the ligands Mg^{2+}, ADP, creatine and nitrate, except the combinations in which ADP, creatine and nitrate were simultaneously present) the second-order plots were linear for at least 90% of the total reaction. The inclusion of sodium acetate had no effect on the reaction of the thiol groups with Nbf-Cl in the presence of Mg^{2+} plus ADP plus creatine (Table I and Fig. 3).

Reaction of Nbs_2 with creatine kinase

Using the published value for the extinction coefficient of the thionitrophenolate anion at 412 nm [20], Nbs_2 was shown to react with 1.8 thiol groups

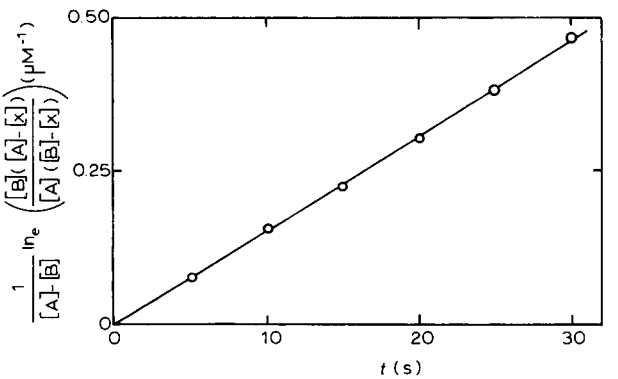

Fig. 4. The reaction of Nbs_2 (9 μM) with creatine kinase (4.5 μM subunits) in 50 mM sodium tricine buffer at pH 7.5 (T = 11°C) analysed as a second-order process. The data cover 90% of the total reaction. On the ordinate, [A] refers to the initial concentration of Nbs_2, [B] to the initial concn. thiol groups reacting, and [x] to the concentration of product formed at time t.

TABLE II

THE REACTION OF RABBIT MUSCLE CREATINE KINASE WITH Nbs_2

The concentration of enzyme (subunits) was 4.5 μM in 50 mM sodium tricine buffer at pH 7.5 (T = 11°C). The concentration of Nbs_2 was 9 μM, except in the reaction in which the effect of ADP was studied, when the concentration was 90 μM. The concentrations of ligands when added were as in Table I.

Additions	Second-order rate constant ($M^{-1} \cdot min^{-1} \times 10^{-4}$)
None	94
ADP	2
Mg^{2+} + ADP	60
Creatine	76
Mg^{2+} + ADP + creatine	46
Mg^{2+} + ADP + creatine + acetate	45

per creatine kinase dimer in all cases studied. In order to facilitate an analysis of the kinetics of the reaction, it was necessary to lower the Nbs_2 concentration to 8—10 μM, the creatine kinase concentration to around 5 μM (subunits), and the temperature of reaction to 11°C.

Under the conditions employed, the reaction of Nbs_2 with the enzyme could be analysed as a second-order reaction (Fig. 4). As before, the linearity of the plot (for at least 90% of the total reaction) shows that the two thiol groups react at the same rate with Nbs_2. The rate constant for the reaction is $9.4 \cdot 10^5$ $M^{-1} \cdot min^{-1}$ compared with a value of $2.6 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ for the reaction of glutathione with Nbs_2 under these conditions.

The effects of inclusion of ligands on the reaction are summarised in Table II. Mg^{2+} plus ADP cause a 35% decrease in the rate constant; ADP alone has a much more pronounced effect. Creatine causes a small (20%) decrease in the rate constant. Magnesium acetate (2 mM) is without effect on the rate constant. All the reactions summarised in Table II follow second-order kinetics for at least 90% of the total reaction.

In the presence of ADP plus creatine plus nitrate (with or without Mg^{2+}), the reaction of Nbs_2 with the enzyme was much slower than in the absence of ligands, and the second-order kinetic plots showed very marked curvature (Fig. 5). It was possible in these cases to perform the reactions under pseudo first-order conditions. This enables the differences in reactivities of the two thiol groups to be analysed in more detail [21] than is the case under second-order conditions [14]. Semi-logarithmic plots for these pseudo first-order reactions performed in the presence and absence of Mg^{2+} are shown in Fig. 6. In each case the analysis [21] showed 0.85 ± 0.05 thiol group per dimer in the fast phase of the reaction and 0.95 ± 0.05 thiol group per dimer in the slow phase. The rate constants derived from the analysis were: (Mg^{2+} present), fast phase $1.2 \cdot 10^5$ $M^{-1} \cdot min^{-1}$, slow phase $2.3 \cdot 10^4$ $M^{-1} \cdot min^{-1}$; (Mg^{2+} absent), fast phase $2.3 \cdot 10^4$ $M^{-1} \cdot min^{-1}$, slow phase $5.2 \cdot 10^3$ $M^{-1} \cdot min^{-1}$.

As in the case of Nbf-Cl, there was no effect of sodium acetate on the reaction of the thiol groups with Nbs_2 in the presence of Mg^{2+} plus ADP plus creatine (Table II).

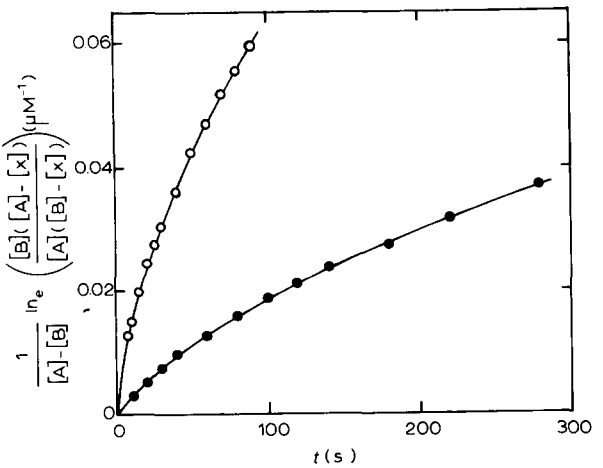

Fig. 5. The effects of ligands on the reaction of Nbs_2 (40 μM) with creatine kinase (5 μM subunits) under the conditions of Fig. 4. ○, reaction performed in the presence of Mg^{2+} plus ADP plus creatine plus nitrate; ●, reaction performed in the presence of ADP plus creatine plus nitrate. The data refers to 90 and 80% of the total reactions, respectively. The significance of the ordinate is as in Fig. 4, and the concentrations of ligands, when added, are as in Table I.

Reaction of iodoacetate with creatine kinase

The reaction of iodoacetate with rabbit muscle creatine kinase has been investigated in some detail by a number of workers [1,15,22]. In agreement with this work, it was found that the reaction follows second-order kinetics until at least 85% of the total reaction is completed (Fig. 7). The rate constant for the

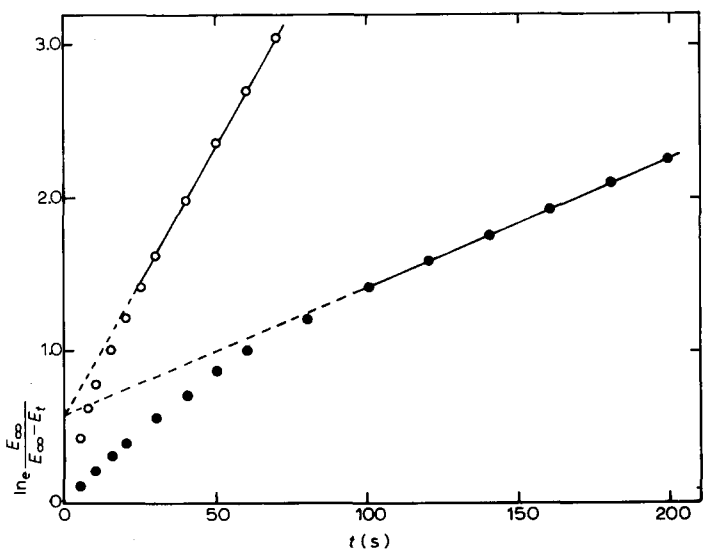

Fig. 6. Semi-logarithmic plots for the reaction of Nbs_2 (90 μM) with creatine kinase (5 μM subunits) in the presence of ligands. ○, reaction performed in the presence of Mg^{2+} plus ADP plus creatine plus nitrate; ●, reaction performed in the presence of ADP plus creatine plus nitrate. Other reaction conditions are as in Fig. 5. On the ordinate, E_∞ is the final absorbance change at 412 nm, E_t is the absorbance change at time t.

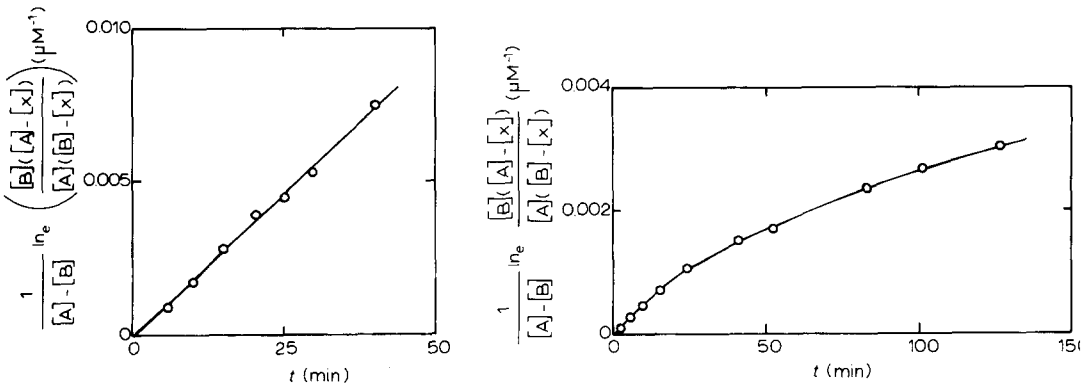

Fig. 7. The reaction of iodoacetate (275 μM) with creatine kinase (105 μM subunits) in 50 mM sodium tricine buffer at pH 7.5 (T = 25°C) analysed as a second-order process. The data cover 85% of the total reaction. On the ordinate, [A] refers to the initial concentration of iodoacetate, [B] to the initial concentration of thiol groups reacting, and [x] to the concentration of carboxymethylated thiol groups formed at time t; determined by back-titration with Nbs_2 as described in the text.

Fig. 8. The effect of ligands on the raction of iodoacetate (760 μM) with creatine kinase (120 μM subunits). The reaction was performed in the presence of Mg^{2+} plus ADP plus creatine plus nitrate (similar data were obtained when Mg^{2+} was omitted). The data cover 85% of the total reaction. Other conditions, and the significance of the ordinate are as in Fig. 7. The concentrations of ligands, when added, are as in Table I.

reaction of the two thiol groups is 180 $M^{-1} \cdot min^{-1}$, compared with a value of 1 $M^{-1} \cdot min^{-1}$ for the reaction of glutathione under these conditions. The effects of ligands on the reaction between iodoacetate and creatine kinase are summarised in Table III, and are substantially in accord with previous observations [15,22]. All these reactions followed second-order kinetics for at least 85% of the total reaction.

In the presence of ADP plus creatine plus nitrate (with or without Mg^{2+}), the second-order plots showed distinct curvature (Fig. 8), again showing that the two thiol groups of the enzyme react at different rates with the reagent in these cases. The reactions in the presence of these combinations of ligands are slower

TABLE III

THE REACTION OF RABBIT MUSCLE CREATINE KINASE WITH IODOACETATE

The concentration of enzyme (subunits) was 100 μM in 50 mM sodium tricine buffer at pH 7.5 (T = 25°C). The iodoacetate concentrations varied from 200 to 500 μM for the different experiments. The concentrations of ligands when added were as in Table I.

Additions	Second-order rate constant ($M^{-1} \cdot min^{-1}$)
None	180
ADP	35
Mg^{2+} + ADP	370
Creatine	190
Mg^{2+} + ADP + creatine	300

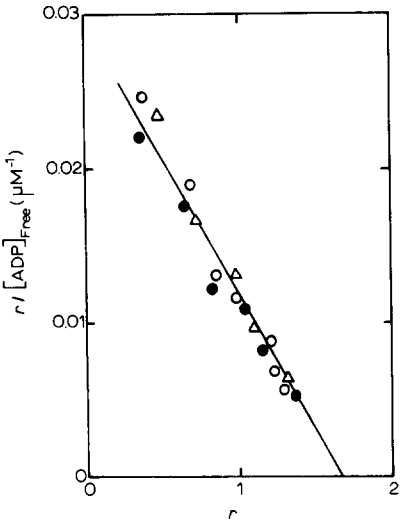

Fig. 9. Scatchard plots for the binding of ADP to creatine kinase in 50 mM sodium tricine buffer at pH 7.5 (T = 18°C). r represents the mol ADP bound per mol creatine kinase dimer. △, binding of ADP; ●, binding of ADP in the presence of 5 mM magnesium acetate; ○, binding of ADP in the presence of 5 mM magnesium acetate plus 30 mM creatine. The enzyme concentration used was 2 mg/ml.

than in the absence of ligands (compare Figs. 7 and 8), but this decrease in rate is not as pronounced as in the case of reaction of iodoacetamide with the enzyme [2].

Binding of ADP to creatine kinase

The results of the studies of binding of ADP to creatine kinase are shown in Figs. 9 and 10 in the form of Scatchard plots [17]. Fig. 9 shows the data for ADP binding (i) in the absence of other ligands, (ii) in the presence of 5 mM magnesium acetate, and (iii) in the presence of 5 mM magnesium acetate plus

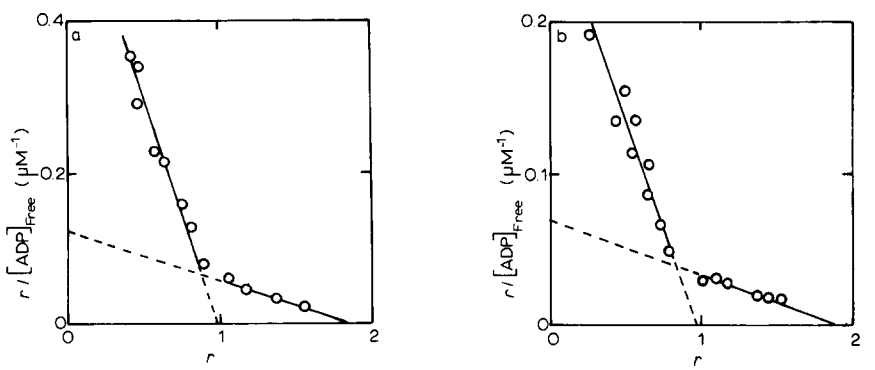

Fig. 10. Scatchard plots for the binding of ADP to creatine kinase. (a) Binding of ADP in the presence of 5 mM magnesium acetate plus 30 mM creatine plus 10 mM $NaNO_3$. (b) Binding of ADP in the presence of 30 mM creatine plus 10 mM $NaNO_3$. The enzyme concentration used was 0.4 mg/ml. Other conditions and the significance of the axes are as in Fig. 9.

30 mM creatine. The binding characteristics are very similar in these three cases, as previously noted [5]. In each case the linearity of the plot shows that the binding is hyperbolic with a stoichiometry of 1.7 sites per creatine kinase dimer, and a dissociation constant of 55 μM. This latter value is in good agreement with other estimates made under comparable conditions [4,5].

In the presence of creatine plus nitrate (in the presence or absence of Mg^{2+}), two main effects are observed (Fig. 10). Firstly, the binding of ADP to the enzyme is considerably tightened. Secondly, the two binding sites are no longer equal and independent. From the plots shown in Fig. 10, the following dissociation constants can be estimated [23]: (Mg^{2+} present), K_1 = 1.5 μM, K_2 = 18 μM; (Mg^{2+} absent), K_1 = 3.5 μM, K_2 = 28 μM. These results are comparable with those obtained using a fluorescent probe technique under slightly different conditions [5].

Discussion

The results reported here show that under conditions in which rabbit muscle creatine kinase forms the postulated "transition state analogue" complex [2] (i.e. enzyme plus Mg^{2+} ADP plus creatine plus nitrate), the two subunits of the enzyme behave non-identically, both in terms of ADP binding and thiol group reactivity towards a number of reagents. If the complex does indeed represent a model of the enzyme in the transition state of the phosphoryl transfer reaction, this non-identical behaviour of the subunits could well have implications for understanding the catalytic and possible regulatory mechanisms of the enzyme.

The non-identical behaviour of the subunits of creatine kinase has been referred to in a previous study of ADP binding to the enzyme using ANS as a fluorescent probe [5]. It is important to note that the interpretation of the ANS fluorescence experiments can be equivocal when the binding is studied in the presence of creatine plus nitrate, since it is necessary to assume that the change in the fluorescence of ANS bound to the enzyme is a linear function of the fractional saturation of the ADP sites. (This point is particularly difficult to establish in those cases where there may be interactions between the subunits of the enzyme). The technique used in this paper (equilibrium dialysis) is free from these difficulties of interpretation, since a direct separation of free from bound ligand is made, and the concentrations of ligands are determined directly from measurements of radioactivity.

This paper also presents clear evidence that the thiol groups on the two subunits of rabbit muscle creatine kinase can, under certain conditions, react at different rates with various modifying reagents. This possibility has also been referred to in a recent paper by Milner-White and Kelly [24]. It is important to analyse as wide a range of the total reaction as possible in order to detect this differential reactivity. If data from only the first portion (e.g. 30%) of the total reaction in the presence of ADP plus creatine plus nitrate are analysed, the reactions may not appear to depart significantly from second-order processes. However, the curvature of the second-order plots becomes clearer as a greater proportion of the total reaction is analysed.

The specificity of nitrate in causing the differential reactivity of the thiol groups towards Nbf-Cl and Nbs_2 was shown by the fact that sodium acetate

(even at concentrations as high as 50 mM) has no effect on the linearity of the second-order kinetic plots when the reactions were performed in the presence of Mg^{2+} plus ADP plus creatine. This difference between the effects of nitrate and acetate is consistent with previous studies using magnetic resonance and kinetic techniques [2—4].

In the case of the reaction of the enzyme with Nbs_2 in the presence of ADP plus creatine plus nitrate it was possible to estimate the actual difference in reactivity of the two thiol groups, since the reactions could be conveniently carried out under pseudo first-order conditions (Fig. 6). The results show that the differences in reactivity are 5.2-fold in the presence of Mg^{2+} and 4.4-fold in the absence of Mg^{2+}. These differences in reactivity are not sufficient, however, to permit the preparation of enzyme specifically modified in only one subunit by reacting the dimeric enzyme with one equivalent of Nbs_2 in the presence of Mg^{2+} plus ADP plus creatine plus nitrate. Using the method outlined previously [14] it can be calculated that under these conditions, 15% of the total protein would be unmodified, 15% would be modified at both thiol groups and 70% would be modified at one thiol group.

The observed non-identical behaviour of the subunits of the enzyme could arise from two possible causes:

(i) In the complex of the enzyme with ADP plus creatine plus nitrate (in the presence or absence of Mg^{2+}), the two subunits have identical tertiary structures. However, on dissociation of ADP from one subunit, or one modification of the thiol group on one subunit, the resulting conformational change is relayed to the second subunit. This would correspond to the type of subunit interactions proposed in the "sequential" model of Kohsland et al. [25], which allows negative cooperativity of ligand binding.

(ii) In the complex of the enzyme with ADP plus creatine plus nitrate (in the presence or absence of Mg^{2+}), the two subunits have different tertiary structures and behave as different types of subunit in the experiments described.

From the results presented here it is not possible to decide between these two possibilities. A choice could be made if data on the symmetry of the enzyme in the presence of ADP plus creatine plus nitrate were available (e.g. from X-ray crystallographic studies). Thus, the presence of a 2-fold axis of symmetry in this complex would indicate the first possibility was correct.

Levitzki [26] has made a detailed study of a related problem in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. This tetrameric enzyme has a rapidly reacting thiol group (Cys-149) in each subunit. With some modifying reagents (e.g. 4-iodoacetamido-salicylic acid) reaction of two out of the four thiol groups leads to complete inactivation of the enzyme whereas with other reagents (e.g. iodoacetamide) all four groups must be modified to cause complete inactivation. From these and related spectroscopic studies [27] it was concluded that the different behaviour was related to the differing abilities of the various modifying groups introduced at Cys-149 to interact with part of a neighbouring subunit. In the case of creatine kinase it would clearly be of value to examine the behaviour of modifying reagents which introduce only a small perturbation at the reactive thiol groups [28], since this might help to distinguish between the two possible causes of the non-identical behaviour of the enzyme under "transition state analogue" conditions.

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