The Reaction of Rabbit Muscle Creatine Kinase with some Derivatives of Iodoacetamide

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The dimeric enzyme creatine kinase from rabbit muscle was treated with three derivatives of iodoacetamide that are capable of introducing fluorescent groups into the enzyme. All three reagents {4-iodoacetamidosalicylate (IAS), 5-[N-(iodoacetamidoethyl)amino]naphthalene-1-sulphonate (IAEDANS) and 6-(4-iodoacetamidophenyl)aminonaphthalene-2-sulphonate (IAANS)} were shown to react at the same single thiol group on each enzyme subunit, leading to complete inactivation of the enzyme. The reaction with IAS was extremely rapid by comparison with the reaction with iodoacetamide or iodoacetate, but various lines of evidence suggest that IAS is not a true affinity label. However, kinetic and binding studies indicate that salicylate itself probably binds at the nucleotide-binding site on the enzyme. As the size of the modifying reagent increased, the first thiol group reacted more rapidly than the second; this trend was more pronounced at 0°C than at 25°C. With the largest modifying reagent used (IAANS), the pronounced biphasic nature of the modification reaction permitted the preparation of a hybrid enzyme in which only one subunit was modified, but a study of the thiol-group reactivity showed that this hybrid enzyme preparation underwent subunit rearrangement.

Rabbit muscle creatine kinase (ATP-creatine *N*-phosphotransferase, EC 2.7.3.2) consists of two very similar, if not identical, subunits each of mol.wt. 41000 and each possessing a reactive thiol group (Watts, 1973). It has been known for some time that reaction of this thiol group with a number of reagents, such as iodoacetamide, iodoacetate or Nbs₂, leads to inactivation of the enzyme (Watts, 1973), although subsequent work (der Terrossian & Kassab, 1976; Maggio *et al.*, 1977), using modifying reagents that

introduce only a small perturbation, has shown that the thiol group cannot be considered 'essential' for activity and probably plays an important role in various substrate-induced conformational changes in the enzyme (Keighren & Price, 1978).

The present paper deals with the reaction of the enzyme with various derivatives of iodoacetamide, which are capable of introducing a fluorescent moiety into the enzyme. The reagents used in the study, shown below, are IAS, IAEDANS and IAANS.

Abbreviations used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); IAS, 4-iodoacetamidosalicylate; IAEDANS, 5-[N-(iodoacetamidoethyl)amino]naphthalene-1-sulphonate; IAANS, 6-(4-iodoacetamidophenyl)aminonaphthalene-2-sulphonate; E-AS, E-AEDANS and E-AANS derivatives refer to the enzyme inactivated by reaction with IAS, IAEDANS and IAANS respectively (in each case the extent of incorporation is between 0.95 and 1.0 group per enzyme subunit); Nbf-Cl, 4-chloro-7-nitrobenzofurazan.

The kinetics of these modification reactions were compared with those of the reaction of the enzyme with iodoacetamide. The results of these studies prompted an examination of the interaction of the enzyme with salicylate. Haugland (1975) has published a short account of the reaction of IAANS with the enzyme, but no attempt was made to establish the sites of reaction or the stoichiometry of modification.

Materials and Methods

Creatine kinase was isolated from rabbit skeletal muscle and routinely assayed as described previously (Price & Hunter, 1976). In the assays, Mg^{2+} was added at an excess of 1 mM over ATP; this ensures that over 84% of the ATP is present as the $MgATP^{2-}$ complex over the range of ATP concentrations used (Storer & Cornish-Bowden, 1976). Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, dithiothreitol, ADP (grade I), iodoacetamide and iodoacetic acid were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Before use the iodoacetamide was recrystallized from hot water and the iodoacetic acid from light petroleum (b.p. 60–80°C). Salicylic acid (AnalaR grade) and 8-anilino-naphthalene-1-sulphonate were purchased from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-50 was purchased from Pharmacia (G.B.), London W5 5SS, U.K. [$8-^{14}C$]ADP (sp. radioactivity 57 Ci/mol) was purchased as the ammonium salt from The Radiochemical Centre, Amersham, Bucks., U.K. IAEDANS was purchased from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K. IAANS was obtained from Molecular Probes, Roseville, MN 55113, U.S.A. Nbf-Cl was purchased from Regis Chemical Co., Morton Grove, IL, U.S.A.

4-Iodoacetamidosalicylic acid was synthesized by the general method of Baker *et al.* (1962) and recrystallized from ethanol/water (2:3, v/v). The recrystallized product had m.p. 206–207°C, identical with the literature value (Rosen *et al.*, 1973).

Reactions of the enzyme with the various reagents were carried out in the dark in 50 mM-Tricine (sodium salt) buffer at pH 8.0, and at 25°C unless otherwise stated. The extents of incorporation of reagent into the enzyme were determined by spectral analysis of the product formed in each case after excess reagent had been removed by dialysis against 50 mM-Tricine (sodium salt) buffer at pH 8.0 or by gel filtration (Sephadex G-50). The appropriate values of the absorption coefficients in the cases of IAS and IAEDANS have been published (Malcolm & Radda, 1970; Hudson & Weber, 1973). For IAANS the absorption coefficient was found to be 26 litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$ at 320 nm for the reagent in 50 mM-Tricine (sodium salt) buffer at pH 8.0, and this value was assumed to be unchanged when IAANS was bound to the enzyme.

The rates of reaction of the reagents with the enzyme were routinely studied by monitoring the loss in enzyme activity of samples withdrawn from the reaction mixtures at known times and diluted into 0.1 M-glycine/NaOH buffer, pH 9.0, containing 2 mM-dithiothreitol (at 0°C) to stop the reaction. Control experiments were carried out to check that this procedure would effectively stop the reaction in each case. In these control experiments, reagent was added to

the enzyme (at the concentrations of both that would prevail after the dilution procedure) in 0.1 M-glycine/NaOH buffer, pH 9.0, containing 2 mM-dithiothreitol. In each case there was no inactivation of the enzyme caused by the reagent under these conditions, showing that the dithiothreitol would effectively react with excess reagent and hence stop the enzyme modification at the time of dilution. In addition, it was found that the enzyme activity of the diluted samples did not change over a 3 h incubation period at 0°C. The kinetic data were analysed as second-order processes, as described previously (Price & Hunter, 1976).

For unmodified enzyme and enzyme inactivated by reaction with iodoacetate or iodoacetamide, the binding of ADP to the enzyme samples was monitored by the 8-anilino-naphthalene-1-sulphonate-fluorescence-quenching method described by McLaughlin (1974). The validity of this method was checked for unmodified enzyme by performing equilibrium-dialysis experiments using [$8-^{14}C$]ADP; the two methods gave very similar results (compare Table 1 and Fig. 6). For the E-AS, E-AEDANS and E-AANS derivatives the binding of ADP was monitored by observing the changes in fluorescence of the attached chromophores (for the E-AS derivative, a control experiment showed that this procedure gave the same result as the 8-anilino-naphthalene-1-sulphonate-fluorescence-quenching method).

The effects of salicylate on the binding of ADP to the unmodified enzyme were studied by equilibrium dialysis, by using [$8-^{14}C$]ADP as described previously (Price & Hunter, 1976). The 8-anilino-naphthalene-1-sulphonate-fluorescence-quenching method could not be used in these experiments, since the fluorescence of the added salicylate effectively masked that from 8-anilino-naphthalene-1-sulphonate.

The effects of salicylate on the kinetics of the enzyme-catalysed reaction were not studied by using the normal coupled assay procedure because of the expected inhibition of the coupling enzymes pyruvate kinase and lactate dehydrogenase by salicylate. Instead the assay method of Kuby *et al.* (1954), based on the production of acid-labile phosphocreatine, was used in these studies and assays were carried out at 25°C in 50 mM-Tricine (sodium salt) buffer at pH 8.0. Although the inclusion of salicylate in the assay mixtures led to the formation of a slight yellow colour on addition of portions of these mixtures to the acidified molybdate solution, this did not interfere with the spectrophotometric determination of phosphate after addition of reducing agent.

Results and Discussion

Site of the reaction of the reagents

Previous studies have shown that Nbs_2 reacts at the same single thiol group on each subunit of creatine

kinase as does iodoacetate or iodoacetamide (Watts, 1973; Price & Hunter, 1976). As shown in Fig. 1(a), modification of the enzyme by IAS, IAEDANS or IAANS led to a proportional decrease in subsequent reaction with Nbs_2 . In each case no more than one group could be incorporated per subunit even after incubation of the enzyme with a 4-fold molar excess of reagent (expressed with respect to the subunit concentration) for 2 h at 25°C. These data suggest very strongly that all three reagents, IAS, IAEDANS and IAANS, react at the same thiol group as does iodoacetamide. 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 enzyme (Quioco & Thomson, 1973; Laue & Quioco, 1977), prior reaction of the enzyme with iodoacetamide has no effect on the stoichiometry of the subsequent modification reaction (Keighren & Price, 1978).

Reaction of the enzyme with IAS, IAEDANS or IAANS leads to inactivation in each case. Fig. 1(b) shows a plot of the residual activity against extent of modification as measured spectrophotometrically, and shows that enzyme that has reacted to the limiting extent of one thiol group per subunit is essentially inactive.

Kinetics of the modification reactions

Table 1 shows the data obtained when the reactions were studied at 25°C and at 0°C. For comparison, the data for reactions of the enzyme with iodoacetamide and iodoacetate are also included in Table 1. The appropriate second-order kinetic plots for the reactions with IAS, IAEDANS and IAANS are shown in Figs. 2–4; in each case the data shown cover at least 85% of the total reaction. For iodoacetamide and iodoacetate the second-order plots were linear for at least 85% of the reaction at 25°C and at 0°C.

The data in Table 1 show that there is a tendency towards a biphasic reaction as the size of the reagent increases. This tendency is particularly pronounced at 0°C, where the non-linearity of the second-order plots in all cases except with iodoacetamide and iodoacetate as modifying reagents shows that the two thiol groups on the enzyme dimer are reacting at different rates with the reagent.

The effect of reagent size on the characteristics of the reaction probably arises from a conformational change induced in the second subunit after incorporation of a bulky aromatic moiety at the thiol group on the first subunit. This situation is somewhat analogous to that described by Levitzki (1974), who studied the reactivity of the thiol groups in the tetrameric enzyme glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle. In this case, it was found that with some large reagents (such as 4-iodoacetamidonaphthol) reaction of only two out of the four thiol groups led to complete inactivation of the enzyme, whereas with small modifying reagents (such as iodoacetamide) reaction of all four thiol groups was required for inactivation. These effects were interpreted in terms of the ability of bulky modifying groups introduced at the thiol group in one subunit to interact with part of a neighbouring subunit (Schlessinger & Levitzki, 1974).

The effect of temperature on the characteristics of the reactions with the reagents of intermediate size (IAS and IAEDANS) is more difficult to interpret, since the changes in the properties of the enzyme over this temperature range are poorly documented

Fig. 1. Reaction of creatine kinase with iodoacetamide derivatives: (●) IAS, (○) IAEDANS, (□) IAANS. Reactions were performed in 50mM-Tricine (sodium salt) buffer at pH 8.0. (a) Extent of incorporation per subunit as a function of the loss in subsequent reaction with Nbs_2 . The extents of incorporation were determined spectrophotometrically after dialysis of reaction mixtures, as described in the text. (b) Relative activity of derivatives as a function of the extent of incorporation per subunit.

Table 1. *Reactions of creatine kinase with iodoacetamide derivatives*

Reactions were carried out in 50 mM-Tricine (sodium salt) buffer at pH 8.0. Concentrations of ligands when added were: ADP, 0.95 mM; magnesium acetate, 2 mM; salicylate, 50 mM.

Reagent	Second-order rate constant ($\text{M}^{-1} \cdot \text{min}^{-1}$)		Second-order rate constant ($\text{M}^{-1} \cdot \text{min}^{-1}$) at 25°C in presence of ligands			Dissociation constant for ADP complex with modified enzyme (μM)
	25°C	0°C	ADP (% change)	Mg ²⁺ + ADP (% change)	Salicylate (% change)	
Iodoacetamide	450	23	440 (−2%)	520 (+15%)	525 (+17%)	20
Iodoacetate	160	8	22 (−86%)	300 (+88%)	27 (−83%)	75
IAS	110000	Biphasic reaction	15000 (−86%)	110000 (0%)	24000 (−78%)	280
IAEDANS	16000	Biphasic reaction	4100 (−74%)	7850 (−51%)	6250 (−61%)	210
IAANS	Biphasic reaction	Biphasic reaction	No effect*	No effect*	No effect*	25
Unmodified enzyme	—	—	—	—	—	45

* Although the kinetics of the biphasic reactions with IAANS were not analysed in detail, there was no effect of the ligands on the rate of inactivation of the enzyme for at least 60% of the total reaction.

Fig. 2. Reactions of creatine kinase with IAS analysed as second-order processes

The reactions were performed in 50 mM-Tricine (sodium salt) buffer at pH 8.0, and the results shown cover 85% of the total reaction in each case. (a) Re-

(Watts, 1973). There may well be differences in the strength and/or type of subunit interactions at 25°C and at 0°C: these possibilities could be examined by studying in more detail the effects of temperature on the parameters of the enzyme-catalysed reaction and on substrate binding. It is possible that a temperature transition exists of the type noted, e.g., for glycogen phosphorylase *b* (Birkett *et al.*, 1971).

Effect of ligands on the modification reactions

The effects of inclusion of the ligands ADP, MgADP and salicylate on the modification reactions at 25°C are included in Table 1. It is clear that these effects are complex and that the effect of a given ligand depends on the nature of the modifying reagent. Thus MgADP can cause a large (iodoacetate) or small (iodoacetamide) increase in rate, no effect (IAS, IAANS), or a marked decrease in rate (IAEDANS). Any explanation based solely on ligand-induced conformational changes is clearly inadequate, and other factors that may need to be

action carried out at 25°C. Enzyme concentration = 2 μM (subunits); IAS concentration = 7.5 μM . (b) Reaction carried out at 0°C. Enzyme concentration = 9 μM (subunits); IAS concentration = 51 μM . On the ordinate $f(x)$ is defined by:

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

where A and B are initial concentrations of the reagent in excess and enzyme subunits respectively, and x is the concentration of product (modified enzyme) formed at a given time.

Fig. 3. Reactions of creatine kinase with IAEDANS analysed as second-order processes

Reactions were performed in 50mM-Tricine (sodium salt) buffer at pH 8.0. The significance of the ordinate is as in Fig. 2. The results shown cover 85% of the total reaction in each case. (a) Reaction carried out at 25°C. Enzyme concentration = 25 μM (subunits); IAEDANS concentration = 54 μM . (b) Reaction carried out at 0°C. Enzyme concentration = 25 μM (subunits); IAEDANS concentration = 170 μM .

taken into account include possible interactions between ligand and reagent on the enzyme and between reagent and enzyme, which may be of a steric, electrostatic or hydrophobic type. Watts (1973) has referred to some of these difficulties in interpreting the effects of ligands on the reactions of the enzyme with iodoacetate and with iodoacetamide. In general, salicylate has effects similar to those caused by ADP (Table 1).

Fig. 4. Reactions of creatine kinase with IAANS analysed as second-order processes

Reactions were carried out in 50mM-Tricine (sodium salt) buffer at pH 8.0. The significance of the ordinate is as in Fig. 2. The results shown cover 85% of the total reaction in each case. (a) Reaction carried out at 25°C. Enzyme concentration = 86 μM (subunits); IAANS concentration = 190 μM . (b) Reaction carried out at 0°C. Enzyme concentration = 86 μM (subunits); IAANS concentration = 300 μM .

Reaction of creatine kinase with IAS

The data in Table 1 show that the rate of reaction of the enzyme with IAS is some 220-fold higher than with iodoacetamide and some 690-fold higher than with iodoacetate. In view of the results of X-ray-crystallographic studies, which have shown that salicylate and various of its iodinated derivatives can bind to the adenine-binding site of liver alcohol dehydrogenase (Einarsson *et al.*, 1974) and pig muscle adenylate kinase (Pai *et al.*, 1977), it was considered possible that IAS might act as an 'affinity label' for creatine kinase, with the salicylate moiety directing the reagent to the adenine-binding site of the enzyme and thus giving a high local concentration of IAS in the region of the thiol group. If IAS does indeed behave as an 'affinity label' the distance between the reactive thiol group and the adenine nucleotide-binding site on the enzyme would be defined.

Fig. 5. Inhibition of creatine kinase by salicylate

Assays were carried out at 25°C in 50 mM-Tricine (sodium salt) buffer at pH 8.0 (in the absence of added sodium acetate). ○, No salicylate; △, 5 mM-salicylate; □, 10 mM-salicylate; ●, 15 mM-salicylate; ▲, 20 mM-salicylate. One unit is that amount of enzyme consuming $1 \mu\text{mol}$ of substrate/min. (a) Double-reciprocal plot with $MgATP^{2-}$ as variable substrate. In these experiments the creatine concentration was 32 mM and the free Mg^{2+} concentration 1 mM. (b) Double-reciprocal plot with creatine as variable substrate. In these experiments the $MgATP^{2-}$ concentration was 2.8 mM (free Mg^{2+} concentration = 1 mM). (c) Secondary plot showing the slopes of the lines in (a) as a function of the salicylate concentration.

Evidence regarding the possibility of IAS acting as an 'affinity label' for creatine kinase can be discussed under the following headings.

(i) *Inhibition of the enzyme by salicylate.* Salicylate was shown to behave as a competitive inhibitor of the enzyme with respect to $MgATP$ and as a non-competitive inhibitor with respect to creatine in the direction of phosphocreatine synthesis (Figs. 5a and 5b). Since creatine kinase has a rapid-equilibrium random-order mechanism (Morrison & James, 1965; Watts, 1973), this behaviour is consistent with salicylate binding to the nucleotide-binding site on the enzyme. It should be noted, however, that the plot of the slopes of the primary plot ($1/\text{velocity}$ against $1/[MgATP]$) against salicylate concentration is non-linear (Fig. 5c), so salicylate should more properly be termed a 'parabolic competitive inhibitor' with respect to $MgATP$ (Cleland, 1963). One possible explanation for this is the existence of more than one salicylate-binding site on the enzyme subunit, occupation of any of which precludes nucleotide binding. Both pig muscle lactate dehydrogenase isoenzyme 5 and yeast phosphoglycerate kinase appear to possess two binding sites for salicylate (Cheshire & Park, 1977; Larsson-Raznikiewicz & Wiksell, 1978). Salicylate does not appear to inhibit the enzyme by chelating the bivalent metal ion, since raising the Mg^{2+} concentration from 5 to 20 mM in the assay mixture did not decrease the inhibition caused by 10 mM-salicylate (in these assays the ATP and creatine concentrations were 4 and 40 mM respectively).

(ii) *Effect of salicylate on nucleotide binding.* Salicylate was shown to weaken the binding of ADP in the absence or presence of Mg^{2+} (5 mM). The data are shown in Fig. 6 in the form of Scatchard plots and it is clear that the lines intersect on the abscissa, showing that salicylate competitively inhibits nucleotide binding. A plot of the apparent dissociation constant against salicylate concentration is non-linear, which may again indicate that there is more than one binding site for salicylate on the enzyme subunit.

(iii) *Effect of modification on nucleotide binding.* The results of studies of ADP binding to the modified enzyme derivatives (summarized in Table 1) show that reaction of the enzyme with IAS has the largest effect on nucleotide binding, the dissociation constant for ADP being some 5 times that for unmodified enzyme. These results would be consistent with the idea that the salicylate moiety in the modified enzyme is occupying at least part of the nucleotide-binding site. It is noteworthy that reaction of the enzyme with the bulkiest reagent (IAANS) does not interfere with nucleotide binding, showing that the effect of modification by IAS does not arise from the size of the modifying group introduced.

(iv) *Dependence of rate on IAS concentration.* The dependence of the rate of inactivation of an enzyme on the concentration of an affinity label should show a 'saturation effect' (Malcolm & Radda, 1970) because of the formation of a 'Michaelis-type' complex between enzyme and reagent before the chemical modification. It was impracticable to study the rate of

Fig. 6. Effect of salicylate on the binding of ADP to creatine kinase

The binding was studied at 20°C in 50mM-Tricine (sodium salt) buffer at pH8.0, with an enzyme concentration of 3mg/ml. ○, No salicylate; □, 5mM-salicylate; ●, 10mM-salicylate. On the axes, r represents the mol of ADP bound/mol of enzyme (dimer of mol.wt.82000). (a) Binding studied in the absence of added Mg^{2+} ions. The dissociation constants with 0, 5mM- and 10mM-salicylate are 55 μ M, 145 μ M and 245 μ M respectively. (b) Binding studied in the presence of 5mM-magnesium acetate. The dissociation constants with 0, 5mM- and 10mM-salicylate are 80 μ M, 170 μ M and 270 μ M respectively.

the reaction in this case at IAS concentrations above 35 μ M (at an enzyme concentration of 2 μ M-subunits), since more than 85% of the reaction would be com-

pleted in 30s. Over the range of IAS concentrations from 8 to 35 μ M (enzyme concentration=2 μ M-subunits) there was no evidence for any variation in the second-order rate constant of the reaction and thus no evidence for any 'Michaelis-type' complex could be obtained. However, since the dissociation constant for the enzyme-salicylate complex is of the order of 5mM (this is the concentration of salicylate that gives half-maximal protection against reaction of the enzyme with iodoacetamide or IAS), it is possible that the 'saturation effect' would only be observed by the use of IAS concentrations in this range, and this would require the use of rapid-reaction techniques.

(v) *Protection by substrate.* One of the criteria suggested to assess a putative 'affinity label' for an enzyme is that the presence of substrates should protect against reaction (Singer, 1967). The data (Table 1) show that although inclusion of ADP or salicylate does lead to substantial protection against modification by IAS, inclusion of MgADP is without effect. Although the difficulties in interpretation of this type of data have already been mentioned, the lack of effect of MgADP makes it unlikely that IAS can be acting as an 'affinity label' with the salicylate moiety directing the reagent to the nucleotide-binding site on the enzyme. It is possible that the salicylate moiety of IAS could interact with another salicylate-binding site on the enzyme, distinct from the adenine-binding site (the existence of which is indicated by the kinetic and binding studies described above), to give rise to the high rate of reaction. Alternatively, the effect of MgADP is 'special' in some way in that the induced conformational change, which would tend to increase the reactivity of the thiol group (as in the reaction with iodoacetate), is counteracted by the occupation of the salicylate-binding site by substrate.

In summary, although there is good evidence for the binding of salicylate at the nucleotide-binding site on the enzyme, in view of the lack of protection by MgADP it would appear that IAS cannot be definitely considered to be an 'affinity label' for creatine kinase. Nevertheless there are interesting possibilities for further investigation of the reactions of IAS with other kinases and dehydrogenases.

Reaction of creatine kinase with IAANS

The data in Fig. 4 show that the reaction of creatine kinase with IAANS is markedly biphasic. At concentrations of enzyme subunits and reagent of 86 and 190 μ M respectively at 25°C, reaction of one thiol group per dimer was complete within 1min, whereas reaction of the second thiol group was only about 70% complete after 60min. Such a marked difference in reactivity should enable 'hybrid' molecules to be prepared in which only one subunit is modified. A routine procedure was to make 100 μ M-

subunits react with $120\mu\text{M}$ -IAANS for 2min in 50mM -Tricine (sodium salt) buffer, pH8.0 (at 20°C), and then to add dithiothreitol (final concentration 5mM) to stop the reaction. Gel filtration through a column ($2.5\text{cm}\times 15\text{cm}$) of Sephadex G-50 was used to remove excess dithiothreitol and the dithiothreitol-IAANS adduct. The enzyme derivative contained 0.96 -AANS group per dimer and possessed 49% of the activity and 50% of the thiol groups reactive towards Nbs_2 compared with unmodified enzyme. Some studies on this derivative were undertaken to assess the extent to which the subunits of creatine kinase might function independently.

Properties of the 'hybrid' enzyme derivative

A detailed analysis of the kinetics of the enzyme-catalysed reaction showed that the K_m values for substrates were identical for the unmodified and the 'hybrid' enzyme derivative. (The values for MgATP and creatine were 0.3mM and 10mM respectively, measured at a free Mg^{2+} concentration of 1mM in 0.1M -glycine/ NaOH buffer, pH9.0, at 30°C .) The V_{max} value for the 'hybrid' derivative was 48% of that for unmodified enzyme. The reactivity of the thiol group in the 'hybrid' enzyme derivative towards iodoacetamide is very similar to the reactivity of the two thiol groups in unmodified enzyme [the respective second-order rate constants are $430\text{M}^{-1}\cdot\text{min}^{-1}$ and $450\text{M}^{-1}\cdot\text{min}^{-1}$ in 50mM -Tricine (sodium salt) buffer, pH8.0, at 25°C]. Also the effect of the ligand combination Mg^{2+} (2mM)+ADP (1mM)+creatine (30mM)+nitrate (10mM), which has been postulated to form a complex resembling the transition state of the enzyme-catalysed reaction (Milner-White & Watts, 1971; McLaughlin *et al.*, 1976; Milner-White & Kelly, 1976), on the reactivity of the thiol group towards iodoacetamide is very similar for the two cases (82% and 85% decreases in the initial rate of reaction for the unmodified enzyme and the 'hybrid' derivative respectively).

A study by equilibrium dialysis of the binding of $[8\text{-}^{14}\text{C}]\text{ADP}$ to the 'hybrid' enzyme derivative in the presence of Mg^{2+} (5mM)+creatine (30mM)+nitrate (10mM) showed that there was a strong nucleotide-binding site [$K_d=2.5\mu\text{M}$ in 50mM -Tricine (sodium salt) buffer at pH8.0, at 20°C], as for unmodified enzyme ($K_d=1.5\mu\text{M}$ under these conditions; Price & Hunter, 1976).

These results might be interpreted as indicating that in the 'hybrid' enzyme derivative the unmodified subunit is capable of functioning as a 'normal' subunit in the unmodified enzyme. However, before such a conclusion could be drawn it would be necessary to establish that the enzyme derivative did in fact consist of 100% 'hybrid' molecules. Two types of experiment were carried out to check whether or not this was the case.

Fig. 7. Reactions of unmodified creatine kinase (●) and 'hybrid' enzyme derivative (○) with IAANS

The reactions were performed in 50mM -Tricine (sodium salt) buffer at pH8.0 (at 25°C). The concentration of IAANS in each experiment was $19.6\mu\text{M}$; the concentrations of reacting thiol groups (as determined by reaction with Nbs_2) were $11.2\mu\text{M}$ (unmodified enzyme) and $9.5\mu\text{M}$ ('hybrid' enzyme derivative).

Fig. 8. Reactions of unmodified creatine kinase (●) and 'hybrid' enzyme derivative (○) with Nbf-Cl

The reactions were performed in 50mM -Tricine (sodium salt) buffer at pH8.0 (at 25°C) in the presence of 2mM -magnesium acetate plus 1mM -ADP plus 30mM -creatine plus 10mM - NaNO_3 . The meaning of the ordinate is as in Fig. 2, and the results shown cover at least 85% of the total reaction in each case. In each experiment the concentration of Nbf-Cl was $47\mu\text{M}$; the concentrations of reacting thiol groups were $3.9\mu\text{M}$ (unmodified enzyme) and $3.6\mu\text{M}$ ('hybrid' enzyme derivative).

(i) *Reaction with IAANS.* If the derivative consisted of 100% 'hybrid' molecules, the further reaction with IAANS should be characterized by a slow rate of

reaction (comparable with the rate of the slow phase of the reaction of unmodified enzyme) with a single second-order rate constant. The results of this type of experiment (Fig. 7) show that in fact reaction of the 'hybrid' enzyme derivative with IAANS follows a time course very similar to that of the unmodified enzyme, with a clearly biphasic second-order kinetic plot. This implies that the hybrid enzyme has, within the time period of preparation and gel filtration (about 30 min), undergone disproportionation to yield a mixture of fully modified and unmodified enzyme, the latter then reacting in a biphasic reaction with IAANS.

(ii) *Reaction with Nbf-Cl*. Previous work (Price & Hunter, 1976) has shown that the thiol groups on the two subunits of unmodified enzyme react at identical rates with Nbf-Cl, both in the absence of ligands and in the presence of combinations of Mg^{2+} , ADP and creatine, but as the transition-state analogue complex (in the presence of $Mg^{2+} + ADP + creatine + NO_3^-$) the two thiol groups react at different rates. For the hybrid enzyme derivative, the reaction with Nbf-Cl (corresponding to one thiol group per dimer) in 50 mM-Tricine (sodium salt) buffer at pH 8.0 and 25°C obeyed second-order kinetics for at least 90% of the total reaction, both in the absence of ligands and in the presence of Mg^{2+} (2 mM) + ADP (1 mM), and the rate constants for these reactions ($35000 M^{-1} \cdot min^{-1}$ and $70000 M^{-1} \cdot min^{-1}$ respectively) were very similar to those previously reported under slightly different conditions (Price & Hunter, 1976). However, as the transition-state analogue complex, the hybrid enzyme reacted with Nbf-Cl in a biphasic fashion and showed a second-order kinetic plot very similar to that of unmodified enzyme (Fig. 8). This biphasic behaviour could not arise from a homogeneous population of hybrid enzyme molecules and therefore this result again implies that disproportionation to yield some unmodified enzyme has occurred.

These two sets of experiments indicate that the 'hybrid' enzyme derivative (which is presumably the initial product after the fast phase of the reaction between enzyme and IAANS) is not stable and, within the 30 min time period (at 20°C) of preparation and gel filtration, has undergone disproportionation to yield a mixture of unmodified enzyme and fully modified enzyme. The disproportionation presumably occurs via dissociation of the 'hybrid' enzyme into subunits, which then combine to form the homodimers, and it is likely that the large -AANS group incorporated into one subunit causes a distortion within that subunit leading to an increased tendency to dissociation.

There are at least two analogous subunit-rearrangement processes known: (i) the equilibrium between the rabbit muscle and yeast glyceraldehyde 3-phosphate dehydrogenase enzymes (R_4 and Y_4

respectively) and the hybrid R_2Y_2 species, which occurs via dissociation to the R_2 and Y_2 dimer species (Osborne & Holloway, 1974, 1975, 1976); (ii) the disproportionation of *Escherichia coli* aspartate transcarbamoylase lacking a regulatory subunit to give a mixture of unmodified enzyme and catalytic trimer subunits (Subramani *et al.*, 1977). The rates of these subunit-exchange processes have been investigated, as have the effects of ligands on these rates. It would be of interest to investigate the rate of the disproportionation process with 'hybrid' creatine kinase; at present it appears that within 30 min the process is substantially completed, since the biphasic characteristics of the reactions with IAANS or with Nbf-Cl in the presence of the 'transition-state analogue' complex are not further altered by standing at 20°C for a further 2 h.

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