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A Study of the 'Reactive' Sulphydryl Groups of Adenosine 5'-triphosphate-Creatine Phosphotransferase

BY D. C. WATTS* AND B. R. RABIN

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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Creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) catalyses the reaction shown in eqn. (1):

with pH optima for the forward reaction (formation of ADP) and reverse reaction of approximately 9.0 and 7.0 respectively (Kuby, Noda & Lardy, 1954b). The enzyme has two 'reactive' sulphydryl groups (Benesch, Lardy & Benesch, 1955), and the reaction of these with a variety of reagents inhibits the phosphate-transfer reaction (Mahowald & Kuby, 1960; Watts, Rabin & Crook, 1961).

* Beit Memorial Fellow.

Most samples of the enzyme, as prepared from individual rabbits, have two active sites, and a single sulphydryl group is associated with each. One sample of the enzyme, with only one active site, has been prepared in this Laboratory. In this preparation only one of the 'reactive' sulphydryl groups is concerned with the catalytic activity, and blocking of the other is without significant effect on the catalytic function (Watts, Rabin & Crook, 1962). Thus the existence of a single 'active' sulphydryl group associated with each catalytic site of the enzyme is a tenable hypothesis. Its function in the catalytic process has proved more contentious (Dixon, 1949; Padieu & Mommaerts, 1960; Ennor & Morrison, 1958; Rabin & Watts, 1960). The

present experiments were undertaken with a view to a further elucidation of this problem. A preliminary account of some of this work has been given (Rabin & Watts, 1960).

MATERIALS AND METHODS

Materials

Crystalline creatine kinase. This was prepared by 'method B' of Kuby, Noda & Lardy (1954a). Residual activity in the absence of added Mg^{2+} ions was removed by dialysing the enzyme solution (approx. 3%, v/v) for 8 hr. against 2 l. of mM-EDTA and then for 24 hr. in each of two changes of de-ionized distilled water. A molecular weight of 81 000 was assumed and the enzyme concentrations were determined spectrophotometrically (Noda, Kuby & Lardy, 1954). For the data presented here, a preparation containing one catalytic site per molecule (Watts *et al.* 1962) was used. The experiments were subsequently checked with preparations containing two catalytic sites per molecule.

Chemicals. Iodoacetamide and iodoacetic acid were prepared and purified as described by Watts *et al.* (1961).

The crystalline disodium salts of ATP and ADP (Sigma) were used without further purification.

Creatine was recrystallized from hot water. Phosphocreatine was prepared by a slight modification of the method of Ennor & Stocken (1948). The phosphoryl chloride was diluted with 3 vol. of sodium-dried light petroleum (b.p. 80–100°). The final product was found by chromatography [with propan-1-ol-aq. ammonia (sp.gr. 0.880)–water (6:3:1, by vol.)] to be free from inorganic phosphate and to contain only a trace of free creatine.

Tris (L. Light and Co. Ltd.) was purified by the procedure of Crook, Mathias & Rabin (1960). *N*-Ethylmorpholine hydrochloride (p*K* 7.65), recrystallized from acetone-ethanol (2:1, v/v), was a gift from Dr C. A. Ross. Recrystallized pentane-3,3-dicarboxylic acid (p*K*₂ 7.29) was a gift from Dr D. G. Herries.

All other reagents were reagent grade or, where obtainable, AnalaR.

Buffers. Boric acid-sodium hydroxide, pH 9.0, was prepared by mixing appropriate amounts of 0.1*N*-boric acid and 0.1*N*-sodium hydroxide.

Tris-nitric acid buffer was prepared by adjusting 0.2*N*-nitric acid to pH 7.0 with saturated tris solution and diluting the final solution to 0.1*N* with respect to nitric acid. The pH was rechecked at the final dilution.

N-Ethylmorpholine-*N*-ethylmorpholine hydrochloride, pH 7.0, 1.0*M*, was made by adding *N*-sodium hydroxide (4.63 ml.) to *N*-ethylmorpholine hydrochloride (3.7908 g.), and diluting the solution to 250 ml.

Pentane-3,3-dicarboxylic acid-sodium hydroxide, pH 7.0, 1.0*M*, was made by diluting 0.1*M*-pentane-3,3-dicarboxylic acid with an equal volume of water containing sodium hydroxide to give a final concentration of 80.1 mM, pH 7.0. The final volume also contained potassium nitrate to give the correct ionic strength.

Methods

The reactions of iodoacetate and iodoacetamide with creatine kinase were followed with a silver-silver iodide electrode assembly coupled to a Radiometer titrimeter

TTTla and titrigraph SBR2B. Creatine-kinase activity was determined by measuring the phosphocreatine formed in the forward reaction at pH 9.0 in 4 min. at 37°. The details of both these assays are described by Watts *et al.* (1961).

In the experiments where the effects of substrates on the inhibition of enzyme activity by iodo compounds were investigated, a control, without substrate, was set up for each substrate combination tested. Samples were removed at the times shown and, after the inhibition had been stopped with 0.01*M*-cysteine, pH 9.0, were assayed for creatine-kinase activity. The control samples, after being mixed with cysteine, were adjusted to contain the same concentrations of substrates as the samples from the test mixture. In this way the composition of the enzyme assay mixtures was identical for both the test and control samples. The controls vary slightly in composition, since they contain small amounts of the different substrates under test. This factor rarely contributed more than an additional 5% to the concentrations of substrates in the assay and, as Fig. 3 shows, causes no significant difference.

RESULTS

The reaction of iodoacetate with creatine kinase is first-order with respect to the enzyme and there is a rapid release of 2 moles of iodide/mole of enzyme (Watts *et al.* 1961). Similar results are obtained with iodoacetamide.

Effect of ionic strength. The effect of ionic strength on the initial velocity of the alkylation reaction is shown for iodoacetamide in Fig. 1. Varying the ionic strength from 0.012 to 0.25 is without effect at either pH 7.0 or 9.0. With iodoacetate (Fig. 2), varying the ionic strength has a marked effect on the initial velocity of inhibition.

Fig. 1. Effect of ionic strength on the reaction of iodoacetamide with creatine kinase at 25°. ●, At pH 7.0 (pentane-3,3-dicarboxylate-NaOH buffer), creatine kinase, 9.65 μM; ○, at pH 9.0 (borate-NaOH buffer), creatine kinase, 4.95 μM. The concentrations of other reagents were: iodoacetamide, 0.1 mM; KI, 1.0 μM; Tween 80, 0.02% (v/v); the ionic strength was adjusted with potassium nitrate. The total volume was 5 ml.

Fig. 2. Effect of ionic strength on the reaction of iodoacetate with creatine kinase at 25°. ●, At pH 7.0 (*N*-ethylmorpholine hydrochloride-NaOH); ○, at pH 9.0 (borate-NaOH buffer). The concentrations of the reagents were: iodoacetate, 1.0 mM; creatine kinase, 1.48 μ M; the ionic strength was adjusted with potassium nitrate. The total volume was 2.5 ml. For enzyme assay, 0.25 ml. samples were withdrawn.

Fig. 3. Effect of substrates, in the absence of Mg^{2+} ions, on the inhibition of creatine kinase by iodoacetamide. The concentrations of the reagents were: creatine kinase, 2.5 μ M; iodoacetamide, 0.45 mM. The final volume was 5 ml. At the times shown 0.5 ml. samples were transferred to 10 mM-cysteine in 40 mM-borate-NaOH buffer to remove unchanged iodoacetamide, and each sample was assayed for creatine-kinase activity. At pH 9.0 (40 mM-borate-NaOH buffer): ▲, control; △, plus creatine (10 mM) and ATP (20 mM). At pH 7.0 (20 mM-tris- HNO_3 buffer): ●, control; ○, plus phosphocreatine (5 mM) and ADP (2 mM).

The effect is more marked at pH 7.0, where a plot of $\log v_i$ against $I^{\frac{1}{2}}$ gives a slope of -2.0 , than at pH 9.0, where the slope is decreased to -0.64 . At $I \approx 0.1$ the rate of inhibition becomes the same at both pH 7.0 and 9.0. The experiment at pH 7.0 was repeated with tris buffer instead of *N*-ethylmorpholine and the same results were obtained.

Effect of pH. Rabin & Watts (1960) showed that the initial velocity of the reaction of iodoacetamide and iodoacetate with creatine kinase was independent of pH over the range 6–10. This finding was confirmed by measuring the inhibition of creatine-kinase activity. Fig. 3 shows that the progress curve with iodoacetamide is the same at pH 7.0 and 9.0.

Effect of substrates. From Fig. 2 it is clear that an apparent protection by substrates against iodoacetate could be observed simply by the addition of substrates which increased the ionic strength of the mixture, particularly combinations involving nucleotides. To avoid this possibility, it is essential to perform these experiments at constant ionic strength.

Marked protection of creatine kinase against inhibition by either iodoacetate or iodoacetamide occurred only in the presence of both substrates and the activating metal ion. The progress curves for activity loss and the alkylation reaction with iodoacetate in the presence and absence of the substrate equilibrium mixture and Mg^{2+} ions at pH 9.0 have been presented by Watts *et al.* (1962). Fig. 3 shows that, in the absence of Mg^{2+} ions, neither ATP plus creatine at pH 9.0 nor ADP plus phosphocreatine at pH 7.0 protects the enzyme from inactivation by iodoacetamide. A summary of the effects of various combinations of substrates and Mg^{2+} ions on the rate of alkylation of creatine kinase by iodoacetate and iodoacetamide at pH 7.0 and 9.0 is given in Table 1. The ionic contribution of added substrates and metal ion was not allowed to exceed 20 % of the total ionic strength and was computed from the stability constants of Smith & Alberty (1956).

Since metal-free creatine kinase is activated to a greater extent by Mn^{2+} ions than by Mg^{2+} ions (Cho, Haslett & Jenden, 1960; Watts, 1961), some substrate combinations were tried in which Mg^{2+} ions were replaced by Mn^{2+} ions. The results confirmed the observations with Mg^{2+} ions although the experiments were complicated by the finding that preincubation of the enzyme with Mn^{2+} ions in the absence of the alkylating agent caused considerable inactivation of the enzyme.

DISCUSSION

The results presented here for the reaction of creatine kinase with iodoacetate (Table 1) are in disagreement with those of Padieu & Mommaerts

Table 1. *Effects of substrates on the reaction of creatine kinase with iodoacetamide or iodoacetate at 25° and ionic strength 0.12*

Experimental details are given in the text. The substrate concentrations used were: Mg^{2+} ion, 1 mM; ADP, 1 mM; ATP, 1 mM; creatine, 10 mM; phosphocreatine, 1.4 mM. Other concentrations were: iodoacetamide, 0.1 mM; iodoacetate, 0.5 mM. Reactions carried out at pH 9.0 were in borate-NaOH buffer, and those at pH 7.0 in pentane-3,3-dicarboxylate-NaOH buffer. Initial velocities are expressed relative to that of the control, which was taken to be 1.00.

pH	Substrates	Relative initial velocity	
		Iodoacetamide	Iodoacetate
9.0	None (control)	1.00	1.00
9.0	Equilibrium mixture* + Mg^{2+}	0.66	0.65
9.0	Equilibrium mixture†	1.05	0.91
9.0	Mg^{2+}	0.97	1.01
9.0	ATP	1.00	0.96
9.0	ATP + Mg^{2+}	1.00	0.81
9.0	ATP + creatine	1.07	0.96
9.0	Creatine	1.07	1.03
9.0	Creatine + Mg^{2+}	0.93	1.01
9.0	ADP	1.00	1.00
9.0	ADP + Mg^{2+}	1.01	1.27
7.0	Mg^{2+}	—	1.00
7.0	ATP	1.00	0.93
7.0	ATP + Mg^{2+}	0.99	0.87
7.0	ADP	1.00	0.78
7.0	ADP + Mg^{2+}	1.00	1.24
7.0	Phosphocreatine	—	0.98
7.0	Phosphocreatine + Mg^{2+}	—	0.98

* Obtained by allowing the reaction mixture containing ATP (1 mM), creatine (10 mM) and Mg^{2+} ion (1 mM) to proceed to equilibrium before the addition of the alkylating reagent.

† Approximate only; the reaction mixture contained ADP (0.5 mM), ATP (0.5 mM), phosphocreatine (0.7 mM) and creatine (5 mM).

(1960), who found that absolute protection was obtained at 0° against iodoacetate by ATP or phosphocreatine alone and some protection by ADP, AMP or pyrophosphate. They did not investigate substrate combinations or the effect of Mg^{2+} ions. We have been unable to establish the reason for the disagreement, which must reflect differences in the experimental approach. Changes in the conformation of the protein in passing from 0° to 25° could account for the different results obtained. We could not obtain complete protection by the addition of any substrate, either alone or in combination with other substrates, at relatively high concentrations (10 mM), and these results have been corroborated by following the alkylation reaction as well as by loss of enzyme activity.

The rate of reaction of creatine kinase with iodoacetamide (Fig. 1) varies little with ionic strength, showing the absence of any primary salt effect, as expected for a reaction involving a

neutral molecule (Bell, 1941). If the reaction were due to the attack of a mercaptide ion on the reagent, as occurs for the reaction of simple thiols with chloroacetamide (Lindley, 1960), a secondary salt effect, due to the displacement of the mercaptan-mercaptide equilibrium, would be expected. This is not observed, suggesting that the reactive species on the protein is not a mercaptide ion.

The unusual nature of the 'reactive' sulphhydryl group is further shown by the effect of pH on the reaction rate (Fig. 3, and Rabin & Watts, 1960). The reactions of the protein with both iodoacetate and iodoacetamide are essentially independent of pH over the range 6.0–10.0. It can be concluded that no ionization of the type mercaptan → mercaptide ion occurs for the 'reactive' sulphhydryl groups of the protein. Further, the reaction rate is intermediate between that expected for a mercaptan and a mercaptide ion. Simple thiols, e.g. cysteine, readily react with the silver-silver iodide electrode at pH 9.0, whereas creatine kinase does not (Watts *et al.* 1961) except in 8M-urea (Watts, 1961). These facts suggest strongly that the sulphhydryl groups of creatine kinase are hydrogen-bonded to a base species on the protein (I).

This would raise the pK of the sulphhydryl group and lower the pK of the conjugate acid of B. The rate of reaction of this hydrogen-bonded pair with alkylating reagents should be greater than a mercaptan but less than a mercaptide ion. The sulphur atom of the combination should be more basic than SH but less basic than S^- . The group B is probably either a carboxylate ion or an imidazole group since, from the pH data, the pK of its conjugate acid is probably below 6. An imidazole group would seem more probable, since the introduction of negative charges in or near the catalytic centre would repel the phosphate chain of ATP. The properties predicted for a hydrogen-bonded thiol-imidazole pair are essentially those found for creatine kinase.

The effect of ionic strength on the reaction of creatine kinase with iodoacetate can be interpreted as being due to electrostatic interaction between the carboxyl group of the reagent and positive charges on the protein which would decrease with increasing ionic strength. The decrease in the ionic-strength effect in passing from pH 7.0 to 9.0 could be due to partial removal of positive charge on increasing the pH. Further interpretation must await a more detailed kinetic analysis.

The effect of various components of the substrate system on the rates of alkylation is more difficult to interpret for iodoacetate than for iodoacetamide because of the complications introduced by the electrostatic interactions. None of the substrates, individually, in the presence or absence of Mg^{2+} ions, significantly alters the reaction of creatine kinase with iodoacetamide. This is definite evidence that the 'reactive' sulphydryl group is not involved in binding the components. It can also be concluded that the 'reactive' sulphydryl group does not bind the metal ion.

The presence of the equilibrium mixture and Mg^{2+} ions reduces considerably the rate of reaction with iodoacetamide, suggesting that the 'reactive' sulphydryl group is involved in the actual catalytic process. This is supported by the effects of ATP or ADP on the reaction of iodoacetate with creatine kinase in the presence but not in the absence of Mg^{2+} ions (Table 1). These show that the sulphydryl group is sufficiently close to the nucleotide- Mg^{2+} ion binding sites to permit electrostatic interactions with iodoacetate but not steric hindrance, as is shown by the analogous experiments with the uncharged iodoacetamide. These results are also in agreement with the report by Kuby & Mahowald (1959) that the binding of ATP or ADP is dependent on the presence of Mg^{2+} ions. Protection of creatine kinase by the complete creatine-kinase system, but not by individual components, has been observed for heat denaturation (Kuby *et al.* 1954*b*) and for the reaction with chicken antibody (Samuels, 1961).

The reaction of creatine kinase with iodoacetate is very much reduced by the presence of the equilibrium mixture plus Mg^{2+} ions. At both pH 7.0 and 9.0, ATP plus Mg^{2+} ions protects the enzyme slightly against iodoacetate. This effect is probably due to the negative charges on the ATP repelling the reagent; the Mg^{2+} ions are probably required for correct orientation of the terminal phosphate group of ATP. The slight effect by ATP alone could well be due to trace metal impurities. The Mg^{2+} ions are probably bound simultaneously by ATP and the protein, but there are almost certainly additional interactions between the enzyme and ATP.

In the presence of Mg^{2+} ions, ADP enhances the rate of reaction of creatine kinase with iodoacetate. An explanation for this effect can be given. Suppose the ATP- Mg^{2+} ion-enzyme complex can be represented by (II), then by the principle of microscopic reversibility the complex for ADP would be (III). The vacant co-ordination site on the Mg^{2+} ion, occupied by the phosphate group of phosphocreatine in the reverse reaction, could bind the carboxyl group of iodoacetate to give the mixed complex (IV). The formation of this mixed

complex might well account for the enhanced reaction rate. Since Mg^{2+} ions alone are without effect on the alkylation reaction (Table 1), this hypothesis implies that the presence of the nucleotide is essential for the binding of the metal ion to the enzyme. A detailed theory of the mechanism of action of this enzyme and related enzymes is proposed below.

Mechanism of action of creatine kinase and of other 'sulphydryl' enzymes of phosphate-group transfer

Many enzymes of phosphate transfer have been shown, by the use of 'sulphydryl reagents', to depend on the presence of 'reactive' sulphydryl groups for their catalytic activity. These enzymes may be classified into two groups according to whether or not a phosphate-enzyme complex is

involved as intermediate. Isotope-exchange criteria (Harrison, Boyer & Falcone, 1955; Noda, Nihei & Morales, 1960) have shown that the creatine-kinase reaction is in the class of direct exchange reactions. This is in contrast with that of glucose phosphomutase, for which a phosphorylated enzyme intermediate, involving a serine hydroxyl group, has been detected (Anderson & Jolles, 1957; Najjar & McCoy, 1958; Kennedy & Koshland, 1957). Both of these groups of enzymes are best pictured as catalysing an exchange of nucleophiles about a phosphorus atom (Cohn, 1959), as shown in eqn. (2). For the creatine-kinase

reaction XO is ADP and YH is creatine; for the glucose-phosphomutase reaction, XO is either 1- or 6-glucosyl and YH an enzyme-bound serine hydroxyl group. The presence of metal ions is essential for the catalytic process.

It would be expected that the forward reaction in eqn. (2) (from left to right) would be favoured by interaction of a hydrogen of YH with a base group and the interaction of XO with an acid group. The reverse reaction requires the catalytic acid-base pair in their conjugate forms (Rabin, 1958). These interactions would involve hydrogen-bonding of the substrates to the catalytic site.

An important property of hydrogen bonds (Pauling, 1945) is best illustrated by considering the structure of ice. The protons are not located mid-way between pairs of oxygen atoms, and for any pair of oxygen atoms the proton can occupy one of two positions separated by 0.76 Å. Thus there

are two positions of minimum energy which the proton can occupy separated by an energy barrier. In the particular instance of ice the energy profile of the proton as a function of O-H distance is symmetrical about the mid-point of the O-O axis. At any instant the proton will be at one of the minima, but it can readily jump to the other position: protons will tend to shift in this way in groups so that whole regions of the structure change (Pauling, 1945; see also various articles in Hadzi, 1957). This concept of the existence of two favoured positions for the proton of a hydrogen bond is very important in considering enzyme reactions, which depend on proton-transfer processes. Where the atoms forming the hydrogen bond are different, the energy profile of the proton will not be symmetrical and there will be a greater probability of finding the proton in one position

than in the other. If multiple hydrogen bonds are involved, as in some enzyme-substrate complexes, it is probable that several proton transfers occur simultaneously, causing considerable changes in structure. As discussed below, if the acid-base pair on the protein is hydrogen-bonded together then a self-enhancing system can exist which would have a catalytic effectiveness without precedent in organic chemistry.

For creatine kinase, evidence has been presented above for a hydrogen-bonded acid-base pair at the active site, probably consisting of the structure shown by (V). Hammond & Gutfreund's (1959) hypothesis for a hydrogen-bonded thiol-carboxyl pair in *ficin* is probably the first definite suggestion of mercaptan hydrogen bonds at the catalytic site of an enzyme.

In the convention adopted here the proton occupies the favoured position nearest the group to which it is joined by a full line. A dotted line joining the proton to a group means that the proton is located at the energy-minimum position farthest from that group. The conjugate species of (V) is shown in (VI).

The transition from (V) to (VI) can take place by the addition of a proton to the sulphur atom and the removal of the proton from the imidazole nitrogen atom, with the proton of the -S-H-N- hydrogen bond simultaneously changing its position. Structures (V) and (VI) should coexist in pH-independent equilibrium, although on chemical grounds the contribution of (VI) would be expected to be small except in the particular circumstance favouring the transfer of a phosphate group to ADP, as discussed below. Rabin & Watts (1960) have suggested that the hydrogen-bonded thiol-imidazole pair acts as an acid-base pair at the catalytic site of the enzyme.

Before considering the mechanism of creatine kinase further it is essential to consider the role of Mg^{2+} ions in the catalytic process. As shown by the model systems of Lowenstein (1958), Mg^{2+} ions catalyse a very slow rate of phosphate transfer in aqueous solution. In this system it is probable that water acts simultaneously, and rather ineffectively, as both acid and base, assisting the attack and departure of the nucleophiles. It is reasonable to assume that in the enzymes of phosphate transfer the catalytic function of the metal is similar to that in the model system, as has been suggested by many authors (Lowenstein, 1958; Kuby *et al.* 1954*b*; Calvin, 1954; Ennor & Morrison, 1958; Morrison, Ennor & Griffiths, 1958; Riehl, 1960). There is no agreement on the detailed structure of the Mg^{2+} ion complexes of ATP. Maciel & Waugh (1961), in contrast with Hottak, Brahms & Morales (1961), find no evidence for the interaction of the adenine ring with the metal, and

the results of Walass (1958) and Cohn & Hughes (1962) support the non-involvement of the adenine ring. Even if interaction between the adenine and metal ion does occur in aqueous solutions it is unlikely to be of importance when the adenine is bound to a specific site on the enzyme. The conflict

of opinion about whether or not a Mg^{2+} ion-nucleotide complex is the 'true substrate' for the enzyme (Noda *et al.* 1960; Morrison, O'Sullivan & Ogston, 1961) is largely irrelevant to considerations of the mechanism of catalysis and will not be considered here.

The enzyme-substrate complexes for the forward reaction, showing the overall charge distribution, is represented by (VII). The catalytic process can then proceed by simultaneous hydrogen-bonding of the thiol-imidazole pair to the substrates. The sulphur, acting as a base, bonds to a guanidino hydrogen, and the imidazole hydrogen, acting as an acid, bonds to a β -phosphate oxygen of ATP to give the transition state (VIII). These interactions could involve considerable conformational changes in the protein, consistent with the 'induced fit' theory of Koshland (1958), and possibly responsible for the rotational changes reported by Samuels,

Nihei & Noda (1961). The formation of the transition state would be favoured by multiple electrostatic factors which could well compensate for the probable decrease in entropy. The broken lines represent the bonds being formed and broken. As the reaction proceeds the electrons shift as indicated by the curved arrows and the protons jump from one potential-energy minimum to the other [from arrow tail to arrow head in an anticlockwise direction in (VIII)]. The γ -phosphate group, moving in the same direction as the protons, shifts from the β -phosphate bridge oxygen of ATP to the guanidino nitrogen. Hydrogen-bonding facilitates

the reaction by simultaneously increasing the nucleophilic reactivity of creatine and decreasing that of ADP. The proton shifts would occur almost simultaneously, since a shift of any one would trigger off the movement of the others. The catalytic process, once it begins, is self-enhancing, although many cycles of proton shifts might occur before the phosphate transfer, and hence the reaction, takes place. The correct steric orientation of the substrates is important for the reaction to occur, resulting in a high degree of specificity. Thus substitution of glycocyamine or negmine for creatine causes almost complete loss of activity (Ennor, Rosenberg & Armstrong, 1954; Tanzer & Gilvary, 1959). The metal ion is essential for the binding of ATP (Kuby & Mahowald, 1959), although a purine-binding site is also important since at a comparable concentration no other nucleotide triphosphate will catalyse the reaction at a significant rate (Nihei, Noda & Morales, 1961). The Mg^{2+} ion functions partly by binding the phosphate in the correct steric orientation and also assists the transfer process by removing some of the negative charge which impedes the exchange of nucleophiles.

The catalytic groups have basicity and acidity approaching those of mercaptide and imidazolium ions respectively. The system is a device for enabling such a pair of ions, which are normally mutually exclusive, to coexist.

When the processes shown in (VIII) are taken to completion, followed by breaking of the hydrogen bonds between the catalytic site and the substrates, the enzyme-products complex, shown in (IX) is formed. For simplicity, charges are pictured as if localized in this representation. In fact, in the prototropic form of phosphocreatine shown, delocalization of the positive charge is not possible and the imino nitrogen should be quite basic. As required by general principles, the acid-base groups at the catalytic site end up in their conjugate forms. A proton ionizes from ADP and dissociation of the products completes the catalytic process. The re-establishment of equilibrium between forms (V) and (VI) of the thiol-imidazole pair is then possible. This equilibrium position will be varied by the presence of the substrates. Form (V) will be favoured by creatine plus ATP and form (VI) by phosphocreatine plus ADP. Thus the formation of the enzyme-substrates complex will induce the formation of the tautomeric form of the thiol-imidazole pair required for catalysis.

The reverse reaction can be pictured as proceeding by the complete reversal of the forward process, since this must necessarily be the pathway of lowest activation energy. The enzyme-substrates complex would be as shown in (IX), form (VI) of the thiol-imidazole pair being favoured by two factors: (i) form (V) would require two protons

to occupy the same position between the imidazole nitrogen and the oxygen of ADP; (ii) electrostatic interaction between the fractional positive charge on the sulphur of form (VI) and the partial negative charge on the imino nitrogen of phosphocreatine. The latter arises by contribution of canonical form (X) to the structure of bound phosphocreatine. The reaction would occur by hydrogen-bonding, similar to that discussed above, through the intermediate shown in (XI). The acid site is a stronger acid than a free sulphhydryl group and the base site has a basicity exceeding that of a free imidazole. As before, the system is self-enhancing.

This type of reaction mechanism may be readily extended to other phosphate-transfer systems. For example, in the pyruvate-kinase reaction enolization occurs only in the presence of both substrates (Rose, 1960). In the reaction leading to the formation of phosphoenolpyruvate the interaction between the sulphhydryl group and pyruvate would be as depicted in (XII) and the imidazole would react with the ATP as shown above (VIII).

The sugar-kinase systems lack the 'attacking' double bond. However, a hydrogen bond between the sulphhydryl group and the sugar hydroxyl group would enhance the nucleophilic reactivity of the oxygen and promote its attack on the phosphate group as shown by (XIII).

Reactions in which a phosphate-enzyme intermediate occur can be pictured in the same way with XOH representing the reactive serine side chain. For the glucose-phosphomutase reaction, the presence at the active site of both a histidine (Ray, Ruscia & Koshland, 1960) and a sulphhydryl group with a pH-independent reactivity towards iodoacetate (Yankeelov & Koshland, 1961) has been demonstrated.

The evolution of catalytic systems and induced enzyme formation could be associated with the juxtaposition of fundamental catalytic entities, such as the thiol-imidazole pair, with pre-existing substrate-binding sites by induced conformational changes in proteins. This should prove a fruitful field for future experiments.

SUMMARY

1. The reactions of iodoacetate and iodoacetamide with the reactive sulphhydryl groups of creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) have been investigated by following the alkylation reaction and by loss of enzyme activity.

2. The initial velocity of the reaction with iodoacetate varies markedly with ionic strength but that with iodoacetamide does not.

3. The rate of inhibition of enzyme activity is independent of pH.

4. Protection against inhibition of enzyme activity is only obtained when both substrates for the reaction and the activating metal ion are present. No protection is obtained when only part of the enzyme reaction mixture is present.

5. The nature of the reactive sulphydryl groups and their role in the phosphate-transfer reaction are discussed in the light of these findings.

6. A mechanism of action is proposed for creatine kinase which is dependent on a hydrogen-bonded acid-base pair, involving a 'reactive' sulphydryl group, on the enzyme.

7. The properties of hydrogen bonds are of great importance in such a system.

8. The possible existence of similar mechanisms in other 'sulphydryl-dependent' enzymes of phosphate-group transfer is discussed.

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