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Studies on the Mechanism of Action of Adenosine 5'-Triphosphate-Creatine Phosphotransferase

INHIBITION BY MANGANESE IONS AND BY *p*-NITROPHENYL ACETATE

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In the reaction catalysed by creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2):

Mn^{2+} ions may replace Mg^{2+} ions as the activating metal ions (Kuby, Noda & Lardy, 1954*b*; Geiger, 1956; Griffiths, Morrison & Ennor, 1957; Cho, Haslett & Jenden, 1960). Ennor & Rosenberg (1954), and Cho *et al.* (1960) also observed that preincubation of creatine kinase with Mn^{2+} ions caused inactivation of the enzyme. During the present studies it was found that the presence of some substrates enhanced the inhibitory effect of added Mn^{2+} ions. This phenomenon has been investigated in greater detail. The reactive sulphydryl groups found to be essential for catalytic activity (Mahowald & Kuby, 1960; Watts, Rabin & Crook, 1961) appear to be the sites of inactivation. The results also provide evidence that these sulphydryl groups are situated close to the substrate-binding sites.

The inhibition of creatine kinase by *p*-nitrophenyl acetate was reported by Watts, Rabin & Crook (1962), and used to distinguish enzyme preparations with one and two catalytic sites/

molecule. Though loss of enzyme activity could be attributed to reaction of the essential sulphydryl group for the enzyme preparation with one catalytic site/molecule, for preparations containing two sites/molecule some reactive sulphydryl groups remained after complete loss of enzyme activity had occurred. The present paper concerns the further investigation of the nature of this loss of activity with the normal enzyme containing two catalytic sites/molecule. The results indicate the presence of an essential amino group, whose integrity is essential for the maintenance of enzyme conformation, which changes its *pK* during the transphosphorylation reaction.

MATERIALS AND METHODS

Materials

Creatine kinase. This was prepared by 'method B' of Kuby, Noda & Lardy (1954*a*). Except where the crystalline enzyme is specified, purification was carried out to the precrystallization stage. The specific activity is given for each sample in the Results section. For the preparations with high specific activity [above 54 'Kuby units', as defined by Kuby *et al.* (1954*a*)] the ethanol used in the purification procedure was redistilled from potassium hydroxide (Nihei, Noda & Morales, 1961), and all solutions

were made up in 10 μ M-EDTA. All preparations behaved in the manner associated with two catalytic sites/enzyme molecule (Watts *et al.* 1962). A molecular weight of 81000 is assumed (Noda, Kuby & Lardy, 1954).

Chemicals. These were purified as described previously (Watts *et al.* 1962; Watts & Rabin, 1962) or were of AnalaR grade.

Buffers. Tris-malate buffer was prepared according to the Tables of Datta & Grzybowski (1961). Tris-hydrochloric acid buffers were prepared by adjusting 0.2N-hydrochloric acid (obtained by diluting constant-boiling hydrochloric acid) to the required pH with 1M-tris solution and diluting the final solution to 0.1N with respect to the acid. The pH was rechecked at the final dilution.

All other buffers were prepared as described by Watts & Rabin (1962).

Methods

Determination of creatine-kinase activity. Kinase activity was determined by measuring the phosphate transferred from ATP to creatine in 4 min. at 37° (Watts *et al.* 1961) in a reaction mixture containing the enzyme sample, creatine (10 mM), ATP (1 mM), cysteine (5 mM), and borate buffer, pH 9.0 and 0.015 *I*, and either magnesium sulphate (1 mM) or the metal salt as described in the Results section.

Inactivation by manganese ions. The initial velocities of the inhibition process have been measured from tangents at the origin of progress curves. Where the effects of substrates were tested the initial velocity is expressed as the percentage of the activity of the uninhibited enzyme lost/min. This formulation is used because the presence of various substrates affected the apparent activity of the uninhibited enzyme. For the same reason separate control curves were determined for each substrate tested.

In the experiment to determine the loss of reactive sulphhydryl groups, replicate tubes containing 2.4 mg. of enzyme were incubated at 25° with ATP (10 mM), manganese chloride (2 mM), borate buffer, pH 9.0 and 0.015 *I*, in a total volume of 15 ml. At suitable time-intervals inhibition was arrested by the addition of 1 ml. of 75 mM-cysteine solution, pH 9.0, and the mixture cooled in an ice bath. The mixtures were then dialysed under reduced pressure (500 mm. Hg) against several changes of borate buffer, pH 9.0 and 0.003 *I*, to remove substrates and cysteine. Each concentrated enzyme solution was adjusted to 5 ml. and the protein concentration measured (extinction at 280 μ ; $\epsilon = 7.3 \times 10^4$ in borate buffer, pH 9.0 and 0.023). After removal of a sample for enzyme assay, the reactive sulphhydryl content of the remainder (4.5 ml.) was determined from the reaction with 1 mM-iodoacetate by using the silver-silver iodide electrode to follow the reaction (Watts *et al.* 1961).

Inactivation by p-nitrophenyl acetate. p-Nitrophenyl acetate was dissolved in redistilled acetone. Preliminary experiments showed that acetone did not affect the reactive sulphhydryl groups of creatine kinase at concentrations of up to 20% (v/v). In practice the acetone concentration was limited to 8% (v/v) at the highest concentrations of p-nitrophenyl acetate used.

The reaction of p-nitrophenyl acetate with creatine kinase was followed spectrophotometrically by using either a Uvispek (Hilger and Watts) or a Cary model 14 recording spectrophotometer thermostatically controlled at 25°. The reference cuvette contained the reaction mixture without p-nitrophenyl acetate. Where this was important, the

aqueous hydrolysis of p-nitrophenyl acetate was determined by a separate experiment. Enzyme activity was followed by transferring samples from a duplicate reaction mixture to 10 mM-cysteine, pH 9.0, in borate buffer, at suitable time-intervals and assaying as described above.

Where the effect of pH on the inactivation of creatine kinase was investigated, just sufficient buffer was used to maintain the pH which was measured immediately after all the samples had been removed. In this way the buffer capacity of the sample was not sufficient to affect the pH of the assay mixture. The four samples, which were transferred to cysteine and assayed as described above, spanned the first 25–30% of the total reaction at the highest rate of inhibition and 3–4% at the lowest rate measured. Initial velocities were measured as described above.

RESULTS

Inhibition by manganese ions. Fig. 1 shows the $p[\text{Mn}^{2+} \text{ ion}]$ -activity curve for creatine kinase. In an assay system containing ATP (1 mM) and optimum concentrations of metal ion (1 mM- Mg^{2+} ion or 0.2 mM- Mn^{2+} ion), the Mn^{2+} ion gave about 30% more activity than Mg^{2+} ion. For investigation of the inhibitory effects of metal ions on creatine kinase the concentration of the metal salt in the reaction mixture was chosen so that the samples removed for assay gave the optimum concentration when diluted tenfold in the assay mixture. The Mg^{2+} ions and Mn^{2+} ions were tried alone and in combination with substrates for the forward reaction at pH 9.0 and the back reaction at pH 7.0. Fig. 2 shows the dramatic effect

Fig. 1. Effect of Mn^{2+} ions on the activity of creatine kinase at 37°. Each mixture contained: MnCl_2 ; ATP, 1 mM; creatine, 10 mM; cysteine, 5 mM; borate buffer, pH 9.0 and 0.015 *I*; crystalline creatine kinase (52 Kuby units), 28 $\mu\text{g./ml.}$

obtained in the presence of phosphocreatine when Mn^{2+} ions replace Mg^{2+} ions in the reaction mixture. The relative initial velocities of the inactivation process in this experiment and for the other substrates tested are listed in Table 1. Table 1 also shows that cysteine completely protects the enzyme against inactivation by added Mn^{2+} ions. Enzyme activity, once lost, could not be restored by the addition of sulphhydryl compounds. The assay system depended on this finding.

Attempts were also made to investigate the effects of inorganic phosphate and pyrophosphate. Inorganic phosphate was found to give a precipitate with Mn^{2+} ions and could not be used. With pyrophosphate the rate of inactivation was not significantly different from that with added Mn^{2+} ions alone (Table 1).

Effects of enzyme concentration and purity. The sensitivity of creatine kinase to inactivation by added Mn^{2+} ions increased with dilution. Table 2 shows the effect with a highly active preparation purified to the precrystallization stage. The initial velocity of inactivation is much less than that for the crystalline preparation used in Fig. 2. Both preparations contained only a single major component as revealed by horizontal starch-gel electrophoresis (Smithies, 1959) with the buffer system of Poulik (1957).

Site of inactivation. The most probable sites of inhibition are the sulphhydryl groups on the enzyme which are essential for activity. To test this hypothesis samples of an inhibition reaction mixture were assayed simultaneously for enzyme activity and reactive sulphhydryl group content (Table 3).

Fig. 2. Effects of Mg^{2+} and Mn^{2+} ions on the inactivation of creatine kinase by phosphocreatine at 20° . Each reaction mixture (total volume 2.5 ml.) contained: tris buffer, pH 7.0 and 0.01 *I*; crystalline creatine kinase (52 Kuby units), 160 μ g./ml.; phosphocreatine, 10 mM; plus ●, $MnCl_2$, 2 mM; or ○, $MgCl_2$, 10 mM; or Δ, control (water added). For assay, 0.25 ml. samples were removed.

The loss of enzyme activity parallels the loss of reactive sulphhydryl groups.

Inhibition by *p*-nitrophenyl acetate. Fig. 3 shows a progress curve for the reaction of *p*-nitrophenyl acetate with creatine kinase at pH 6.3, followed by measuring the production of nitrophenolate ion. The reaction rate decreases to about 38% of the initial rate after 65 min. and then becomes almost linear. Extrapolation of the linear part of the progress curve (Fig. 3, *B*) to zero-time yields 15 ± 1 molecules of *p*-nitrophenol produced/mole-

Table 1. *Effects of substrates on the stability of creatine kinase at 20°*

Experimental details are given in the text. The final concentrations used were: creatine, 10 mM; ATP, 10 mM; ADP, 10 mM; pyrophosphate, 10 mM; $MgCl_2$, 10 mM; $MnCl_2$, 2 mM; phosphocreatine, 1.5 mM; cysteine, 15 mM; crystalline creatine kinase (52 Kuby units), 160 μ g./ml. Reactions carried out at pH 9.0 were in borate buffer, 0.015 *I*; and those at pH 7.0 in tris buffer, 0.01 *I*. Separate controls were run for each substrate tested and initial velocities are expressed relative to the control. A 0% inhibition indicates full activity in the presence of that particular combination of substrates.

Substrate	Percentage of initial activity lost/min.	
	At pH 7.0	At pH 9.0
None	0.13	0
Mg^{2+} ion	0	0
Mn^{2+} ion	1.81	1.81
ATP	—	0
ATP + Mg^{2+} ion	—	0
ATP + Mn^{2+} ion	—	6.6
Creatine	—	0
Creatine + Mg^{2+} ion	—	0.6
Creatine + Mn^{2+} ion	—	2.0
ADP	2.07	—
ADP + Mg^{2+} ion	2.32	—
ADP + Mn^{2+} ion	5.36	—
Phosphocreatine	0.45	—
Phosphocreatine + Mg^{2+} ion	0.26	—
Phosphocreatine + Mn^{2+} ion	5.5	—
ADP + Mn^{2+} ion + cysteine	0	0
Pyrophosphate	—	0
Pyrophosphate + Mn^{2+} ion	—	0.92

Table 2. *Effect of enzyme concentration on the inhibition of creatine kinase by manganese ions plus adenosine triphosphate*

Conditions were as in Table 1 except that creatine kinase (72 Kuby units) was used.

Concn. of enzyme (mg./ml.)	Percentage of initial activity lost/min.
0.8	0.7
0.16	2.04
0.16	0.07
(without Mn^{2+} ion)	

cule of enzyme for the primary reaction. Loss of enzyme activity does not correlate with the overall production of *p*-nitrophenol after correction for the contribution by aqueous hydrolysis but relates closely to the primary reaction, obtained by subtracting the extrapolated linear part of the curve (Fig. 3, A–B), as shown in Fig. 4.

The inactivated enzyme could not be reactivated by the addition of cysteine or by dialysis overnight in the presence or absence of cysteine.

The initial velocity of *p*-nitrophenol liberation by creatine kinase is linear with enzyme concentration (Fig. 5) for any fixed concentration of *p*-nitrophenyl acetate. The initial velocity is also linear with *p*-nitrophenyl acetate concentration for any particular concentration of enzyme (Fig. 6).

Table 3. Inactivation of creatine kinase by manganese ions plus adenosine triphosphate at 25°: relationship between the loss of enzyme activity and the loss of reactive sulphhydryl groups

The experimental details are given in the text. Creatine kinase (72 Kuby units) was used.

Incubation time (min.)	Percentage of enzyme activity lost/min.	Initial velocity of reaction with iodoacetate (% of control)
0	100	100
12	72.3	74.3
25	51.8	55.8

Fig. 3. Reaction of *p*-nitrophenyl acetate with creatine kinase at 25°. The reaction mixture contained *N*-ethylmorpholine-HCl buffer, pH 6.8 and 0.076 *I*; creatine kinase (72 Kuby units), 2 mg.; KNO₃, 0.097 *I*; *p*-nitrophenyl acetate, 5.08 mM in 8.07% (v/v) acetone; the total volume was 3.1 ml. Measurements of *p*-nitrophenolate ion (as $E_{440\text{ m}\mu}$) are taken from a Cary recording spectrophotometer trace.

Effect of pH. The effect of pH on the rate of inactivation of creatine kinase by *p*-nitrophenyl acetate, plotted as the pseudo-first-order velocity constant, k_1 , is shown in Fig. 7. The effect of pH on the liberation of *p*-nitrophenol could not be determined since at pH values greater than 7.0 the rapid

Fig. 4. Inhibition of creatine kinase by *p*-nitrophenyl acetate at 25°: comparison of the nitrophenol produced by the initial reaction calculated from Fig. 3 (A–B) (continuous line) with loss of enzyme activity (●). Enzyme activity was measured by transferring 0.025 ml. samples of a duplicate reaction mixture to that used for measuring nitrophenol production (see the legend to Fig. 3) to cysteine, pH 9.0, at time-intervals and assaying as described in the Methods section.

Fig. 5. Reaction of *p*-nitrophenyl acetate with creatine kinase at 25°: effect of enzyme concentration on the rate of *p*-nitrophenol production. Each reaction mixture (total volume 2.5 ml.) contained: creatine kinase (62 Kuby units); tris buffer, pH 7.0 and 0.064 *I*; KNO₃, 0.1 *I*; *p*-nitrophenyl acetate, 1 mM in 4% (v/v) acetone.

increase in the rate of aqueous hydrolysis rendered accurate measurement impossible.

Effect of ionic strength. This was very small. Variation of the ionic strength from 0.01 to 0.2 in borate buffer, pH 9.0 (concn. of *p*-nitrophenyl acetate, 0.42 mM), or in tris buffer, pH 8.15 (concn. of *p*-nitrophenyl acetate, 4.2 mM), decreased the rate of inactivation of creatine kinase by not more than 5%, which was within the limits of experimental error.

Effects of substrates. Protection against inactivation of creatine kinase by *p*-nitrophenyl acetate was obtained only when the complete substrate reaction mixture was present so that transphosphorylation could occur (Fig. 8). No significant difference in the degree of protection was observed whether the inhibitor was added immediately after the enzyme to the rest of the reaction mixture or whether addition of inhibitor was delayed until the transphosphorylation reaction had gone to equilibrium. Because of the high enzyme concentration used the combination of substrates is best regarded as an equilibrium mixture. The omission of a substrate resulted in no protection. Thus at pH 9.0 (borate buffer) no protection was obtained with creatine (10 mM) alone, ATP or ADP (1 mM), or phosphocreatine (0.8 mM) alone or in the presence of 1 mM-Mg²⁺ ion.

The effect of pH on the rate of inactivation of the enzyme in the presence of the substrate equilibrium mixture is shown in Fig. 9.

Fig. 6. Effect of *p*-nitrophenyl acetate concentration on the reaction with creatine kinase at 25°. Each reaction mixture contained: creatine kinase (72 Kuby units), 0.59 mg./ml.; tris buffer, pH 7.0 and 0.29 *I*; KNO₃, 0.1 *I*; *p*-nitrophenyl acetate in 8% (v/v) acetone; the total volume was 2.5 ml. ○, Aqueous hydrolysis.

Fig. 7. Effect of pH on the inhibition of creatine kinase by *p*-nitrophenyl acetate at 25°. Each reaction mixture (total volume 3 ml.) contained: creatine kinase (72 Kuby units), 153 μg./ml.; KNO₃, 0.1 *I*; acetone, 8% (v/v); plus ○, tris buffer, 0.04 *I*; *p*-nitrophenyl acetate, 834 μM; or ●, borate buffer, 0.025 *I*; *p*-nitrophenyl acetate, 167 μM; or △, tris-maleate buffer, 0.025 *I*; *p*-nitrophenyl acetate, 4.17 mM. For enzyme assay, 0.1 ml. samples were removed as described in the Methods section. The arrows and broken line indicate the correction for spontaneous loss of enzyme activity.

Fig. 8. Effect of an equilibrium mixture of substrates on the inhibition of creatine kinase by *p*-nitrophenyl acetate at 25°. Each reaction mixture (total volume 3 ml.; 0.12 *I*) contained: ○, creatine kinase (72 Kuby units), 160 μg./ml.; borate buffer, pH 9.0; *p*-nitrophenyl acetate, 834 μM in 4% (v/v) acetone; or ●, plus creatine, 84 mM; ATP, 0.067 mM; MgSO₄, 0.67 mM, and allowed to proceed to equilibrium (10 min.) before adding the inhibitor.

Fig. 9. Effect of pH on the protection of creatine kinase by the substrate equilibrium mixture against inactivation by *p*-nitrophenyl acetate at 25°. ○, Control, conditions as given in Fig. 7; ●, plus creatine, 10 mM; ATP, 1 mM; MgSO₄, 1 mM; allowed to react for 5 min. before adding the inhibitor. For pH 6.67–8.4, tris buffer was used; for pH 8.85–10.05, borate buffer.

Reactivity towards enzyme sulphydryl groups. The reactivity of *p*-nitrophenyl acetate towards the two essential sulphydryl groups of the creatine-kinase molecule was determined by a mixed inhibitor experiment with iodoacetamide. Iodoacetamide does not react with *p*-nitrophenyl acetate. It reacts almost exclusively with the two reactive sulphydryl groups, other reactions taking place to the extent of not more than 2% under the conditions used (Watts *et al.* 1962). Also, this reaction can be continuously and sensitively followed with the silver-silver iodide electrode. The concentration of *p*-nitrophenyl acetate was chosen so that the rate of inactivation of the enzyme was small at low pH and approximately 5 times that for the iodoacetamide at pH 10. If the reaction of the sulphydryl groups were responsible for the pH-inactivation curve with *p*-nitrophenyl acetate (Fig. 7) then the increasing rate of reaction as the pH is raised should, by competition, decrease the rate of reaction of the sulphydryl groups with the iodoacetamide. Thus the initial velocity of the reaction with iodoacetamide should be progressively decreased as the pH is raised and the asymptote of the progress curve progressively lowered. Fig. 10 shows the result of this experiment. Fig. 11 shows the effect of pH on the initial velocity of the reaction and on the velocity of the reaction after 17 min. when, in the absence of *p*-nitrophenyl acetate, 95% of the sulphydryl groups would have reacted.

Fig. 10. Effect of pH on the simultaneous reaction of iodoacetamide and *p*-nitrophenyl acetate with creatine kinase at 25°. Each reaction mixture (total volume 5 ml.) contained: creatine kinase (72 Kuby units), 0.38 mg./ml.; Tween 80, 0.02% (to stabilize the electrode); KI, 1 μM; KNO₃, 0.1 *I*; buffer, 0.04 *I*. The reaction was started by the addition of the inhibitors contained in acetone (50 μl.) to give final concentrations of: iodoacetamide, 170 μM; *p*-nitrophenyl acetate, 210 μM. The buffers used were: tris-maleate for pH 6.45 and pH 7.4; borate for pH 8.18–9.7. Only the reaction with iodoacetamide was followed by automatically recording the liberation of I⁻ ions. The short vertical bars indicate the range of variation observed over the range pH 6.45–9.17. A, pH 6.45; B, pH 7.4; C, pH 8.35; D, pH 9.17.

Fig. 11. Simultaneous reaction of iodoacetamide and *p*-nitrophenyl acetate with creatine kinase at 25°: results from Fig. 10 show the effect of pH on: ●, the initial velocity of liberation of I⁻ ions; ○, the velocity of liberation of I⁻ ions after 17 min.

DISCUSSION

The results presented above are in agreement with the pH-stat findings of Cho *et al.* (1960) in that they show greater activation of creatine kinase with Mn^{2+} ions than with Mg^{2+} ions. However, the enzyme-inactivation results show two differences. First, no significant degree of inactivation was found in the presence of Mg^{2+} ions at pH 9.0 (Table 1). Secondly, enzyme activity could not be recovered by the addition of sulphhydryl compounds.

Cho *et al.* (1960) found that loss of activity occurred in the presence of the complete substrate reaction mixture with Mg^{2+} ions as the activating metal ions, but activity was completely restored by the addition of sulphhydryl compounds. Since inactivation occurs only when the enzyme is 'working', this suggests that the reactive sulphhydryl group is transiently labilized at some stage in the transphosphorylation reaction. Thus, in common with other sulphhydryl enzymes, creatine kinase appears to be inactivated by a two-step process, only the first step of which is reversible. This is supported by observations on aged enzyme preparations, which go through a phase where cysteine causes reactivation before irreversible inactivation occurs. Fresh preparations of creatine kinase are not affected by cysteine (Kuby *et al.* 1954*b*). Analogous situations exist for yeast alcohol dehydrogenase (E. P. Whitehead, personal communication) and for glyceraldehyde phosphate dehydrogenase (Krebs, 1955). The presence of the complete substrate reaction mixture may prevent inactivation from proceeding beyond the first stage.

Aged preparations of creatine kinase have been found to contain less than the expected two moles of sulphhydryl group/mole of enzyme (Watts, 1961). Since cysteine prevents enzyme inactivation it is reasonable to infer that loss of activity results from oxidation of the reactive sulphhydryl groups (Table 3) catalysed by Mn^{3+} ions. Solutions of manganous chloride do not contain more than traces of Mn^{3+} ion. The enhancement of enzyme inactivation by substrates does not appear to be due to an increase in the concentration of Mn^{3+} ions after chelation of the Mn^{2+} ions by phosphate groups. ADP and phosphocreatine, with widely different stability constants (Smith & Alberty, 1956), are equally effective, and no enhancement is seen with pyrophosphate (Table 1). It seems more reasonable to conclude that the metal ion is bound in close proximity to the sulphhydryl group by the chelating substrate, with the subsequent production of the Mn^{3+} ion and oxidation of the sulphhydryl group. This scheme does not demand any preferred order of complexing between enzyme, metal ion and substrate. It does, however, require the sulphhydryl

group to be close to the substrate-binding sites. The whole inhibitory process must also be dependent on traces of oxidizing agents introduced, presumably, during the purification procedure. This is compatible with the finding that enzyme preparations with a low specific activity are more sensitive to inactivation than those with a high specific activity (cf. Fig. 2 and Table 3), and that the rate of inactivation increases with dilution (Table 2). Great care in removing contaminating metal ions and oxidizing agents during purification results in a high specific activity and a low sensitivity towards added Mn^{2+} ions.

Table 3 also explains, in terms of loss of reactive sulphhydryl groups, the 46% inactivation of creatine kinase in 3 min. at 37° found by Ennor & Rosenberg (1954) and attributed to 10 mM- Mn^{2+} ion alone. The inactivation was carried out in the presence of 0.33 mM-phosphocreatine to facilitate the subsequent assay.

The recent evidence that a change in the conformation of creatine kinase occurs only when transphosphorylation takes place (Samuels, Nihei & Noda, 1961) requires that chemical groups that participate in the catalysis are distinguished from those essential for maintaining enzyme conformation. The enhancement of Mn^{2+} ion-inactivation of creatine kinase by single substrates suggests that the essential sulphhydryl groups are close to the active centre and are associated with catalysis rather than maintenance of protein structure, in agreement with the catalytic mechanism proposed by Watts & Rabin (1962).

Inhibition by p-nitrophenyl acetate. Figs. 3 and 4 show that the progress curve for the interaction of p-nitrophenyl acetate with creatine kinase at pH 6.8 is complex but may be divided into two parts, an initial reaction and a steady-state catalysis that continues after completion of the initial reaction. The second-order rate constant for the catalysis (Fig. 3, B):

$$k_2 = \frac{v}{[p\text{-nitrophenyl acetate}][\text{creatine kinase}]}$$

where v is the initial velocity and $[p\text{-nitrophenyl acetate}]$ and $[\text{creatine kinase}]$ are the initial concentrations of inhibitor and enzyme respectively, is 94 moles/l./min. after correction for the contribution from aqueous hydrolysis. This formulation appears justified since the reaction was first-order with respect to both enzyme (Fig. 5) and inhibitor (Fig. 6) over the concentration ranges used. Since creatine kinase contains 33 histidine residues/molecule (Noltmann, Mahowald & Kuby, 1962), and histidine side chains in synthetic polypeptides have k_2 values of 3.5–9.5 moles/l./min. (Katchalski *et al.* 1960), it seems improbable that the enzyme contains amino acid side chains with special

catalytic properties for hydrolysing *p*-nitrophenyl acetate at this pH.

The rate of nitrophenol liberation by the 14–16 reacting groups/enzyme molecule in the initial reaction (Figs. 3*a*, and 3*b*) yields an average k_2 of 9.5–11 moles/l./min. per group. From the correlation with activity loss (Fig. 4) this represents seven or eight groups/catalytic site, if it is assumed that there are two sites/enzyme molecule (Watts *et al.* 1962). If it is assumed that the reaction of particular groups completely destroys enzyme activity then not more than one or two groups/molecule can be so involved or a significant disagreement would be observed between the rate of nitrophenol production and loss of enzyme activity on the 'percentage reacted' basis used in Fig. 4. Also, the reaction velocity of these involved groups must not be significantly faster than that of the slowest-reacting group or a discrepancy would be observed. A single group/catalytic site involved in the catalysis would be accounted for by the essential sulphhydryl group, shown to react with loss of enzyme activity by Watts *et al.* (1962).

As the pH is raised, new groups appear which cause loss of activity on reaction with *p*-nitrophenyl acetate (Figs. 7 and 9). The log plot (Fig. 7) shows that these are groups with a normal ionization over the range investigated. Although the curve is tending to flatten out at high pH the very rapid increase in the rate of spontaneous inactivation above pH 10 renders precise determination impossible.

The ionization curve indicates a pK of about 9.0 for the reacting groups, and the lack of effect of ionic strength on the inhibition process suggests a cationic-neutral type of dissociation. Since the inhibition is also irreversible the experimental results show a strong resemblance to those found for amino groups in simple peptides and other compounds (Koltun & Gurd, 1960; Koltun, Ng & Gurd, 1963). The k_2 value for the inhibition process at pH 9.7, where the reacting groups might be expected to approach complete ionization, is 10^3 moles/l./min., or 500 moles/l./min. per catalytic site. Koltun & Gurd (1960) and Koltun *et al.* (1963) found k_2 values of up to 100 moles/l./min. for α -amino groups in a variety of dipeptides, but obtained a k_2 of 600 moles/l./min. for ϵ -aminocaproic acid. Mahowald & Kubly (1960) were unable to locate an *N*-terminus in creatine kinase, which suggests that lysine side chains may be responsible for the phenomenon observed. This is not incompatible with a pK near 9.0, as the pK is raised by 0.85 in the presence of the substrate equilibrium mixture.

Figs. 10 and 11 show that the reactive sulphhydryl groups are not associated with the increased sensitivity of the enzyme to *p*-nitrophenyl acetate as the pH is raised. The initial velocity of the reaction

with iodoacetamide is not depressed as the pH is raised in the presence of *p*-nitrophenyl acetate, and the asymptote of the progress curve is not lowered. At pH 9.7 (Fig. 10) the enzyme would be completely inhibited by *p*-nitrophenyl acetate in the first 3–4 min. This behaviour of the two essential sulphhydryl groups/molecule is what would be expected in the light of the finding that the reaction with iodoacetamide is unaffected by pH (Rabin & Watts, 1960).

Fig. 11 shows that above pH 9.0 the reaction with *p*-nitrophenyl acetate causes a sharp increase in the rate of the secondary reaction with iodoacetamide when the primary reaction with the two essential sulphhydryl groups/molecule is almost complete. More than four moles of I^- ion/mole of enzyme are rapidly liberated (Fig. 10), and this can only come from a reaction with the sulphhydryl groups which are buried and unreactive in the native protein. No reaction of this sort occurs in the absence of the *p*-nitrophenyl acetate. This suggests that the group on the enzyme reacting with *p*-nitrophenyl acetate is associated with the maintenance of protein conformation.

The failure of part of the substrate reaction mixture to protect creatine kinase against inactivation by *p*-nitrophenyl acetate excludes the possibility that the reacting group is directly associated with substrate-binding, or is sufficiently close to the substrate-binding site to be protected by steric hindrance or influenced by a charged group on the substrate.

Fig. 9 shows that the protection of creatine kinase by the substrate equilibrium mixture against inactivation by *p*-nitrophenyl acetate (Fig. 8) is due to raising the pK of the reacting group by 0.85 pH unit. The most reasonable interpretation of this finding is that, during transphosphorylation, a change in the conformation of the enzyme alters the juxtaposition of charged groups on the protein and consequently their degree of ionization. The magnitude of the shift in pK expected from a change of this sort would easily encompass the experimental value found. This conclusion is in complete agreement with the optical-rotatory-dispersion results of Samuels *et al.* (1961) mentioned above. This would appear to be the first indication of a change in the pK of a group on the enzyme associated with a conformational change during catalysis.

The present results suggest that the integrity of these *p*-nitrophenyl acetate-reacting groups is essential for the maintenance of enzyme conformation. However, the normal ionization of these groups (Fig. 7) is incompatible with the maintenance of protein conformation by forming a hydrogen bond. By analogy with the change in charge observed to accompany the mutation of

haemoglobin A to the less soluble sickle-cell mutant (Ingram, 1958), a more probable role would be that of controlling the solubility of the protein. Loss of charge would affect the conformation by changing slightly the solubility of the protein. If the presence of the cationic species is necessary for catalytic activity, then ionization and the concomitant change in conformation could be responsible for the descending limb at high pH of the normal pH-reactivity curve. A comparison of the pH-*p*-nitrophenyl acetate-reactivity curve in the presence of substrates (Fig. 9) with the pH-phosphotransferase-activity results of Kuby *et al.* (1954*b*) for the forward reaction shows that the similarity is great enough for this indeed to be the case.

A possible alternative although less probable explanation of the results could be that the reacting group is required to stabilize the transferring phosphoryl group during the transition state of the catalysis. Interaction of a negatively charged phosphoryl group with an amino group would tend to raise the *pK* of the latter by stabilizing the cationic form. From the effect of ionic strength on the reaction of iodoacetate with creatine kinase at pH 7.0 and pH 9.0, Watts & Rabin (1962) have indicated a cationic-neutral type of dissociation occurring near the reactive sulphydryl group. However, their results indicate a *pK* of about 8.0, which is too low for the group reacting with *p*-nitrophenyl acetate. Also, this hypothesis does not explain the change in conformation found to occur in the mixed inhibitor experiment (Fig. 10).

In summary, the evidence for the *p*-nitrophenyl acetate-reacting group, tentatively identified as a lysine ϵ -amino group, favours its association with maintenance of protein conformation. On the other hand, information on the reactive sulphydryl groups suggests participation in the catalytic mechanism, and gives encouragement that the difficult problem of distinguishing catalytic from structural side chains in enzymes may be overcome.

Finally, the results underline the importance of investigating the pH-dependence of any protection phenomena observed with inhibitor studies.

SUMMARY

1. Creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) is irreversibly inhibited by incubation with Mn^{2+} ions. The rate of inhibition varies with the enzyme preparation and the protein concentration, and is stopped but not reversed by the addition of cysteine.

2. Inhibition is markedly enhanced by ATP, ADP and phosphocreatine, but not by creatine or pyrophosphate.

3. A progressive diminution in the rate of the subsequent reaction with iodoacetate indicates that inhibition by Mn^{2+} ions causes loss of the reactive sulphydryl groups.

4. The results are interpreted to indicate a Mn^{3+} ion-catalysed oxidation of the sulphydryl groups and to provide further evidence that the sulphydryl groups are in close proximity to the substrate-binding sites.

5. The reaction of *p*-nitrophenyl acetate with creatine kinase is first-order with respect to both enzyme and inhibitor over the range investigated.

6. The progress curve for the reaction at pH 7.0 can be divided into an initial reaction, causing loss of activity, and a steady-state catalysis.

7. The inhibition at pH 7.0 can be attributed mainly to the modification of the reactive sulphydryl groups.

8. Above pH 7.0 the ionization of new more-rapidly-reacting groups, with *pK* values of about 9.0, is revealed whose modification also causes loss of activity.

9. An increased rate of reaction of the sulphydryl groups is excluded and the reacting groups are tentatively identified as ϵ -amino groups of lysine.

10. The presence of the complete substrate reaction mixture gives strong protection against inactivation by *p*-nitrophenyl acetate at pH 9.0. Single substrates alone or plus Mg^{2+} ions are without effect.

11. The pH profile of the inhibition process in the presence of a substrate equilibrium mixture reveals that protection is achieved by raising the *pK* of the reacting groups by 0.85 pH unit.

12. The results are interpreted to indicate that a conformational change occurs in creatine kinase during the transphosphorylation reaction and that the integrity of the groups reacting above pH 7.0 are essential for the maintenance of conformation.

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The Conversion of Shikimic Acid into Certain Aromatic Compounds by Cell-Free Extracts of *Aerobacter aerogenes* and *Escherichia coli*

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One of the major unsolved problems in the biosynthesis of aromatic compounds by micro-organisms is the process by which shikimic acid 5-phosphate is converted into anthranilic acid. This involves the formation of an aromatic ring and the introduction of nitrogen into the molecule and might therefore be expected to take place in several steps. Although tryptophan auxotrophs that appear to have a metabolic lesion in this region of the pathway have been known for many years (Yanofsky, 1955; Tatum, Bonner & Beadle, 1943; Nyc, Mitchell, Leifer & Langham, 1949; Gots, Koh & Hunt, 1954), there have been no reports of the accumulation of possible intermediates by such cells. Investigations of accumulations by whole cells of certain tryptophan auxotrophs yielded mixtures of phenolic compounds (Pittard, Gibson & Doy, 1961, 1962; Doy & Gibson, 1961). The relation of these compounds to tryptophan synthesis, however, was not clear, and it was decided to seek further information on the conversion of shikimic acid 5-phosphate into anthranilic acid by using cell-free extracts of bacterial auxotrophs.

Srinivasan (1959) reported briefly on the enzymic synthesis of anthranilic acid from shikimic acid 5-phosphate by cell-free extracts of a mutant strain of *Escherichia coli*. He found that, with shikimic

acid 5-phosphate as substrate, glutamine and NAD, NADH₂ or NADP were required for optimum synthesis.

The present work confirms and extends the results of Srinivasan. The experimental system used was also suitable for examining the biosynthesis of phenylalanine and tyrosine. Part of this work has been briefly reported (Morgan, Gibson & Gibson, 1962).

MATERIALS AND METHODS

Organisms. Various auxotrophic strains of *Escherichia coli* and *Aerobacter aerogenes* were used. These strains were obtained by penicillin selection (Davis, 1948; Lederberg & Zinder, 1948) after ultraviolet irradiation. The wild-type strain of *A. aerogenes* was first irradiated and a non-capsulated variant isolated, this variant being used as the parent strain for the auxotrophs used. Some details of the strains used are set out in Table 1.

All strains were maintained on meat infusion-agar slopes and subcultured monthly.

Preparation and fractionation of cell extracts. The cells were grown in the citric acid-mineral salts mixture described by Vogel & Bonner (1956), supplemented with 0.16% of glucose, 0.005% of acid-hydrolysed casein and indole (2 µg./ml.). The concentration of indole was made limiting to encourage de-repression on the tryptophan pathway.