The Role of Thiol Groups in the Structure and Mechanism of Action of Arginine Kinase

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1. A detailed study of the reaction of iodoacetamide with arginine kinase has been carried out. 2. The enzyme contains five reactive thiol groups per 37 000 g. of protein, all of which can be alkylated. 3. Below pH 8.5 loss of activity is substantially independent of pH and can be correlated with the alkylation of a single pH-independent thiol. 4. One catalytic site per enzyme molecule is inferred. 5. The progress curves of the alkylation reaction are polyphasic and reveal a pH- and time-dependent sequential release of thiols which is dependent upon the alkylation of the first pH-independent thiol. This is supported by electrophoretic investigations. 6. Comparison of alkylation rate and rate of loss of activity suggests that two thiol groups are not essential for catalytic activity. Variability in enzyme preparations with respect to alkylation rate appears to be associated with these two groups. 7. A complex protection pattern is revealed by the effects of various substrate combinations on rates of alkylation and of loss of activity. It is inferred that two thiol groups participate in conformational changes and nucleotide interactions. 8. Comparison with creatine kinase suggests a fundamentally similar catalytic mechanism, although for arginine kinase certain additional restrictions are necessary because of the protection observed with nucleotide substrates.

Most phosphotransferases appear to contain reactive thiol groups, which are essential for catalytic activity. For arginine kinase (EC 2.7.3.3) this was first inferred by Elodi & Szorenyi (1956) and by Morrison, Griffiths & Ennor (1957) from the inhibitory effects of a wide range of thiol reagents. By studying the effect of iodoacetamide on the enzyme purified from lobster (*Homarus vulgaris*) muscle, Ruiz Cruz, Virden & Watts (1963) confirmed this finding. On the basis of protection afforded by substrates, they suggested that arginine kinase has some features similar to those of creatine kinase. They also showed that similar essential thiol groups appear to be present in the arginine kinases of animals from several different phyla. These findings suggested that the phosphagen phosphotransferases might have evolutionary relationships which were reflected in their reaction mechanisms.

A detailed hypothesis for the mechanism of action of creatine kinase, based on a study of its reactive thiol groups, has been presented by Watts & Rabin (1962). The present study of the reactive thiol groups of lobster arginine kinase was undertaken with three aims in mind: first, to determine the number and properties of the thiol groups and their role in the catalytic mechanism; second, to

establish whether there were sufficient features in common with the thiol groups of creatine kinase to infer a common type of catalytic mechanism; third, if a common type of mechanism seemed probable, to test further the validity of the mechanism of action proposed for creatine kinase by a study of the differences between the two enzymes.

The results indicate a complex picture with essential and non-essential thiol groups, some of which are involved in catalysis and some in the maintenance of tertiary protein structure. A catalytic mechanism basically similar to that for creatine kinase is suggested but it is necessary to impose certain additional restrictions on enzyme behaviour to explain the nature of protection by substrates against alkylation.

MATERIALS AND METHODS

Source of enzyme and definition of enzyme activity. Arginine kinase preparations were obtained by the method of Virden, Watts & Baldwin (1965). As an indication of enzyme purity the specific activity of each preparation, determined in a separate experiment, is given in the legends to the Figures. One unit of enzyme activity is defined as that amount of enzyme which, in 1 ml. of reaction mixture, will catalyse phosphoryl transfer from 1 mM-ATP to 25 mM-arginine in the presence of 6 mM-MgSO₄ in glycylglycine

buffer, pH 8.5, at 30° and yield an apparent second-order velocity constant of $1 \text{ ml. } \mu\text{mole}^{-1} \text{ min.}^{-1}$. This procedure was adopted so that the specific activity (units/mg. of protein) is directly comparable with that described for creatine kinase by Kuby, Noda & Lardy (1954). The molecular weight of the enzyme was assumed to be 37000 (Virden, Watts, Watts, Gammack & Raper, 1966). Protein concentration was determined as described by Virden & Watts (1966).

Iodoacetamide. This was recrystallized five times as described by Watts, Rabin & Crook (1961).

Buffers. Quartz-distilled water was used throughout. Unless otherwise stated, pH measurements were made at room temperature with an E.I.L. model 23a pH-meter fitted with a temperature compensator.

Potassium *p*-phenolsulphonate-NaOH buffer, pH 8.22 (30°) and *I* 0.1, was prepared by making a solution 68.9 mM in potassium *p*-phenolsulphonate (recrystallized anhydrous salt, given by Dr S. P. Datta) and 0.016 N in NaOH. The measured pH was 8.28 (30°). Other potassium *p*-phenolsulphonate-NaOH buffers were prepared by dissolving the salt in quantities calculated from the Tables of Datta & Grzybowski (1961) and adjusting to the required pH value with saturated NaOH before making up to the final volume to give *I* 0.05. The pH was checked at the final dilution. Tris buffers were prepared by dissolving the calculated quantities of tris (recrystallized in the presence of EDTA) and HCl (AnalaR, redistilled) in a solution of final *I* 0.05. Disodium malonate-HCl buffers were prepared by dissolving the calculated quantities of disodium malonate (anhydrous salt given by Dr D. G. Herries, recrystallized from aq. ethanol) and HCl in a stock solution of final *I* 0.1. Sodium acetate-HCl buffer, pH 5.32 and *I* 0.1, was prepared by adjusting a solution containing 0.681 g. of sodium acetate (AnalaR) to the required pH with HCl before diluting to 100 ml. Glycine-NaOH buffer, pH 9.4 and *I* 0.05, was prepared by dissolving 1.162 g. of glycine (recrystallized) in water and adjusting to the required pH with saturated NaOH, before diluting to 100 ml.

The pH values shown in the Figures are those of the actual reaction mixtures, measured immediately after the end of the experiment and, when necessary, corrected to give the actual pH at the temperature of the reaction.

Measurement of iodide concentration and of initial rate of iodide production. A silver-silver iodide electrode coupled to a calomel reference electrode by a saturated KNO_3 bridge was employed in the manner described by Watts *et al.* (1961). The temperature of the reaction vessel was controlled at either 25° or 30° as indicated in the legends to the Figures. The volume of the experimental solution was 4 ml. Experiments were conducted in the presence of 0.02% (v/v) Tween 80 and $1 \mu\text{M}$ -KI and reactions were started by the addition of iodoacetamide. Under these conditions arginine kinase did not interact with the electrode at a significant rate. The recorder gave a continuous plot of $\log [I^-]$ as a function of time. The results were replotted as progress curves for the production of I^- ions, and the initial velocities were obtained by drawing tangents to the curves at zero time.

Measurement of enzyme activity. Specific activities were determined as described above. Where loss of enzyme activity during alkylation experiments was being followed a single tube assay with incubation time of 10 min. was used. The volume of the reaction mixture was either 2 ml.,

as described by Virden *et al.* (1965), or 0.4 ml. as described by Virden & Watts (1964). The measured extinction in the colorimetric determination of the inorganic phosphate derived from phosphoarginine was corrected for the non-linearity of product formation as a function of enzyme concentration by the use of calibration curves. Since Tween 80 interferes with the phosphate determination these measurements were always carried out on samples from duplicate reaction mixtures without Tween 80. By a titrimetric assay it was found that Tween 80, at the concentration used, was without effect on enzyme activity. Varying the concentration of Tween 80 between 0 and 0.1% (v/v) was without effect on the rate of alkylation of the enzyme by iodoacetamide.

RESULTS

In preliminary experiments on the reaction of arginine kinase with iodoacetamide the initial velocity of I^- ion production was found to be first order with respect to both enzyme (Fig. 1b) and

Fig. 1. Effects of variation of (a) iodoacetamide concentration in the presence of $1.89 \mu\text{M}$ -arginine kinase, and (b) arginine kinase concentration in the presence of 4 mM -iodoacetamide, on the initial velocity of alkylation of arginine kinase by iodoacetamide. Reaction mixtures were at 25° and contained: *p*-phenolsulphonate buffer, pH 8.4 and final *I* 0.025; KI, $1 \mu\text{M}$; Tween 80, 0.02% (v/v); arginine kinase solution, dissolved in glycylglycine buffer, pH 7.8 and *I* 0.001; total volume, 4 ml. Controls contained glycylglycine buffer in place of enzyme solution. The reaction was followed by using the silver-silver iodide electrode as described in the text.

iodoacetamide (Fig. 1a). Varying the ionic strength from I 0.025 to I 0.275 had no effect on the rate of I^- ion production.

Specificity of the reaction with iodoacetamide. Amino acid analyses of hydrolysates of the alkylated enzyme showed that only cysteine side chains reacted and no other carboxymethylamino acid derivative could be detected. This was also the case when the enzyme was alkylated either under conditions where some of the thiols were unreactive, or under conditions where all the thiols that could be exposed by denaturation with sodium lauryl sulphate reacted with iodoacetamide (Table 1).

Effect of pH and temperature on the extent of alkylation. The course of the reaction of iodoacetamide with arginine kinase became increasingly complex as the pH was raised. Fig. 2 shows progress curves at various pH values and 25°. At pH 5.6 one equivalent of I^- ion was rapidly produced (Fig. 2, A). At pH 8.5, five thiol groups reacted rapidly (Fig. 2, E). Between these two pH values there was evidence for the progressive appearance of additional thiol groups (Fig. 2, B–D). Only one equivalent of I^- ion was rapidly released below

Fig. 2. Effects of pH on the alkylation of arginine kinase by iodoacetamide at 25°. Reaction mixtures contained: iodoacetamide, 4mM; KI, 1μM; Tween 80, 0.02% (v/v); arginine kinase (specific activity 232), 0.224mg./ml. (6.05μM); buffer, final I 0.025, giving pH in the reaction medium □, pH 5.6 (malonate buffer), ▲, pH 6.9 (tris buffer), △, pH 7.6 (*p*-phenolsulphonate buffer), ○, pH 8.3 (*p*-phenolsulphonate buffer), ●, pH 8.5 (tris buffer). The series of experiments was completed in 3 days.

Table 1. Yields of S-carboxymethylcysteine in acid hydrolysates of alkylated arginine kinase

Arginine kinase was treated with iodoacetamide and the enzyme was isolated and hydrolysed as described by Virden & Watts (1966). The extent of alkylation was measured with the Ag–AgI electrode. Samples of the hydrolysates were subjected to amino acid analysis in the Technicon Autoanalyzer.

Conditions of reaction	Total alkylation (moles/37000g. of protein)	Carboxymethylcysteine recovered	
		(moles/37000g. of protein)	Recovery as a percentage of total groups alkylated
Native enzyme*	0	0.01†	—
Partially alkylated enzyme‡	4.35	3.94§	90
Enzyme alkylated in presence of 1% sodium lauryl sulphate	5.31	5.09	96

* Solution dialysed against water before hydrolysis.

† Methionine sulfoxide runs chromatographically in the same position as carboxymethylcysteine and a small amount is usually detected.

‡ Two reaction mixtures pooled after reaction with 4mM-iodoacetamide, concentrated by vacuum dialysis against water before hydrolysis.

§ Value based on a single hydrolysis (22hr.).

|| Value based on three hydrolysis times (22, 42 and 71 hr.).

pH 7, but if the reaction was allowed to continue all five thiol groups were alkylated after 7hr.

Variability of enzyme preparations. In one preparation, at pH 8.3, three groups reacted rapidly but two further groups reacted slowly so that a biphasic curve was produced. In another preparation, at pH 8.2, three groups reacted rapidly at 25° with two more groups reacting slowly, and five groups reacted rapidly at 30°. When the enzyme was stored the observed total number of reactive groups at 30° progressively decreased by about one equivalent. All these preparations had almost identical specific activities.

Effect of pH on rate of activity and correlation with iodide production. Over the range pH 5.6–9.1, and at 25°, the apparent second-order rate constant (k_2) for the alkylation process increased tenfold while the rate constant for loss of enzyme activity increased only fourfold. The progress curve for the alkylation of arginine kinase at pH 7.1 (Fig. 5a) consisted of a rapid reaction, which was complete in 5–10min., followed by a complex secondary reaction which gradually accelerated after 25–30min. (cf. Fig. 2). If, as a first approximation, the secondary phase is assumed to be linear for the first 40min., then subtraction of the value of the I^- ion liberated in the secondary phase (obtained by extrapolation) from that produced in the overall reaction gives a curve for the primary reaction which reaches a maximum after a little less than one equivalent of iodide has been liberated (Fig. 5b).

A semi-log plot of this rapid phase yields a straight line which correlates closely with the curve for loss of activity (Fig. 4). At pH 8.2 the rate of loss of activity was the same as at pH 7.1 (Fig. 4) but approximately four equivalents of I^- ion were produced per mole of enzyme in the rapid initial phase. The secondary phase was now almost negligible and so had very little influence upon the derivation of the curve for the rapid phase alone, which gives a linear semi-log plot (Fig. 4). However, on the basis of percentage reaction it indicates a fourfold reduction in the rate of I^- ion production as compared with the rate of loss of activity (Fig. 4 and Table 3).

In a second series of experiments at 30° the essentially pH-independent nature of the rate of inactivation of arginine kinase by iodoacetamide was confirmed for the range pH 5.9–9.2 (Fig. 3).

Effects of arginine and ATP + Mg²⁺ ions on the alkylation reaction at pH 7.1 and 30°. Under these conditions only one equivalent of iodide was rapidly produced. The addition of either arginine or ATP plus Mg²⁺ ions to the reaction mixture modified the rate of iodide liberation in both the primary and secondary reactions (Fig. 5a) when the progress curves were analysed as described above. Arginine reduced the rate of the primary reaction to a greater extent than did ATP + Mg²⁺ (Fig. 5b), but whereas the latter also reduced the rate of the secondary reaction, arginine was found to enhance it (Table 2). Loss of activity always seems to correlate with the iodide produced in the primary reaction (Fig. 6).

Fig. 3. Reaction of arginine kinase with iodoacetamide. Effects of pH on the apparent second-order rate constants for loss of activity and alkylation. Reaction mixtures contained the components shown in Fig. 2 except that Tween 80 and KI were omitted from the inhibition experiments. □, Loss of activity at 25° in the absence of added inhibitor; ○, loss of activity at 25° in the presence of 4 mM-iodoacetamide; △, release of iodide at 25° in the presence of 4 mM-iodoacetamide; ●, loss of activity at 30° in the presence of 1 mM-iodoacetamide.

However, the rate of the second reaction was relatively great in comparison with the primary reaction (Fig. 5a). Because of the relatively large correction involved it is not clear whether the

Fig. 4. First-order log plots for the inhibition and alkylation of arginine kinase by iodoacetamide at 30°. Results are expressed as percentage of initial activity and percentage of reactive thiol respectively. Reaction mixtures for iodide production contained: KI, 1 μ M; Tween 80, 0.02% (v/v); arginine kinase specific activity 221, 0.205 mg./ml. (5.54 μ M); either (●) malonate buffer, final pH 7.1, or (▲) *p*-phenolsulphonate buffer, final pH 8.2. Reaction mixtures for measurements of loss of activity were identical except for the omission of Tween 80 and KI; ○, final pH 7.1; △, final pH 8.2. In all experiments the final buffer ionic strength was 1.05 and the total ionic strength was adjusted to 1.01 with sodium acetate.

Fig. 5. Effects of substrates on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 7.1 and 30°. Reaction mixtures contained: KI, 1 μ M; Tween 80, 0.02% (v/v); arginine kinase specific activity 221, 0.205 mg./ml. (5.54 μ M); malonate buffer, final pH 7.1, 1.05; no substrate (●); arginine, 10 mM (▲); ATP and MgSO₄, each 10 mM (▼). The total ionic strength was adjusted to 1.01 with sodium acetate. (a) Progress curves after subtraction of blank values in the absence of enzyme. (b) The same curves with the secondary reaction subtracted as described in the text.

Table 2. Rate constants for the reaction of arginine kinase with iodoacetamide at pH 7.1 and 30°

Second-order rate constants were calculated by dividing the apparent first-order rate constant by the iodoacetamide concentration (4 mM). The conditions for the experiments are described in the text and the legends to Figs. 6 and 7.

Substrate added	Apparent second-order rate constant for primary rapid reaction (l.mole ⁻¹ min. ⁻¹)	Rate of secondary release of I ⁻ ions (μg.ion l. ⁻¹ min. ⁻¹)
None	111.0	0.252
10 mM-Arginine	14.4	0.325
10 mM-ATP plus 10 mM-MgSO ₄	59.8	0.133

Fig. 6. First-order log plots for the effects of substrates on the alkylation and inhibition of arginine kinase by 4 mM-iodoacetamide at 30° and pH 7.1. The curves for alkylation are derived from the primary reactions shown in Fig. 5, as described in the text. ●, Control; ▲, arginine, 10 mM; ▼, ATP and MgSO₄, each 10 mM. Reaction mixtures for inhibition were identical except for the omission of Tween 80 and KI: ○, control; △, arginine, 10 mM; ▽, ATP and MgSO₄, each 10 mM.

alkylation process remains first order in the presence of substrates.

Effect of arginine, ADP and ATP alone and with Mg²⁺ ions on the alkylation reaction at pH 8.2 and 30°. Under these conditions a maximum of five thiol groups reacted. The greatest protection against loss of activity by single substrates was again obtained in the presence of arginine (Fig. 7). The concomitant progress curve for the production of I⁻ ion (Fig. 8) shows that protection was only partial, and that after 70 min. the same number of thiol groups had been alkylated as in the control. However, although loss of activity in the presence of arginine remained a first-order process up to

Fig. 7. First-order log plots for the effects of substrates on the inhibition of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. Reaction mixtures contained: arginine kinase specific activity 221, 0.205 mg./ml. (5.54 μM); *p*-phenolsulphonate buffer, final pH 8.2 and *I* 0.05; no substrate added (○); ATP, 5 mM (●); ATP and MgSO₄, each 5 mM (□); ADP, 5 mM (△); ADP and MgSO₄, each 5 mM (▲); arginine, 5 mM (▽); substrate equilibrium mixture, from preincubation with arginine, ATP and MgSO₄, all 5 mM (▼). The total ionic strength was adjusted to *I* 0.1 with sodium acetate. The lines for the last two substrate combinations were regressed to include additional points at 20 min.

Fig. 8. Effect of arginine on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. Reaction mixtures contained: KI, 1 μM; Tween 80, 0.02% (v/v); arginine kinase specific activity 221, 0.205 mg./ml. (5.54 μM); *p*-phenolsulphonate buffer, final pH 8.2 and *I* 0.05; either no added substrate (●) or arginine, 5 mM (▲). The total ionic strength was adjusted to *I* 0.1 with sodium acetate.

40 min., when the reaction was 90% complete, the iodide production was a more complex reaction for which the semi-log plot deviated markedly from linearity (Fig. 9). Similar results were obtained at

25° in *p*-phenolsulphonate buffer, pH 8.4. Arginine plus Mg^{2+} ions behaved in the same way as arginine alone.

In contrast with this, the alkylation pattern in the presence of nucleotide substrates was quite different. ATP alone was almost without effect (Figs. 10 and 11), as were Mg^{2+} ions alone (Fig. 10). In the presence of ATP plus Mg^{2+} ions there was a small reduction in the rate of loss of activity (Fig. 7). However, the concomitant curve for iodide production (Fig. 12) showed that approximately one thiol was completely protected against alkylation. The semi-log plot was no longer completely linear (Fig. 11). The effect with ADP alone at 30° (Figs. 10 and 11) was superficially similar to that with ATP plus Mg^{2+} ions, but there was slightly

greater protection against loss of activity (Fig. 7) and a large deviation from linearity in the semi-log plot (Fig. 11). Addition of Mg^{2+} ions to ADP further enhanced with protection against loss of activity (Fig. 7) but now two thiols were completely protected against alkylation (Fig. 10) and the semi-log plot for iodide production was again first order.

In the presence of an equilibrium mixture of substrates three thiol groups reacted at 30° (Fig. 12) as was observed in the presence of ADP plus Mg^{2+} ions, but the initial velocity of iodide production was reduced (Fig. 9). Protection against loss of activity was similar to that with arginine alone (Fig. 7) as was the first-order semi-log plot for iodide production (Fig. 9).

The apparent second-order velocity constants for the different processes are listed in Table 3.

Fig. 9. First-order log plots for the effects of substrates on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. The curves are derived from the progress curves shown in Figs. 9, 11 and 13. ○, Control; ▲, ADP and MgSO_4 , each 5 mM; ▽, arginine, 5 mM; ▼, substrate equilibrium mixture, from preincubation with arginine, ATP and MgSO_4 , all 5 mM. The solid line is drawn to run through the initial part of each curve.

Fig. 11. First-order log plots for the effects of substrates on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. The curves are derived from the progress curves shown in Figs. 10 and 12. ○, Control; ●, ATP, 5 mM; ▽, ADP, 5 mM; ▲, ATP and MgSO_4 , each 5 mM. The solid line is drawn to run through the initial part of each curve.

Fig. 10. Effects of substrates on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. Reaction mixtures were as in Fig. 9 except for the added substrates. ○, Control; △, MgSO_4 , 5 mM; ●, ATP, 5 mM; ▽, ADP, 5 mM; ▼, ADP and MgSO_4 , each 5 mM.

Fig. 12. Effects of substrates on the alkylation of arginine kinase by 4 mM-iodoacetamide at pH 8.2 and 30°. Reaction mixtures were as in Fig. 9 except for the added substrates. ○, Control; ●, ATP and MgSO_4 , each 5 mM; ▽, substrate equilibrium mixture, from preincubation with arginine, ATP and MgSO_4 , all 5 mM.

Dissociation constant for the enzyme-arginine complex. Because of the high degree of protection by arginine against alkylation by iodoacetamide it was possible to determine the dissociation constant for the arginine kinase-substrate complex. It was assumed that the enzyme-arginine complex was completely unreactive towards iodoacetamide, as

Fig. 13. Protection by arginine against inhibition and alkylation of arginine kinase by iodoacetamide. Reaction mixtures contained: either Δ , for initial rate of alkylation at 25° [KI, 1 μ M; Tween 80, 0.02% (v/v); arginine kinase specific activity 222, 0.211 mg./ml. (5.7 μ M); *p*-phenol-sulphonate buffer, pH 8.4, final *I* 0.025; iodoacetamide, 2 mM], or $\circ$, for initial rate of loss of activity at 30° [arginine kinase specific activity 221, 0.205 mg./ml. (5.54 μ M); *p*-phenolsulphonate buffer, final pH 8.2 and *I* 0.05; iodoacetamide, 4 mM; the final ionic strength was adjusted to *I* 0.1 with sodium acetate].

proposed by Whitehead & Rabin (1964). Two sets of conditions were used (Fig. 13): pH 8.2 and 30° for loss of activity and pH 8.4 and 25° for iodide production. Dissociation constants of 1.6 mM and 1.5 mM respectively were obtained from measurements of intercept/slope in the plots shown in Fig.

13, assuming the relationship $\frac{1}{V} = \frac{1}{V_0} + \frac{[A]}{KV_0}$, where

V is the measured velocity of alkylation or loss of catalytic activity, V_0 is the velocity in the absence of added substrate, $[A]$ is the concentration of the protecting substrate and K is the dissociation constant for the enzyme-substrate complex.

Effect of alkylation on electrophoretic mobility. Plate 1(a) shows a starch-gel electrophoretogram of arginine kinase after reaction for a fixed time with various concentrations of iodoacetamide. Plate 1(b) shows the analogous experiment with iodoacetate.

DISCUSSION

Under our experimental conditions the alkylation of arginine kinase follows first-order kinetics with respect to both iodoacetamide and arginine kinase (Fig. 1).

When an enzyme contains only one or two reactive thiol groups there is generally no problem in estimating what this number is since an error of 20% in their determination will not affect the conclusion. If, as in lobster arginine kinase, more than four thiol groups are involved it becomes difficult to establish the exact number. An error of more

Table 3. *Rate constants for reaction of arginine kinase with iodoacetamide at pH 8.2 and 30°*

Experiments were carried out as described in the text and in the legends to the Figures. Second-order rate constants were calculated by dividing the value of the apparent first-order rate constant by the initial iodoacetamide concentration (4 mM). When substrates were present the concentration of each was 5 mM.

Substrate added	Apparent second-order rate constant (l. mole ⁻¹ min. ⁻¹)			Nearest whole no. of groups protected completely
	Loss of activity	Iodide ion production		
		Molar enzyme basis	Reactive thiol basis	
None*	121	119	27	—
Arginine	21	49	11	0
ATP	100	111	24	0
ADP	79	74†	20†	1
MgSO ₄	—	111	25	0
MgSO ₄ + arginine	—	43	10	0
MgSO ₄ + ATP	84	80†	21†	1
MgSO ₄ + ADP	63	49†	20†	2
Substrate equilibrium mixture	18	36†	12†	2

* Mean of two determinations.

† Calculated from tangent of rate at zero time of semi-log plot, which was non-linear.

Plate 1. Starch-gel electrophoresis of arginine kinase after reaction with (a) iodoacetamide, (b) iodoacetate, at 25° and pH 8. For iodoacetamide the reaction was carried out for 1 hr. in the presence of the iodoacetamide concentrations shown. For iodoacetate the reaction time was 15 hr. At the end of the reaction the mixtures were cooled and immediately applied on filter-paper inserts to the gel cooled to 4°. Electrophoresis (constant current 35 mA) was carried out for 5 hr. with the discontinuous buffer system, pH 8.5, described by Virden & Watts (1964), before staining with Amido Black.

than 10% may alter the final estimate by up to one group. In addition to normal experimental errors, two other errors may influence the results: the purity of the enzyme and its molecular weight. Various criteria suggest that the impurities in our enzyme preparation did not exceed 5% (Virden *et al.* 1965). An estimate of molecular weight is subject to greater error. A value of 37 000 was deduced by Virden *et al.* (1966) but the margin of error could be great enough to introduce an error of one in the total number of thiol groups per enzyme molecule. Differences between the numbers of reactive groups where protection is observed (Figs. 10 and 12) are much more reliable since the possible errors mentioned above become less significant.

While the present work was in progress Pradel, Kassab, Regnouf & Thoai (1964) published data on the reactivity and stoichiometry of the thiol groups in lobster arginine kinase based on its reactions with *N*-ethylmaleimide and *p*-chloromercuribenzoate. However, their determinations are based on the molecular weight of 43 000, which was found by Elodi & Szorenyi (1956) for the enzyme from the Hungarian marsh crab (*Potamobius astacus*). If an allowance is made for the difference in molecular weight our work is substantially in agreement with theirs.

Under suitable conditions all five thiol groups in native arginine kinase can be specifically alkylated by iodoacetamide (Table 1) whereas other amino acid side chains do not react to a significant extent. None of these thiol groups (Tables 2 and 3) reacts at a rate comparable with that of a freely reacting thiol. Hollaway, Mathias & Rabin (1964), for example, found that the free thiol in ficin reacts with an equimolar concentration of iodoacetamide at a rate too fast to measure above pH 8.2. The reaction of arginine kinase may be readily followed above this pH (Fig. 3) in the presence of a much higher iodoacetamide concentration. This suggests that all the thiol groups of arginine kinase have their reactivity modified in some way as the result of interactions within the protein molecule. This is emphasized by the pH-independence of the rate of loss of activity (Fig. 3) in a reaction which is normally a function of the pH-dependent mercaptide ion concentration.

The first requirement for understanding the role of thiol groups in enzyme catalysis is a knowledge of the number of catalytic sites per enzyme molecule. If one excludes the possibility of several interacting catalytic sites where alkylation of a thiol at one site causes profound changes in the properties of thiols at other sites, then Figs. 4 and 5 indicate that lobster arginine kinase contains one catalytic site which includes one thiol with unique properties. At pH 7.1, only this thiol reacts rapidly

(Fig. 5) and the alkylation is exactly paralleled by loss of enzyme activity (Fig. 4). Below pH 7.0 the reactivity of this thiol is somewhat modified (Fig. 3) and this is again paralleled by changes in the rate of loss of activity. These changes are not compatible with normal ionization and must result from interactions with other dissociating groups in the protein molecule. As the pH is raised above 7.5 the apparent k_2 for activity loss is relatively unaffected until pH 8.5 is reached but k_2 for iodide production becomes increasingly greater (Fig. 3). This appears not to be due to an alteration in the properties of the unique catalytic thiol, whose reactivity hardly alters (as indicated by the curve of pH against loss of activity) but to the progressive appearance of new, additional thiol groups (Fig. 2). Above pH 8.5, k_2 for loss of activity increases and the whole reaction becomes very complex.

Since the additional thiol groups, which appear as the pH is raised, react without affecting the rate of loss of activity it might be inferred that at least one of them is not essential. However, alkylation experiments at pH 8.2 and 30° (Fig. 4) reveal that when four equivalents of iodide per mole of enzyme are produced the pseudo-first-order reaction rate (on a reactive thiol basis) is one-quarter of that for loss of activity. This implies that four thiol groups are essential for catalytic activity at this pH. This paradox can be resolved only if the four thiol groups are, in fact, not equally reactive, as is suggested by the linear semi-log plot in Fig. 4, but take part in a sequential reaction which is dependent on reaction of the first, pH-independent thiol.

Fig. 14 shows hypothetically how an overall first-order process could result from the sum of a first-order reaction and the complex ordered reaction of other groups dependent on it. This hypothesis explains the close correlation between the rate constants for loss of activity and iodide production (Fig. 3 and Table 3). Above about pH 8 the initial velocity of iodide production increases more rapidly than that of loss of activity. Between pH 8.2 and pH 9.2 the rate constant for the additional increase in iodide production, after subtraction of the rate constant for the iodide production associated with loss of activity, increases approximately tenfold (Fig. 3). This suggests that at least one of the thiol groups which shows the sequential release behaviour below pH 8.2 begins to ionize and react independently at higher pH values. Inspection of the difference between the progress curves in Fig. 2 (D and E) suggests that two thiol groups could be involved in this ionization process, but probably not more than two. The fact that there is no concomitant increase in the rate of loss of activity indicates that at least one thiol is not essential for enzyme activity.

Fig. 14. Hypothetical progress curves for alkylation of an enzyme with four reactive thiol groups. The complete first-order progress curve (—) is composed of the sum of a first-order process (---) involving one group and leading to the loss of activity, and a complex secondary reaction (-.-.-) which is dependent on the reaction of the first group. Since the velocity of the secondary reaction is zero at the beginning of the reaction, defining the overall reaction as first-order also defines the ratio of rate of loss of activity to the overall rate of alkylation. Thus for the curves shown here this ratio is 1:1 and would become 4:1 if both processes were presented on the basis of percentage reacting, with the total reaction for each process equal to 100%.

Support for this interpretation is provided by the polyphasic character of the progress curve for iodide production as the pH is raised (Fig. 2). Also, in the presence of arginine, ADP, ATP + Mg^{2+} ions, or the substrate equilibrium mixture, the semi-log plots for iodide production deviate markedly from linearity (Figs. 9 and 11), although linearity of the curves for the concomitant loss of activity remain unaltered. Further evidence for the sequential nature of the thiol release is supplied by analysing the enzyme on starch gels, as it is progressively alkylated. With iodoacetamide (Plate 1a) only one new slowly migrating electrophoretic component is produced.

Assuming pseudo-first-order kinetics (Fig. 1), comparison with Fig. 2(E) indicates that the electrophoretic pattern after incubation with 0.19 mM-iodoacetamide is roughly comparable with the production of 1.5 equiv. of I^- ion. With 0.38 and 0.75 mM-iodoacetamide the values are 2.4 and 3.6 equivalents respectively, and the calculated loss of activity for each iodoacetamide concentration is 50%, 80% and 95% respectively. However, although 3.6 thiol groups have reacted, only one, that at the catalytic site, has contributed to the electrophoretic mobility of the protein. The change observed in electrophoretic mobility is similar to that obtained for creatine kinase after the alkylation of a single thiol (Watts, 1964). As the other thiol groups are successively revealed their charges must be rapidly blocked by further reaction with the neutral iodoacetamide. If this interpretation

is true then faster-migrating components should be revealed by the reaction with iodoacetate. This is shown in Plate 1(b).

Alkylation of the first group does not alter the charge on the protein but as the buried thiol groups are exposed their charge is fixed by alkylation with iodoacetate so that at least two more rapidly migrating protein species are produced. Beyond this stage the process becomes too complex for analysis as the protein presumably unwinds to produce eventually the broad slowly migrating band of denatured enzyme. By comparison, when creatine kinase was investigated by the same procedure (Watts, 1964), its electrophoretic mobility was unaffected by iodoacetate, but iodoacetamide progressively formed one, and then a second, slowly migrating protein band as the two catalytic thiol groups are progressively blocked. Thus the pseudo-first-order nature of the reaction at pH 8.2 (Fig. 4) is clearly fortuitous and loss of activity occurs concomitantly with the alkylation of a single pH-independent thiol. Two thiol groups are probably not essential for the activity of the enzyme *in vitro* and these may perhaps be equated with the two thiol groups that were released by raising the temperature from 25° to 30° at pH 8.2 and with the thiol whose reactivity was lost on storage. This offers a ready explanation for the observed enzyme variability without any significant difference in catalytic activity.

In the light of this conclusion it is reasonable to infer that the two thiol groups yet unaccounted for are those which are protected by nucleotide substrates. It is not possible to decide whether they are essential for activity. However, their function, and that of the pH-independent thiol, may be partially elucidated from a detailed analysis of the data on substrate protection. One group is protected completely by ATP + Mg^{2+} ions (Fig. 12) but not by arginine (Fig. 8). It may tentatively be inferred that this thiol is located among the peptide chains connected with the nucleotide-binding site. ATP alone does not protect this thiol but ADP alone does (Fig. 10), although the possible presence of a limiting amount of Mg^{2+} ions as contaminant in the ADP has not been excluded. Since the only difference between these nucleotides is a single phosphoric acid group it is possible that there is a charged group on the enzyme which interacts with the γ -phosphoric acid group of ATP to prevent a conformational change which is induced by ADP. Addition of Mg^{2+} ions blocks the extra charge on the ATP and permits the change to occur. With ADP + Mg^{2+} a second thiol is also protected.

The extra difference between ADP + Mg^{2+} and ATP + Mg^{2+} as opposed to the free nucleotides lies mainly in the location of the metal ion on the polyphosphate chain (Cohn & Hughes, 1962). A

possible explanation of this finding is that a second thiol becomes exposed when the nucleotide is bound to the enzyme and that it interacts with the ADP-Mg^{2+} complex but not with the ATP-Mg^{2+} complex. This in turn suggests a mechanism whereby the ATP-Mg^{2+} complex changes into the ADP-Mg^{2+} complex in the transition state of the transphosphorylation reaction. An alternative, less satisfactory, explanation is that ADP-Mg^{2+} binds in a different way from ATP-Mg^{2+} and induces a different type of conformational change, which may result in the protection of a different number of thiol groups in an unspecified way.

It is of interest to compare the reactive thiols of arginine kinase with those of rabbit-muscle creatine kinase. Both enzymes contain a single thiol, for a sub-unit molecular weight of approx. 40 000, which is pH-independent in the reaction with iodoacetamide and is essential for catalytic activity. Both enzymes also contain a group reactive towards hydroxylamine, which is associated with catalysis (Thoai, Kassab & Pradel, 1963; Pradel *et al.* 1964). On this basis it appears reasonable to suggest that the mechanism of action proposed for creatine kinase by Watts & Rabin (1962) may be equally applicable to arginine kinase and that the pH-independent thiol forms part of the acid-base pair at the catalytic site. However, there are differences between the reactions of the two enzymes. Unlike that in arginine kinase (Figs. 5-13), the pH-independent thiol in creatine kinase is not protected by single substrates when iodoacetamide is the inhibitor although protection against inhibition by iodoacetate has been reported (Padieu & Mommaerts, 1960; Mahowald, Noltmann & Kuby, 1962). The strong protection by arginine (Figs. 5-9) may be partly explained by its lower apparent equilibrium constant (5.2 mM) (Virden *et al.* 1965), compared with 19 mM for creatine and creatine kinase (Nihei, Noda & Morales, 1961). This difference is reasonably explained by the charged α -amino group present in arginine but absent from creatine. The finding of protection by arginine provides support for the hypothetical mechanism of action postulated by Watts & Rabin (1962), which requires interaction of the essential thiol with the guanidino group (Fig. 15A).

In contrast, protection by nucleotide substrates alone (Figs. 5-7 and 9-12) does not, at first sight, accord with the hypothesis since the electron-donating power of the β -phosphoryl oxygen of the nucleotide substrate should, by the tendency depicted by the arrows in Fig. 15(B), increase the nucleophilic power of the thiol rather than decrease it. At this point a further complication in the arginine kinase reaction must be considered. Unlike creatine kinase its two substrate-binding sites are

Fig. 15. Effect of arginine and ATP-Mg^{2+} on the reactivity of the thiol group in the thiol-imidazole catalytic acid-base pair postulated to occur in the catalytic site of guanidine phosphotransferases.

markedly interdependent; binding one substrate to the enzyme influences the apparent equilibrium constant for the binding of the other substrate (Virden *et al.* 1965). Further evidence for this secondary effect is revealed by the observation that arginine and ATP-Mg^{2+} not only protect the pH-independent thiol (Figs. 5 and 6) but also affect the reactivity of the other thiol groups in the enzyme to different extents and in different directions (Table 2). Pradel, Kassab, Terrossian & Thoai (1965) have reported a similar phenomenon in the reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Thus it appears that the interpretation depicted in Fig. 15(B) is over-simplified. If the hypothesis of Watts & Rabin (1962) is to hold it must be assumed that interaction of the nucleotide with the thiol-imidazole pair also involves a conformational change which alters the reactivity of the thiol but it is not possible to predict whether this will be increased or decreased. A consideration of the rate data for the various processes supports the Watts & Rabin (1962) hypothesis (Table 3). Arginine reduces the rate of loss of activity sixfold. ATP-Mg^{2+} , which one might presume to be a good protecting agent, is, in fact, less efficient than ADP-Mg^{2+} . Even so, the rate of loss of activity in the presence of ADP-Mg^{2+} is three times that in the presence of arginine. Thus it is highly improbable that these three substrates all protect by direct interaction with the thiol which is modified (Figs. 6 and 7). The complex changes in the rates

of iodide production which accompanies protection by the nucleotide substrates (Table 3) also tend to exclude simple electrostatic effects as an explanation of these phenomena. The complexity of the rate data is most compatible with the view that induced conformational changes in the enzyme completely protect some thiol groups and alter the reactivity of others in an unpredictable manner. The rate of the secondary reaction in Table 2, which is increased 30% by arginine and decreased 47% by ATP-Mg²⁺, is difficult to explain on any other basis.

Thus while the hypothesis of Watts & Rabin (1962) for the mechanism of action of creatine kinase may be generally applied to arginine kinase the greater flexibility and complexity of the latter requires an additional restriction, in terms of a conformational change, upon binding of the nucleotide substrate if the data are to be adequately explained. If this additional restriction is rejected then the hypothesis for the mechanism of action fails and the only possible role for the pH-independent thiol can be in mediating a conformational change without actually participating in the transphosphorylation process. In this circumstance the protection by arginine alone against alkylation is more difficult to explain, as is the observation that this thiol clearly contributes to the net change on the enzyme whereas other thiol groups which are demonstrably associated with maintenance of structure do not (Plate 1). On either hypothesis for the role of the pH-independent thiol it must be assumed that the groups to which the thiol is linked are on a different part of the protein chain since alkylation breaks the link and leads to a progressive unwinding of the protein chain (Fig. 2).

Determinations of the binding constant for arginine by measurements of loss of activity and by following the alkylation reaction (Fig. 13) are in very good agreement (1.6 and 1.5 mM respectively). This is evidence that the two methods are following the same chemical process and helps justify the analytical procedure used in Figs. 5 and 6. It also reveals that in the protection experiments insufficient arginine was present to saturate the enzyme and that had the enzyme been saturated complete protection might have been achieved, as is suggested by Fig. 15(A). As stated above, the K_m for arginine is modified by the binding of the nucleotide substrate so that the K_i for arginine alone is higher than K_m . Virden *et al.* (1965) obtained 5.2 mM for K_i by an extrapolation procedure assuming equilibrium kinetics. This is in good agreement with the values obtained by studies with inhibitors and supports the assumption that equilibrium kinetics apply.

In summary, the present work suggests that arginine kinase contains five reactive thiol groups

per 37 000 molecular weight. One of these shows a characteristic pH-independence in the reaction with iodoacetamide, is essential for enzyme activity and probably forms part of the catalytic mechanism at the single catalytic site. Two other thiol groups are probably essential for activity, are involved in conformational changes of the protein associated with the binding of nucleotide substrates and one of them might be involved with the function of the Mg²⁺ ion in the transition state. The two remaining thiol groups are probably not essential for enzyme activity *in vitro* and show some variability in behaviour from preparation to preparation.

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