

Inhibition of Adenosine 5'-Triphosphate-Creatine Phosphotransferase by Substrate-Anion Complexes

EVIDENCE FOR THE TRANSITION-STATE ORGANIZATION OF THE CATALYTIC SITE

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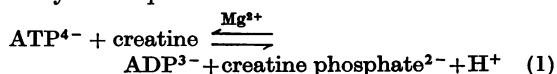
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1. The substrate combination creatine-MgADP does not significantly protect creatine kinase against inhibition by iodoacetamide in the absence of small anions. 2. Small anions can be divided into three groups according to the way in which they affect creatine kinase: I, acetate reversibly increases enzyme activity in the forward reaction but does not affect the rate of inhibition by iodoacetamide in the presence of creatine plus MgADP; II, planar anions and some halides (HCO_3^- , HCO_2^- , NO_3^- , NO_2^- , Cl^- , Br^- , F^-) in the presence of creatine plus MgADP protect the enzyme from inhibition by iodoacetamide; III, tetrahedral anions (SO_4^{2-} , HPO_4^{2-} , ClO_4^- , BF_4^-) and iodide do not affect the rate of inhibition by iodoacetamide in the presence of creatine plus MgADP but may decrease the protection by class II anions under these conditions. Anions of class II and class III also reversibly inhibit enzyme activity. 3. It is concluded that class II anions form a stable and inactive quaternary enzyme-creatine-MgADP-anion complex and this is responsible for the effect attributed by previous workers to the ternary complex lacking anion. Formation of this complex, particularly in the forward reaction, can lead to markedly non-linear enzyme progress curves. Some previous observations are re-appraised in the light of these findings. 4. From the behaviour of chloride and nitrate ions, and the marked lowering of the K_i values for creatine and MgADP they produce, it is inferred that planar or monoatomic anions act in the quaternary complex by simulating the transferable phosphoryl group in the transition state (or another intermediate state) of the reaction. 5. It is suggested that, in the course of the reaction, the tetrahedral phosphate-binding site for the transferable phosphoryl group of the substrate (that also binds class II and class III anions) changes into a trigonal bipyramid site (also occupied by class II anions). This strains the phosphoryl group to adopt the transitional sp^3d hybridized state and must contribute significantly to the low activation energy of the reaction. 6. Catalysis is deduced to proceed by an 'in line' transfer reaction and from the effects of class II anions it is possible to estimate the approximate dimensions of the anionic site in the transition-state complex. 7. The specific protecting effect of an equilibrium mixture of substrates against inhibition by iodoacetamide provides further evidence for the conformational change suggested above as a step in the catalytic process.

The inhibition of adenosine 5'-triphosphate-creatine phosphotransferase (EC 2.7.3.2) by simple inorganic anions is well known. By using sulphate or orthophosphate ions inhibition was found to be competitive with respect to ATP and creatine phosphate but non-competitive with respect to ADP and creatine, and it was inferred that the inhibitory anion binds to the site on the enzyme occupied by the transferable phosphoryl group of the substrate (Nihei, Noda & Morales, 1961; Kumudavalli, Moreland & Watts, 1970). Other anions might be expected to act in the same way and their relative

efficacy related to the extent to which they simulate the tetrahedral structure of a phosphate anion.

Creatine kinase can be assayed by a pH-stat method that is particularly advantageous because it allows the progress of the reaction (1) to be continuously followed. Some improvements in the assay technique are described.



The investigation reported here was initiated because it was noticed that the presence of certain

anions affected both the initial velocity of the reaction and the shape of the progress curve in a way that did not seem compatible with the accepted explanation of anion inhibition. This has led to a re-appraisal of the effects of small anions; as a result it is proposed that in the presence of bound creatine and MgADP* a modified anion binding site is formed on the enzyme that favours the binding of a different spectrum of anions from that which competes with separate substrates.

The rate of reaction of the essential thiol groups on the enzyme with iodoacetamide has been found to be affected by the presence of certain substrate combinations. Watts & Rabin (1962) showed that an equilibrium mixture of substrates protected the enzyme against alkylation, whereas O'Sullivan, Diefenbach & Cohn (1966) found an even greater protection with the 'dead-end complex' creatine-MgADP. Evidence is now presented that such substrate protection phenomena depend largely on the concentration and type of anion present and correlates with the type of inhibition caused by anions in kinetic experiments.

The variety of anions available make useful probes to investigate the geometry of the anion binding site and this is considered in relation to the mechanism of enzyme action. Some aspects of this work have been the subject of a preliminary communication (Milner-White & Watts, 1970).

MATERIALS

ATP and AMP were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., ADP from Seravac Laboratories (Pty) Ltd., Feltham, Middx., U.K. and creatine phosphate from Sigma (London) Chemical Co. Ltd., London W.6, U.K. Creatine (British Drug Houses Ltd., Poole, Dorset, U.K.) was recrystallized from 1 mM-EDTA, pH 7.5, and then again from water. Doubly distilled water was used for all solutions.

NaCl, KCl, MgSO₄ and CaO were of Specpure grade from Johnson Mathey and Co. Ltd., London E.C.4, U.K. Other salts were of AnalaR grade except for NaBF₄ and LiCl which were laboratory grade. Iodoacetamide was recrystallized five times from aq. 50% ethanol.

Buffers. *NN*-Di-(2-hydroxyethyl)glycine (bicine) was prepared as described by Datta, Grzybowski & Bates (1964), recrystallized twice from 80% (v/v) redistilled methanol, once from 1 mM-EDTA, pH 7.5, and finally from water.

Tris (British Drug Houses Ltd.) was recrystallized from 1 mM-EDTA, pH 7.5, and then from water.

Recrystallized diethylmalonic acid was a gift from Dr D. G. Herries. Buffers were prepared from carbonate-free saturated NaOH which was diluted with CO₂-free water before use.

* The magnesium ion-nucleotide complex is the true substrate of the enzyme and throughout this paper is treated as a single substance, abbreviated MgADP or MgATP.

Creatine kinase. Creatine kinase (specific activity 50–60 units, or 130 μmol H⁺ released/min per mg of protein under the pH-stat assay conditions for the forward reaction described in the Methods section) was prepared from rabbit muscle by method B of Kuby, Noda & Lardy (1954), by using AnalaR ethanol treated as described by Nihei, *et al.* (1961). Protein concentration was measured by the biuret method (Gornall, Bardawill & David, 1949) or spectrophotometrically at 280 nm in 5 mM-tris-HCl buffer, pH 8.0, assuming a molecular extinction coefficient of 7.45×10^4 and a molecular weight of 81000 (Noda, Kuby & Lardy, 1954).

METHODS

Enzyme assay. In the forward reaction (eqn. 1) the enzyme was assayed in a pH-stat assembly (Titrigraph, SBR2C; Titrator TTT11a; and pH-meter 25, from Radiometer, Copenhagen) in a reaction volume of 2 ml thermostatically controlled at 8.8°C and magnetically stirred. For routine measurements the assay mixture contained: 40 mM-creatine; 5 mM-magnesium acetate; 4 mM-ATP; 100 mM-sodium acetate; 1 mM-cysteine; 0.01 mM-EDTA. The pH was brought to 8.8 with 0.5 M-CO₂-free NaOH and finally adjusted to 9.0 with the titrator with the 10 mM-CO₂-free NaOH. After allowing the pH-stat to run for 2 min to provide a base-line the assay was started by adding 0.1 ml of the enzyme suitably diluted in 5 mM-cysteine-NaOH buffer, pH 9.1. Experiments in the reverse direction were carried out under similar conditions but at pH 8.0 by using 10 mM-HCl as titrant. The enzyme was diluted in 5 mM-cysteine-NaOH buffer, pH 8.0. The total chloride added in the course of the reverse reaction gave a final concentration of not more than 0.3 mM after 1 min of reaction. The dilution error caused by the addition of titrant was not more than 3% and was ignored. The rate of proton titration in the absence of ATP or enzyme was 1.5–2.5% of the maximum velocities measured. This rate was not affected by the addition of either ATP or enzyme and was attributed to CO₂ uptake.

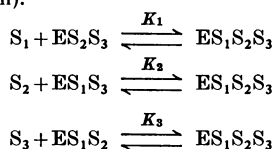
Initial-velocity measurements were obtained from tangents drawn to the progress curves at zero time and the value for the rate without enzyme subtracted. In all cases magnesium acetate was added so that the calculated free Mg²⁺ ion concentration exceeded the total nucleotide concentration by 1 mM, as suggested by Cleland (1967). When ADP was used the concentration of MgADP was calculated assuming a stability constant of 4000 M⁻¹ (Morrison & Uhr, 1966). For MgATP, dissociation was assumed to be negligible.

The cysteine added in low concentration to the reaction mixture serves two functions: (1) as a reducing agent; (2) as a buffer. The latter may be more important because 1 mM-cysteine can be replaced by 1 mM-boric acid-NaOH buffer at pH 9.0 with a similar effect. By using a pH-stat method Cho, Haslett & Jenden (1960) reported that thiol reagents or cyanide activated the enzyme activity and Mahowald, Noltman & Kuby (1962) used 1% albumin for the same purpose. Since creatine kinase is rapidly inactivated at extremes of pH, and both thiol reagents or cyanide, and inert protein are buffers at pH 8–10, it seems likely that the inactivation reported was due to insufficient buffering. Moderate buffer concentrations (up to 10 mM-cysteine) do not lower the sensitivity of the pH-stat assay.

Iodoacetamide inhibition experiments. These were normally carried out in bicine-NaOH buffer, pH 9.0 and 10.0206, at 25°C. The reaction of iodoacetamide with creatine kinase is not affected by ionic strength (Watts & Rabin, 1962) an observation that was confirmed (see the Results section). Hence it is possible to compare the effects of various anions over a range of concentrations. The enzyme (0.5 mg/ml) was allowed to equilibrate with any substrates or anions present for 15 min in a total volume of 1 ml. A sample (0.1 ml) was then transferred to 0.9 ml of 5 mM-cysteine as a control and then 0.1 ml of aqueous iodoacetamide was added to the rest of the reaction mixture. The progress of the reaction was followed by transferring samples to cysteine solution at the appropriate times in the same way as for the control. All the samples were then assayed for enzyme activity in the forward direction, a correction being made for the difference in dilution between the control and other samples. The samples in cysteine solution did not change their enzyme activity for at least 2 h at room temperature so that no other corrections for spontaneous changes in enzyme activity were necessary.

In experiments where the pH of the reaction mixture was varied the buffering power of the cysteine solution was insufficient to hold the pH at 9.1 (the pH used for all other assays in the forward direction). Hence it was necessary to alter the pH at which the enzyme assay was carried out. However, it was found that the initial velocity of enzyme activity did not alter significantly over the pH range 8.0-9.8 with the pH-stat conditions described so that no correction is necessary for the slight differences of assay pH used in these experiments.

Calculation of apparent dissociation constants. These were determined for the three ligands of the quarternary complex, enzyme-creatine-MgADP-anion (see the Discussion section).



where K_1 , K_2 and K_3 are equilibrium constants. The apparent values for K in each case were derived from the

values of the rate constants for iodoacetamide reaction with the enzyme in the presence of various amounts of the particular ligand. It is assumed that the observed reaction rate is the sum of the reaction rate of the enzyme as a quarternary complex and that of the free enzyme, and that no intermediate state exists as far as iodoacetamide reactivity is concerned. This is supported by experiments showing that the combination of any two ligands has no effect on this reactivity. The derivation has been described by O'Sullivan & Cohn (1966) except that here there are considered to be four components instead of three in the complex. If k_1 = first-order rate constant for enzyme inhibition in the absence of ligands, k_2 = first-order rate constant for enzyme inhibition when the three ligands are bound to the enzyme, and k^* = first-order rate constant observed, then

$$\Delta k^* = k_1 - k^*$$

Now let K = apparent dissociation constant for compound S (i.e. K_1, K_2, K_3 for S_1, S_2 and S_3 respectively) then

$$\frac{1}{\Delta k^*} = \frac{K}{k_1 - k_2} \cdot \frac{1}{[S]} + \frac{1}{k_1 - k_2}$$

For each component of the complex $1/\Delta k^*$ is plotted against $1/[S]$, giving $-1/K$ as the intercept on the x -axis.

RESULTS

Effects of inorganic anions on the enzyme reaction

Forward reaction. Fig. 1 shows pH-stat traces of progress curves for the forward reaction in the absence and presence of various small anions and Table 1 shows the effect on the specific activity of the enzyme. With the enzyme concentration used and in the absence of small anions or with added acetate almost linear progress curves were obtained over the first 3 min of reaction. Acetate ions (0.1 M) activated the enzyme by 15-30% and for this reason acetate was routinely used as the counter ion to Mg^{2+} throughout this work, and for controlling the ionic strength of the assay mixture (see the Methods

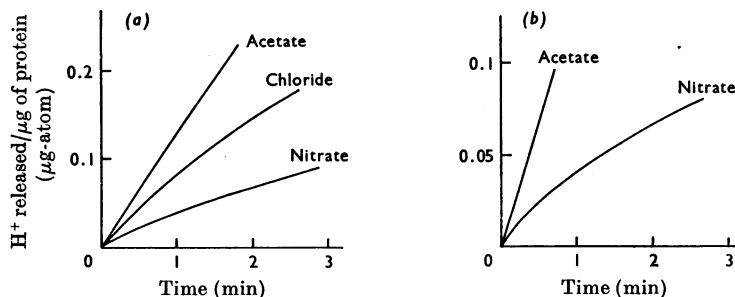


Fig. 1. Effects of anions on progress curves for the forward reaction of creatine kinase at pH 9.0 and 30°C. The curves are traced from pH-stat recordings. Assay conditions are described in the Methods section. Sodium acetate, NaCl and KNO_3 were used to give a final concentration of 0.1 M. In (a) the enzyme concentration was 2.5 $\mu\text{g/ml}$ and in (b) 7.9 $\mu\text{g/ml}$.

Table 1. *Effects of various anions on the specific activity of creatine kinase measured in the forward direction*

Assays were carried out under the standard conditions described in the Methods section.

Source of Anion	Concn. (M)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)
None	—	114
Sodium acetate	0.1	131
Potassium acetate	0.1	130
Sodium fluoride	0.1	118
Sodium arsenate	0.05	111
Sodium sulphate	0.05	99
Sodium sulphite	0.05	96
Potassium bromide	0.1	95
Potassium iodide	0.1	93
Sodium perchlorate	0.1	91
Sodium chloride	0.1	83
Sodium nitrate	0.1	38
Potassium nitrite	0.1	22

section). Other anions such as SO_4^{2-} and ClO_4^- depressed the rate of reaction without noticeably affecting the linearity of the progress curve, but with Cl^- and NO_3^- both inhibition and a marked increase in the curvature of the progress curves occurred. The effect of NO_3^- at high concentrations was so pronounced that it was extremely difficult to make valid initial velocity movements and the effect could not be relieved by increasing the enzyme concentration. Inhibition was independent of the monovalent cation present and lithium chloride, sodium chloride and potassium chloride, for example, all gave identical results over a range of concentrations (Fig. 2). In other experiments it was found that none of the anions used caused irreversible inhibition of the enzyme. Fig. 3 shows as a Lineweaver-Burk plot that the inhibition by Cl^- ions is not of the simple competitive type as compared with acetate when ATP is the variable substrate. Heyde & Morrison (1969) reported that Cl^- ions acted as a non-competitive inhibitor. Inhibitor constants for Cl^- ions measured in the forward reaction are given in Table 2.

If the product of the reaction, MgADP, is added to the reaction mixture, the inhibition that this causes is greatly enhanced by substituting Cl^- ions for acetate ions in the reaction mixture (Fig. 3). It was also noticeable in the absence of added ADP that as the concentration of MgATP was lowered the progress curves in the presence of Cl^- ions became noticeably more curved compared with those in acetate ions. It would seem that the MgATP/MgADP ratio during the reaction is an important feature of this effect.

Fig. 4 shows similar results when creatine is the

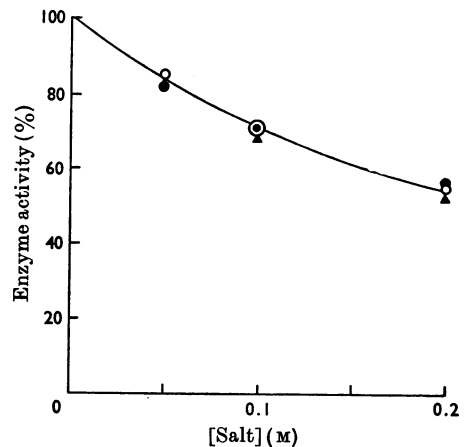


Fig. 2. Effect of varying the cation type on the inhibition of creatine kinase by chloride ions at pH 9.0 and 30°C. The cations used were: \circ , K^+ ; \bullet , Na^+ ; \blacktriangle , Li^+ . The enzyme concentration was 2.5 $\mu\text{g}/\text{ml}$. Other conditions were as described in the Methods section.

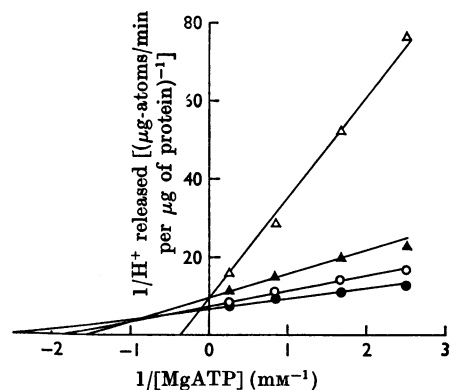


Fig. 3. Comparison of the inhibition of the forward reaction of creatine kinase by MgADP plus either acetate ions or chloride ions at pH 9.0 and 30°C. The creatine concentration was held constant at 40 mM and the initial MgATP concentration varied so that the free Mg^{2+} ion concentration was always 1 mM. The enzyme concentration was 2.5 $\mu\text{g}/\text{ml}$. Other conditions were as described in the Methods section. \bullet , Plus sodium acetate, 100 mM; \circ , plus sodium acetate, 100 mM, and MgADP, 0.161 mM; \blacktriangle , plus NaCl, 100 mM; Δ , plus NaCl, 100 mM, and MgADP, 0.161 mM.

variable substrate and creatine phosphate is the added inhibitor. Unlike the inhibition by MgADP that by creatine phosphate is not measurably enhanced by Cl^- ions.

Calculation of the inhibition constants from these results show that the presence of Cl^- ions markedly

Table 2. *Inhibitor constants for chloride and sulphate ions at 30°C*

The values were derived from the results shown in Figs. 3-7.

pH	Variable substrate	Inhibitor	K_i (mM)
9.0	MgATP	Chloride	140
9.0	Creatine	Chloride	230
8.0	MgADP	Chloride	150
8.0	Creatine phosphate	Chloride	180
9.0	MgATP	Sulphate	30

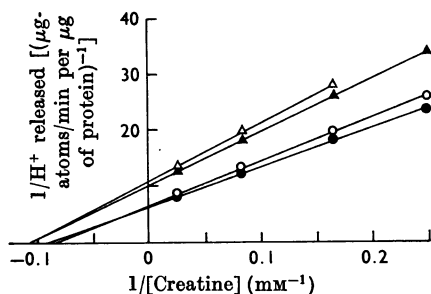


Fig. 4. Comparison of the inhibition of the forward reaction of creatine kinase by creatine phosphate plus either acetate or chloride ions at pH 9.0 and 30°C. The MgATP concentration was held constant at 4 mM, and the creatine concentration was varied. The enzyme concentration was 2.5 μg/ml. Other conditions were as described in the Methods section. ●, Plus sodium acetate, 100 mM; ○, plus sodium acetate, 100 mM, and creatine phosphate, 6 mM; ▲, plus NaCl, 100 mM; Δ, plus NaCl, 100 mM, and creatine phosphate, 6 mM.

lowers the K_i for MgADP but not that for creatine phosphate. The Michaelis constants for creatine and MgATP are only slightly affected by Cl^- ions. The kinetic constants are listed in Table 3.

When sulphate or phosphate ions are added to the assay a predominantly competitive type of inhibition is observed when MgATP is the variable substrate. These anions do not affect the inhibition caused by adding MgADP (Fig. 5 and Tables 2 and 3).

Back reaction. Unlike the forward reaction the curvature of the initial phase of the back reaction is not markedly affected by the addition of Cl^- ions. But as with the forward reaction inhibition by Cl^- ions is not of the simple competitive type either when MgADP (Fig. 6) or creatine phosphate (Fig. 7) is the variable substrate. Inhibitor constants are listed in Table 2. Michaelis constants for the substrates are only slightly affected by Cl^- ions as was observed for the forward reaction and inhibition by the non-phosphorylated product, creatine, is enhanced in exactly the same way as was that by MgADP (Fig. 7 and Table 3).

Inhibition by iodoacetamide

Preliminary observations. The inhibition of creatine kinase by iodoacetamide was found to be first order with respect to both inhibitor and enzyme over the range of conditions used, and in the absence of modifiers was found to yield an apparent second-order rate constant of $670\text{M}^{-1}\cdot\text{s}^{-1}$ at 25°C

Table 3. *Effect of anions on the Michaelis and inhibitor constants for creatine kinase at 30°C*

The concentrations of fixed substrates were creatine, 40 mM; MgATP, 4 mM; creatine phosphate, 10 mM; MgADP, 0.5 mM. Other conditions are given in the text.

pH	Variable Substrate	Inhibitor	Anion (0.1M)	K_m (mM)	K_i (mM)
9.0	MgATP	—	Acetate	0.4	—
9.0	MgATP	—	Chloride	0.65	—
9.0	Creatine	—	Acetate	10.7	—
9.0	Creatine	—	Chloride	9.7	—
8.0	MgADP	—	Acetate	0.14	—
8.0	MgADP	—	Chloride	0.19	—
8.0	Creatine phosphate	—	Acetate	3.3	—
8.0	Creatine phosphate	—	Chloride	4.1	—
9.0	MgATP	MgADP	Acetate	—	0.36
9.0	MgATP	MgADP	Chloride	—	0.05
9.0	Creatine	Creatine phosphate	Acetate	—	46.0
9.0	Creatine	Creatine phosphate	Chloride	—	50.0
8.0	MgADP	MgATP	Acetate	—	1.2
8.0	MgADP	MgATP	Chloride	—	1.4
8.0	Creatine phosphate	Creatine	Acetate	—	40.0
8.0	Creatine phosphate	Creatine	Chloride	—	6.0
9.0	MgATP	MgADP	Acetate	—	0.34
9.0	MgATP	MgADP	Acetate plus 6 mM-sulphate	—	0.39
9.0	MgATP	MgADP	Acetate plus 5 mM-phosphate	—	0.31

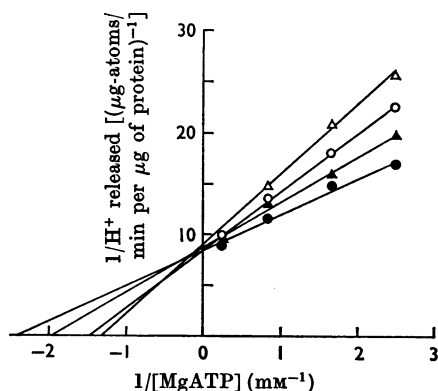


Fig. 5. Effect of SO_4^{2-} on the inhibition of the forward reaction of creatine kinase by MgADP at pH 9.0 and 30°C . The concentration of creatine was kept constant at 40 mM. Other conditions were as described in Fig. 3 except that 0.1 M sodium acetate was present in all cases. ●, No additions; ○, plus MgADP, 0.161 mM; ▲, plus Na_2SO_4 , 6 mM; ▲•, plus Na_2SO_4 , 6 mM, and MgADP, 0.161 mM.

that did not vary between pH 7.0 and 9.0. This compares with other values for the rabbit enzyme of 600 at 20°C (Hooton & Watts, 1966) and 700 at 20°C (O'Sullivan *et al.* 1966).

Effect of anions alone. A variety of anions at various concentrations were found to be without significant effect on the rate of inactivation (Table 4). These experiments, incidentally, confirmed the lack of an ionic-strength effect with iodoacetamide (Watts & Rabin, 1962).

Effect of anions on the protection by creatine plus MgADP. The rate of inhibition of creatine kinase by iodoacetamide is not significantly modified by addition to the reaction mixture of high concentrations of creatine plus MgADP with acetate ions either at 10 or 100 mM (Fig. 8a). However, a small unreproducible degree of protection, up to 11%, was sometimes observed; this is probably due to contamination of the reaction mixture by other small anions such as bicarbonate. This lack of effect on the rate of inhibition by iodoacetamide was found to be independent of pH over the range 7–9 (Table 5).

When certain other anions, in particular Cl^- ions, were used instead of or in addition to acetate ions the protection reported by other workers (Lui & Cunningham, 1966; O'Sullivan *et al.* 1966) was now manifest (Fig. 8b). Results with a wide variety of anions are listed in Table 4 in order of their ability to facilitate protection by creatine plus MgADP. Replacement of bicine-NaOH buffer, pH 9.0, by pentane-3',5'-dicarboxylate-NaOH or boric acid-NaOH buffers at various pH values did not affect the results (Table 5), neither did the type of cation (Table 4).

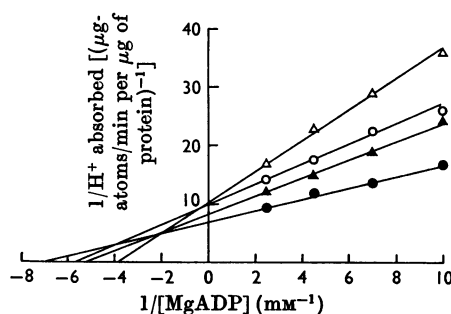


Fig. 6. Comparison of the effects of acetate ions and chloride ions on the reverse reaction of creatine kinase at pH 8.0 and 30°C . The creatine phosphate concentration was kept constant at 10 mM. The free Mg^{2+} ion concentration was maintained at 1 mM and the enzyme concentration was $2\ \mu\text{g}/\text{ml}$. Other conditions were as described in the Methods section. ●, Plus sodium acetate, 100 mM; ○, plus sodium acetate, 100 mM, and MgATP, 1 mM; ▲, plus NaCl, 100 mM; ▲•, plus NaCl, 100 mM, and MgATP, 1 mM.

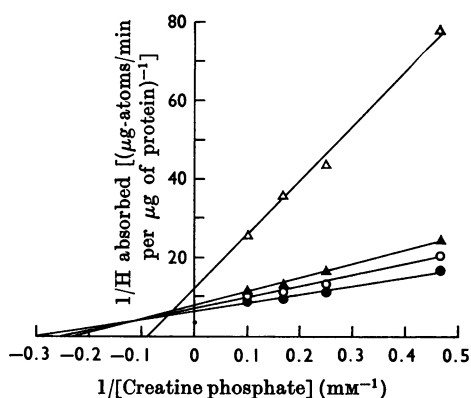


Fig. 7. Comparison of the inhibition of the reverse reaction of creatine kinase by creatine plus either acetate or chloride ions at pH 8.0 and 30°C . The MgADP concentration was kept constant at 0.5 mM. Other conditions were as in Fig. 6. ●, Plus sodium acetate, 100 mM; ○, plus sodium acetate, 100 mM, and creatine, 20 mM; ▲, plus NaCl, 100 mM; △, plus NaCl, 100 mM, and creatine, 20 mM.

With Cl^- ions the presence of high or low concentrations of acetate ions did not affect the degree of protection obtained with creatine plus MgADP. However, other anions, that inhibit in the normal assay mixture, did decrease the extent of protection (Table 6).

The apparent dissociation constants for the components of the quaternary enzyme-creatine-MgADP-anion complex (Table 7) were derived from plots of $1/\text{protection}$ obtained against $1/\text{component}$ when each component of the complex was varied

Table 4. Ability of anions to protect creatine kinase against inhibition by iodoacetamide in the absence and presence of the dead-end complex (creatine 40 mM; ADP, 1 mM; magnesium acetate, 10 mM) at pH 9.0 and 25°C

Experimental conditions are described in the Methods section. The enzyme concentration was 0.45 mg/ml and the iodoacetamide concentration was 0.1 mM. These results were estimated from rate constants derived from experiments like those in Fig. 8(b).

Source of anion	Concn. (M)	% Protection	
		Without substrates	Plus dead-end complex
None	—	0	0-11
Potassium nitrate	0.1	-5	98
Sodium nitrite	0.1	9	100
Potassium chloride	0.1	4	82
Sodium chloride	0.1	0	82
Lithium chloride	0.1	3	80
Sodium formate	0.1	4	70
Sodium bicarbonate	0.1	-10	62
Potassium bromide	0.1	-2	60
Sodium fluoride	0.1	-1	40
Sodium acetate	0.1	-4	3
Sodium tetrafluoroborate	0.1	—	8
Sodium perchlorate	0.1	—	-3
Sodium arsenate	0.05	—	-4
Sodium sulphate	0.05	—	-8
Sodium phosphate	0.005	—	0
Potassium iodide	0.1	—	0

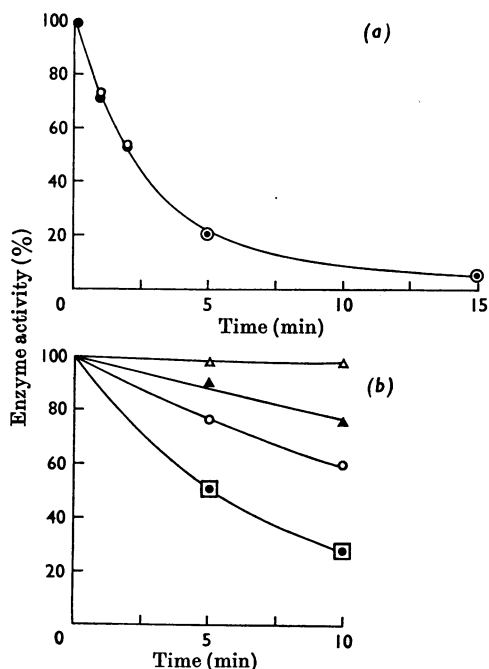


Fig. 8. Rate of inactivation of creatine kinase by iodoacetamide (at pH 9.0 and 25°C), (a) in the presence (○) and absence (●) of the dead-end complex, creatine (40 mM) plus MgADP (1 mM); (b) in the presence of the dead-end complex plus (□), no added anion; ●, sodium acetate (100 mM); ○, KBr (100 mM); ▲, NaCl (100 mM); △, KNO₃ (100 mM). In (a) the iodoacetamide concentration was 0.476 mM. In (b) it was 0.0952 mM. The enzyme concentration was 0.45 mg/ml. Other conditions are described in the Methods section.

in turn (Fig. 9). The underlying theory is outlined in the Methods section. The most noticeable result is the very low dissociation constant obtained with NO₃⁻ ions whereas those for Cl⁻ and Br⁻ increase in a manner that might be expected from the percentage protection experiments (Table 4). The dissociation constants for MgADP and creatine in Cl⁻ ions agree well with the inhibitor constants derived from normal Lineweaver-Burk plots (Table 3) and again are higher than those obtained in the presence of NO₃⁻ ions.

Protection against iodoacetamide inhibition by an equilibrium mixture of substrates. Protection of creatine kinase by an equilibrium mixture of substrates against inhibition by iodoacetamide was first reported by Watts & Rabin (1962). However, they used magnesium sulphate and NO₃⁻ ions to control the ionic strength of the reaction mixture. Table 5 summarizes a re-investigation of this problem under conditions where the type of small anion present is carefully controlled.

The possibility that protection could result from creatine plus MgADP present in the substrate equilibrium mixture was simultaneously checked by using similar concentrations of these components in the controls. It is also important to use a saturating concentration of Mg²⁺ ions since creatine plus ADP protect a little in the absence of any small anion (Table 7).

With acetate as the only added anion in the reaction mixture a protection was consistently obtained that was 30% more than controls containing creatine plus MgADP (Fig. 10). Protection was not particularly affected by varying the pH or buffer

Table 5. *Effect of pH at 25°C on the ability of the dead-end complex and an equilibrium mixture of substrates in the absence and presence of various modifiers to protect creatine kinase against inhibition by iodoacetamide*

The dead-end complex contained creatine (40 mM), ATP (1 mM) and magnesium acetate (10 mM). The equilibrium mixture of substrates was prepared by mixing with the enzyme creatine (40 mM), ATP (4 mM) and magnesium acetate (10 mM) and allowing the reaction to proceed to equilibrium before adding the iodoacetamide. Bicine-NaOH buffers were used for pH 8.0 and 9.0 and pentane-3',5'-dicarboxylate-NaOH buffer for pH 7.0.

pH	Substrates present	Other additive	% Protection
7.0	Creatine plus MgADP	Nil	11
8.0	Creatine plus MgADP	Nil	10
9.0	Creatine plus MgADP	Nil	0-11
7.0	Creatine plus MgADP	Chloride (100 mM)	80
8.0	Creatine plus MgADP	Chloride (100 mM)	84
9.0	Creatine plus MgADP	Chloride (100 mM)	81
7.0	Substrate equilibrium mixture	Nil	35
8.0	Substrate equilibrium mixture	Nil	41
9.0	Substrate equilibrium mixture	Nil	44
9.0	Substrate equilibrium mixture	Creatine phosphate (5 mM)	57
9.0	Substrate equilibrium mixture	Sulphate (10 mM)	23

Table 6. *Ability of some anions to affect the degree of protection by the dead-end complex, creatine-MgADP-chloride, in the reaction of iodoacetamide with creatine kinase at 25°C*

The concentrations of substrates in the dead-end complex were creatine, 40 mM; ADP, 1 mM; magnesium acetate, 10 mM; so that all measurements were made against a background concentration of 10 mM-acetate ions. Other conditions are described in the Methods section and in Table 4.

Concn. of Cl ⁻ (mM)	Second anion	Concn. (M)	% Protection
10	Nil	—	63
10	Acetate	0.1	61
20	Nil	—	67
20	Acetate	0.1	69
20	Sulphate	0.05	49
20	Perchlorate	0.1	40

Table 7. *Apparent dissociation constants for the three ligands of the quaternary complex, enzyme-creatine-MgADP-anion, at pH 9.0 and 25°C*

Except when varied the concentrations were creatine, 40 mM; MgADP, 1 mM; anion as the sodium or potassium salt as in Fig. 8, 100 mM. Other conditions are described in the Methods section and in the legend to Fig. 9.

Ligand	Anion	K for ligand (mM)
Anion	Nitrate	0.95
Anion	Chloride	4.0
Anion	Bromide	10.9
MgADP	Nitrate	0.012
MgADP	Chloride	0.022
Creatine	Nitrate	0.60
Creatine	Chloride	6.2

type but the addition of extra creatine phosphate, that has a rather low concentration in the equilibrium mixture relative to its K_m , enhanced protection. Replacing magnesium acetate by magnesium sulphate decreased protection (Table 5).

Effects of anions and other substrate combinations. Results with various other anion and substrate combinations are summarized in Table 8. Of these, as already mentioned, only creatine plus ADP in the absence of Mg²⁺ ions gives protection. Unlike the same combination with Mg²⁺ ions (Table 4) protection occurs without the participation of small anions and the addition of Cl⁻ ions only slightly enhances protection (Table 8).

Other combinations either had no effect or enhanced the rate of inhibition. An unexpected finding was that ATP plus magnesium acetate enhanced the rate of inhibition; this enhancement was not significantly affected by addition of NO₃⁻ ions or creatine phosphate.

DISCUSSION

The pH-stat assay for creatine kinase is particularly useful because it gives a continuous progress curve. The routine assay system described here overcomes difficulties encountered by pre-

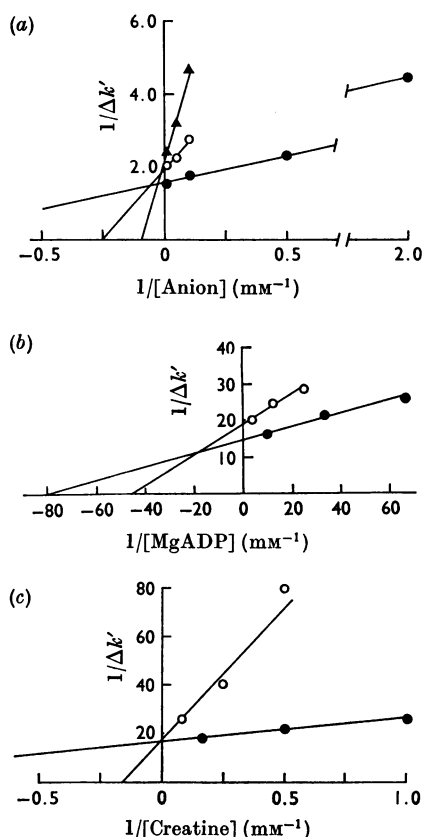


Fig. 9. Determination of the apparent dissociation constants of the three ligands, creatine, MgADP and anion, in the quaternary dead-end complex; this was measured from the ability of the complex to protect creatine kinase against iodoacetamide inhibition at pH 9.0 and 25°C when one ligand in the complex was varied. Details of the method and the conditions used are given in the Methods section. Each point on the graph was determined from plots like those in Fig. 8(b). The concentrations of the non-varied substrates were: creatine, 40 mM; MgADP, 1 mM; and anion as the sodium or potassium salt, 100 mM as in Fig. 8. In (a) the anions whose binding is measured are: \blacktriangle , Br⁻; \circ , Cl⁻; \bullet , NO₃⁻. In (b) the binding of MgADP is measured in the presence of: \circ , Cl⁻; \bullet , NO₃⁻. In (c) the binding of creatine is measured in the presence of: \circ , Cl⁻; \bullet , NO₃⁻.

vious workers (Cho *et al.* 1960; Mahowald *et al.* 1962). In particular the use of 1 mM-cysteine rather than 1% albumin in the assay mixture resulted in reproducible and reasonably linear progress curves with low blank values. Cysteine appears to act as a combined mild buffer and reducing agent without actively absorbing carbon dioxide as was found in preliminary experiments with albumin. The presence of low concentrations of buffer appears to be

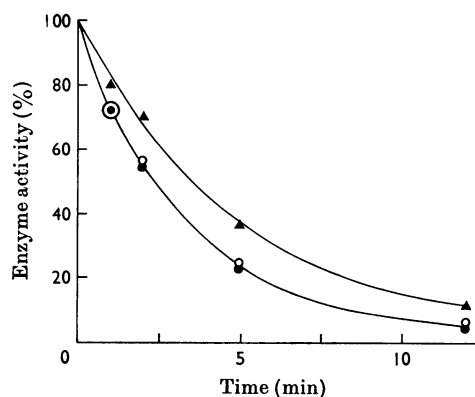


Fig. 10. Effect of a substrate equilibrium mixture on the rate of inactivation of creatine kinase by iodoacetamide at pH 9.0 and 25°C. No small anions were added. An equilibrium mixture of substrates was produced by mixing the enzyme (0.45 mg/ml) with magnesium acetate (10 mM), ATP (4 mM) and creatine (40 mM) for 20 min in bicine-NaOH buffer, $I = 0.0206$, before adding iodoacetamide to give a final concentration of 0.476 mM. Other conditions are described in the Methods section. \blacktriangle , Substrate equilibrium mixture; \circ , dead-end complex as described in the legend to Fig. 8; \bullet , no substrates.

important in preventing local fluctuations in pH when the titrant is added causing enzyme denaturation.

Anion kinetics. Cl⁻ ions (Figs. 3, 4, 6 and 7), unlike sulphate or phosphate ions (Fig. 5) do not act by simple competitive inhibition for MgATP or creatine phosphate. Further, Cl⁻ ions greatly magnify the inhibitory effects of MgADP and creatine by causing a pronounced decrease in their K_i values compared with those in acetate ions (Table 3); sulphate and phosphate ions do not. Thus although we agree with Nihei *et al.* (1961) 'that added anions interact with the same active groups on the enzyme molecule, more specifically where the γ -phosphate of ATP and the phosphoryl group of creatine phosphate interact' this in no way explains all the observed phenomena. The present results allow small anions to be divided into three classes in terms of their effects on creatine kinase. Class I contains acetate ions that activate the enzyme (in the forward reaction) and have no effect on the inhibition by MgADP and creatine; class II contains anions like Cl⁻ and NO₃⁻ that are not competitive inhibitors of MgATP and creatine phosphate and enhance inhibition by MgADP and creatine; Class III contains sulphate and phosphate, are competitive inhibitors and do not affect the inhibition by creatine and MgADP.

The class II anions have the most unexpected behaviour. Morrison & James (1965) first postulated the formation of a ternary 'dead-end complex'

Table 8. *Protecting effects of various substrate and anion combinations against inhibition of creatine kinase by iodoacetamide at pH 9.0 and 25°C*

A negative value for the % protection indicates an enhancement of the rate of inhibition. Added anions were used at a concentration of 0.1M. Other concentrations were as described in Table 4.

Substrates	Anion	% Protection
Creatine (40mM)	Nitrate	-5
Creatine (40mM), ADP (1mM)	None	31
Creatine (40mM), ADP (1mM)	Chloride	40
ADP (2mM), Mg ²⁺ (acetate) (10mM)	None	4
ADP (1mM), Mg ²⁺ (acetate) (10mM)	Nitrate	-16
ATP (4mM), Mg ²⁺ (acetate) (10mM)	None	-31
ATP (4mM), Mg ²⁺ (acetate) (10mM), creatine phosphate (20mM)	None	-27
ATP (4mM), Mg ²⁺ (acetate) (10mM), creatine phosphate (20mM)	Nitrate	-35
ATP (4mM), creatine phosphate (20mM)	None	-11
Creatine phosphate (20mM)	None	-2

consisting of enzyme-creatine-MgADP and it is significant that their assay mixtures contained approx. 50mM-Cl⁻ ions. The unexpectedly low K_i values for creatine and MgADP in the presence of Cl⁻ ions (Table 3) indicate the formation of such a complex. It is now clear that Cl⁻ ions play a specific role in forming the complex whereas anions from classes I and III, acetate and sulphate, are unable to do so. The complex, enzyme-creatine-MgADP, may therefore be more accurately described as a quaternary complex, enzyme-creatine-MgADP-chloride.

This finding explains the unusual curvature of normal progress curves for the forward reaction, caused especially by Cl⁻ and NO₃⁻. At the start of the assay the creatine concentration is already high whereas that of MgADP, the other component of the dead-end complex, approaches the value of K_i when the reaction has only proceeded to the extent of 1% in our assay system. In the reverse reaction the much smaller increase in curvature of the progress curve caused by Cl⁻ ions is to be expected because, although the initial concentration of MgADP is relatively high, the K_i for creatine (6mM) is much higher than the concentration of creatine that can be liberated during the course of the reaction.

Because MgADP and MgATP compete for the same binding site on the enzyme, in the forward reaction formation of the quaternary complex, enzyme-creatine-MgADP-anion, is favoured as the initial MgATP concentration is lowered. This phenomenon is manifest as a progressive and unusually marked increase in the curvature of the progress curves as the MgATP concentration is decreased. In the presence of acetate ions the curvature is much less and presumably due only to the normal product inhibition and substrate

depletion. Variation of the creatine/creatine phosphate ratio in the reverse reaction has a similar but much milder effect because of the higher K_i value for creatine.

It is obvious that in kinetic experiments where the substrate concentrations are being varied the presence of a class II anion can introduce progressive quantitative errors, the seriousness of which depends on the conditions of the experiment. It is unfortunate that high concentrations of Cl⁻ ions have been widely used in the most thorough kinetic analysis of creatine kinase, very often in conjunction with the use of spot assays at a few time-intervals to determine initial velocities (Morrison, O'Sullivan & Ogston, 1961; Morrison & O'Sullivan, 1965; Morrison & James, 1965; Morrison & Uhr, 1966; James & Morrison, 1966; Morrison & Cleland, 1966; Heyde & Morrison, 1969).

In the presence of acetate ions the apparent K_i values for substrates acting as inhibitors are higher than the Michaelis constants for the same substrates (Table 3). This suggests that the binding of creatine to the enzyme does not promote the binding of MgADP as an inhibitor and vice versa, assuming K_m to be a measure of the dissociation constant of the enzyme-substrate complex; in the presence of each other they behave like single substrates. This is consistent with the proposal (Morrison & James, 1965) that the binding of single substrates is weak but that the binding of one substrate to the enzyme enhances the binding of the other substrate. However, in the presence of Cl⁻ ions the apparent K_i values for creatine and MgADP are lower than the corresponding K_m values as a result of unusually strong binding in the quaternary enzyme complex.

The high K_i values for ATP and creatine phosphate in both acetate and chloride solutions as compared with their K_m values are an indication of the

relative inability of these substrates to form a phosphorylated dead-end complex with the enzyme.

Reaction with iodoacetamide. The most surprising result of this work is that, when creatine plus MgADP is added to the reaction mixture to test for protection of the enzyme against iodoacetamide inhibition, virtually no such protection is seen unless chloride or other class II anions are also added (Table 4). The protected species must be the same quaternary complex, enzyme-creatine-MgADP-anion, that leads to inhibition in kinetic experiments. With nitrate, under the conditions used (Table 1) the protection is almost complete; the list of class II anions can be enlarged, in order of effectiveness, to $\text{NO}_2^- > \text{NO}_3^- > \text{Cl}^- > \text{HCOO}^- > \text{HCO}_3^- > \text{Br}^- > \text{F}^-$. In passing they draw attention to the importance of using bicarbonate-free solutions. Measurement of the binding of the components of the complex to the enzyme shows that the more effective the anion the more tightly each of the three components (including the anion) binds to the enzyme. The apparent dissociation constants in chloride are in accord with those measured kinetically.

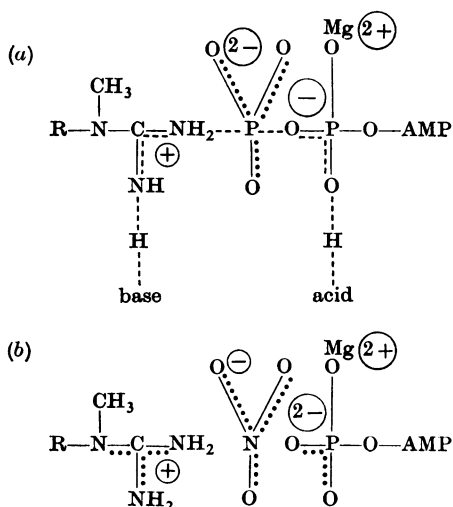
Also important are the anions that do not enhance protection with creatine plus MgADP. All class III anions (that inhibit enzyme activity reversibly) behave in this way; these are SO_4^{2-} , AsO_4^{2-} , ClO_4^- , I^- , HPO_4^{2-} , BF_4^- . Also, SO_4^{2-} and ClO_4^- have been shown to decrease the protection by class II anions, which may be a general effect of class III anions. Class I anions (which do not inhibit enzyme activity) also do not enhance protection and include acetate and the buffer anions borate $[\text{B}(\text{OH})_4^-]$, bicine, and diethyl malonate. High concentrations of acetate ions do not affect the protection by class II anions. Class I anions seem to be either too large or sterically unsuitable for binding at the catalytic site.

Since creatine plus MgADP and acetate ions do not significantly protect creatine kinase against iodoacetamide inhibition it is possible to check if an equilibrium mixture of substrate protects in the absence of class II anions. Table 5 shows that, under conditions where the possibility of protection due to quaternary complex formation is excluded, a protection of about 30% can be attributed specifically to the substrate equilibrium mixture. The extent of protection is complicated and, among other factors, depends on the substrates/products ratio as is indicated by perturbing the equilibrium with extra creatine phosphate. If the binding of substrates and products is rapid, random and equilibrium the formation of the non-protecting enzyme-creatine-MgADP complex would be expected to prevent protection ever becoming complete. As would be expected from Table 3, replacing magnesium acetate by magnesium sulphate simply

decreases the magnitude of the effect by decreasing the binding of creatine phosphate and MgATP in the competitive manner originally envisaged by Nihei *et al.* (1961). Addition of Cl^- ions, on the other hand, allows formation of the quaternary enzyme-creatine-MgADP-chloride complex, and strong protection results.

The protective effect of the quaternary dead-end complex and the substrate equilibrium mixture are not significantly affected by pH over the range 7.0-9.0. In this respect rabbit muscle creatine kinase is markedly different from the enzyme of primate muscle where the usual pH-independent reaction with iodoacetamide is converted into a reaction reflecting an ionization with a pK near 8.6 by the addition of creatine plus MgADP (the sulphate ions used in their experiments would not be expected to affect the qualitative findings). The ionization is attributed to the reactive thiol groups on the enzyme and it would seem that their environment can be changed in a different way in the two enzymes. Undoubtedly conformational changes are involved.

Other experiments in which the unanticipated participation of anions have probably influenced experimental results are those on the reaction of *p*-nitrophenyl acetate with creatine kinase in the presence of substrates. Watts (1963) observed protection by an equilibrium mixture of substrates in the presence of 0.1M- NO_3^- ions; Lui & Cunningham (1966) obtained a similar result by using creatine plus MgADP in 0.17M-tris-HCl buffer. Watts (1963) also observed that protection was associated with the increase in pK of the reacting group, inferred to be lysine, by 0.85 of a unit. Although this finding remains valid and is supported by the work of Clark & Cunningham (1965) and Jacobs & Cunningham (1968), the associated conformational change can now be seen to result predominantly from the formation of the enzyme-creatine-MgADP-anion complex; the protection brought about by transphosphorylation in a substrate equilibrium mixture would be minimal under these conditions. Jacobs & Cunningham (1968) also inferred conformational changes to explain their finding that creatine plus MgADP protected creatine kinase against trypsin digestion in excess of either substrate alone. However, the experiments were carried out in the presence of 87.5mM-sodium chloride, so that a similar quaternary complex was the effective combination. Their reported constants of 13 ± 3 and 0.07 ± 0.03 mM at 30°C for the dissociation of creatine from the enzyme-MgADP complex and MgADP from the enzyme-creatine complex respectively agree well with our own values of 6 and 0.022mM, also obtained in the presence of a saturating concentration of Cl^- ions but at 25°C (Table 7).



Scheme 1. Diagrammatic representation of (a) the postulated transition-state complex of the creatine kinase reaction and (b) the quaternary dead-end complex showing how this can simulate the transition state. For details see the Discussion section.

Mechanism of anion inhibition and structure of the catalytic site. A mechanism for the stabilization of the enzyme-creatine-MgADP complex by NO_3^- and Cl^- ions is suggested by the planar structure of NO_3^- ions. All the polyatomic anions that behave similarly are also planar, whereas no tetrahedral anions studied act in this way. Transphosphorylation probably involves direct transfer of a phosphoryl group by an S_N2 type of reaction, with the phosphoryl group forming a planar sp^3d hybrid in the transition state (Scheme 1a), in combination with creatine and MgADP, could inhibit by mimicking this transition-state complex (Scheme 1b).

Pauling (1946) pointed out that a molecule resembling the transition state in the conversion from substrate into product might be expected to bind more tightly to the enzyme than the substrate molecules alone. Investigations of various other enzymes (Jencks, 1969a; Grass & Meister, 1970; Johnson & Wolfenden, 1970) are beginning to support this view. The results show that the binding of both creatine and MgADP as components of the enzyme-creatine-MgADP-anion complex is stronger than their apparent binding as substrates. This is remarkable as this quasi-transition-state complex unlike those postulated in other enzymes, comprises three components. In normal circumstances the binding of three specific components together on an enzyme surface would be expected to be thermodynamically less favourable than that

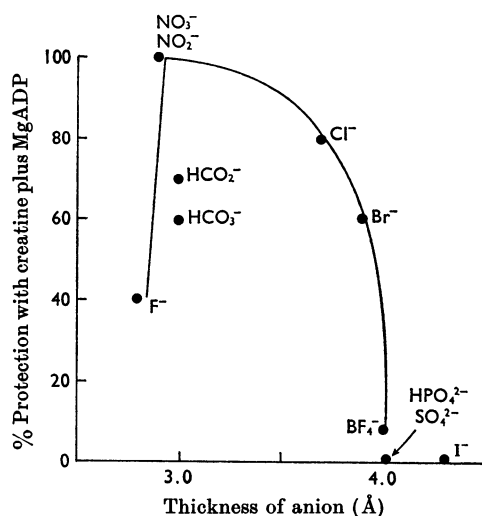
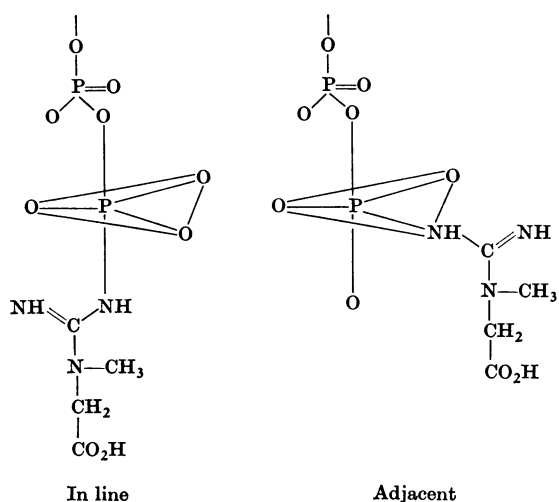


Fig. 11. Estimate of the size of the gap between creatine and ADP that can be effectively occupied by class II anions in the quasi-transition-state complex. The percentage protection values with creatine plus MgADP are taken from Table 4. The sizes of monoatomic anions used are double the van der Vaal's radius, and those of planar polyatomic anions are direct measurements of the thickness through the plane of Cori-Pauling-Kolton atomic models. The values for tetrahedral anions, also taken from models, are the maximum distances across van der Vaal's envelopes.

of two components. However, the fact that the binding of one component is only maximal when the other two components are also present indicates the very special nature of this complex.

Experiments by several workers (Desnoyers & Conway, 1964; Padova, 1964; Robinson & Jencks, 1965) have led to the conclusion that anions in solution do not have a tightly bound water shell. Such investigations have also shown that the magnitude of many non-specific effects of anions on proteins varies according to the Hoffmeister series ($\text{ClO}_4^- < \text{I}^- < \text{NO}_3^- < \text{Br}^- < \text{Cl}^- < \text{SO}_4^{2-} < \text{F}^-$) in which the planarity of the anion is not an important feature. This situation contrasts with the different order of effectiveness of class II anions in the quaternary complex and the separation of tetrahedral anions into a separate group (class III) with different properties.

It is improbable that anions simulating a transition state phosphoryl group would be hydrated so that an estimate of the effective size of the anion site in the quasi-transition-state complex can be obtained from a plot of the overall thickness of the unhydrated anion as a function of its ability to facilitate protection in the quaternary complex



Scheme 2. 'In line' and 'Adjacent' modes of interaction of substrates with a trigonal bipyramidal phosphoryl group in the transition-state complex.

(Table 4). Fig. 11 shows such a plot, using measurements of anions from Pauling (1960) and from direct measurement of Cori-Pauling-Koltun molecular models, from which it would appear that the distance between creatine and MgADP lies between 2.8 and 4.0 Å when both substrates are bound to their sites on the enzyme. Model building shows that this distance is about right for the space required for a tetrahedral phosphoryl group to invert via the sp^3d hybrid.

Fig. 11 also explains why tetrahedral anions, such as sulphate, perchlorate and phosphate, and also the monoatomic iodide ion, cannot inhibit in this system; they are much too large to fit the transition-state anionic site.

In a discussion of the mechanism of action of ribonuclease Usher (1969) distinguished between two stereochemical alternatives for a reaction pathway that involves a transitional planar phosphoryl group. For creatine kinase, the corresponding 'in line' and 'adjacent' transition-state structures are shown in Scheme 2. In the 'adjacent' structure the guanidine nitrogen atom of creatine forms part of the equatorial plane and the oxygen atoms of the phosphoryl group are not planar with the phosphorus atom. This structure could not be simulated by a planar anion such as nitrate and hence can be discarded in favour of the 'in line' orientation where the requirement for a planar phosphoryl group is fulfilled. The second step in the mechanism of action of ribonuclease A is also reported to proceed via an 'in line' mechanism (Usher, Richardson & Eckstein, 1970).

It seems probable that the function of the conformational change in creatine kinase is to convert the tetrahedral anionic site into one that binds a trigonal bipyramid. This would strain the phosphoryl group into the planar sp^3d transition state, thereby facilitating transfer to the receiver substrate by an S_N2 type of reaction. This mechanism for lowering the activation energy could be common to many phosphate-transferring enzymes.

An alternative hypothesis (see Jencks, 1969b) that transphosphorylation proceeds by an S_N1 type of reaction, with the planar PO_3^- anion as a stable intermediate, is also compatible with our results and might offer an explanation of the protection observed with an equilibrium mixture of substrates.

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