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THE INTERACTION OF Mg^{2+} AND ATP^{4-} WITH ATP:CREATINE PHOSPHOTRANSFERASE

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SUMMARY

1. The forward reaction catalysed by creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) has been studied under conditions where Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ were each present in significant concentration. The results indicate the presence of significant concentrations of enzyme–Mg and enzyme–ATP complexes, as well as of the enzyme–MgATP complex, under these conditions.

2. The inhibition of the forward reaction by concentrations of Mg^{2+} or ATP^{4-} in excess of the $MgATP^{2-}$ concentration has been shown to be less effective than would be expected on the basis of the results obtained when Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ are all present. Excess Mg^{2+} causes noncompetitive inhibition with respect to both creatine and $MgATP^{2-}$, and it is concluded that detectable inhibition is caused only by the interaction of Mg^{2+} at a site on the enzyme distinct from the active site. This conclusion is in agreement with previous results obtained in the reverse reaction.

3. From comparison of these two types of kinetic data and previous thermodynamic data, it appears probable that Mg^{2+} does not interact directly with creatine kinase at the active site, and that the enzyme–Mg complex is formed only by the dissociation of ATP^{4-} from the enzyme–MgATP complex.

4. ATP^{4-} is a much weaker inhibitor with respect to $MgATP^{2-}$ than ADP^{3-} was previously found to be with respect to $MgADP^{-}$ in the reverse reaction. This indicates that, while ADP^{3-} may react with free creatine kinase at the active site, a similar reaction involving ATP^{4-} is unlikely.

INTRODUCTION

The mechanism of the overall reaction catalysed by creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2) has been shown to be of the rapid equilibrium random type[1,2], under experimental conditions where the metal–nucleotide complexes could be regarded as the substrates and where the kinetic effects of both the metal ion activator and the uncomplexed nucleotides could be neglected. However, details of the interactions of Mg^{2+} and ATP^{4-} with creatine kinase in the forward reaction have

not been established, although they are of basic interest in a system of this type.

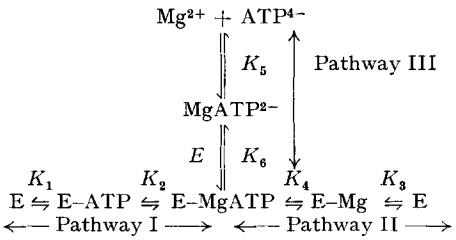
The results of thermodynamic experiments have indicated that the divalent metal ions Mg^{2+} (ref. 3) and Mn^{2+} (ref. 4) are bound to only a small extent. Kinetic experiments on the reverse reaction, where the Mg^{2+} concentration was considerably in excess of the $MgADP^-$ concentration[5], have confirmed a weak and inhibitory reaction between Mg^{2+} and creatine kinase and furthermore, have shown that Mg^{2+} does not compete at the site at which $MgADP^-$ is bound. However, the reverse reaction has also been studied kinetically under conditions where the Mg^{2+} concentration was not considerably greater than the $MgADP^-$ concentration, and where there were significant concentrations of all three species Mg^{2+}, $MgADP^-$ and ADP^{3-} (refs. 5 and 6) Under these conditions there was evidence for a much higher concentration of the enzyme–Mg complex than would be expected from the previous observations.

There is a similar discrepancy in the results for ATP^{4-}. NODA, NIHEI AND MORALES[7] reported kinetic evidence for a weak inhibitory interaction of ATP^{4-} with creatine kinase, whereas the thermodynamic results of KUBY, MAHOWALD AND NOLTMANN[3], and especially of COHN[4], indicated that ATP^{4-} combined quite strongly with the enzyme. In contrast, the kinetic evidence of MORRISON AND O'SULLIVAN[5] for strong inhibition by ADP^{3-} in the reverse reaction is in agreement with thermodynamic results[3].

In order to ascertain if kinetically significant concentrations of the enzyme–ATP and enzyme–Mg complexes can be formed, kinetic investigations have been made of the forward reaction. For this purpose, experimental conditions have been adjusted so that Mg^{2+} and ATP^{4-} as well as $MgATP^{2-}$ are present. The general approach was similar to that used by MORRISON, O'SULLIVAN AND OGSTON[6] and by MORRISON AND O'SULLIVAN[5] to study the reverse reaction, but a more rigorous theoretical treatment of the reaction kinetics has been made. The results are similar to those reported for the reverse reaction in that enzyme–Mg and enzyme–ATP complexes, as well as an enzyme–MgATP complex, appear to be formed in kinetically significant concentrations and in that excess Mg^{2+} acts as a weak noncompetitive inhibitor with respect to $MgATP^{2-}$. They differ in that ATP^{4-} causes only weak inhibition of the forward reaction, whereas ADP^{3-} is a potent inhibitor of the reverse reaction. It is plausible that the enzyme–Mg and enzyme–ATP complexes may be formed only by the breakdown of the enzyme–MgATP complex.

THEORY

By analogy with the theoretical treatment of the reverse reaction by MORRISON, O'SULLIVAN AND OGSTON[6] and MORRISON AND O'SULLIVAN[5], the conversion of creatine and ATP to phosphocreatine and ADP by creatine kinase (E) in the presence of Mg^{2+} has been considered to occur *via* the formation of an active enzyme–Mg–ATP–creatine complex. It is proposed that this complex might be formed by sets of bimolecular reactions taking place in a number of parallel sequences as given in Scheme I. The dissociation constants are represented by K_1, K_2, K_3, K_4, K_5 and K_6. K_5 is the inverse of the stability constant for $MgATP^{2-}$. Because the results of MORRISON AND JAMES[1] indicated that the combination of creatine at a separate site on the enzyme is influenced by the previous combination of the nucleotide substrate, $MgATP^{2-}$, it has been postulated here, in contrast with earlier treatments[5,6], that the dissociation



Scheme I.

constants for the reactions of creatine with the different enzyme forms in the above scheme are not identical. The reactions which have been considered are:

$$\begin{aligned} E + \text{creatine} &\rightleftharpoons E\text{–creatine} && (K_{ip}) \\ E\text{–Mg} + \text{creatine} &\rightleftharpoons E\text{–Mg–creatine} && (K_{ip}') \\ E\text{–ATP} + \text{creatine} &\rightleftharpoons E\text{–ATP–creatine} && (K_p') \\ E\text{–MgATP} + \text{creatine} &\rightleftharpoons E\text{–MgATP–creatine} && (K_p) \end{aligned}$$

Each of these complexes could also be formed by the reaction of E–creatine with Mg^{2+}, ATP^{4-} or $MgATP^{2-}$. However, on the assumption that all of these steps are in rapid equilibrium, such alternative pathways would not involve any independent dissociation constants.

In deriving the initial velocity equation given below, it has been supposed that: (i) ATP^{4-} and $MgATP^{2-}$ are bound at the same site, as also are Mg^{2+} and $MgATP^{2-}$; (ii) the same active E–MgATP–creatine complex is formed irrespective of the pathway, and the rate of breakdown of this complex limits the reaction velocity, all other steps being in rapid equilibrium; (iii) the reaction does not occur in the absence of magnesium; (iv) the steady-state concentrations of the various enzyme complexes are not sufficient to have a significant effect on the concentrations of Mg^{2+}, ATP^{4-}, $MgATP^{2-}$ or creatine.

The initial velocity equation may be written:

$$v = \frac{V}{\dfrac{K_6}{[MgATP^{2-}]}\left(\dfrac{K_p}{K_{ip}} + \dfrac{K_p}{[Cr]}\right) + \dfrac{K_6}{[MgATP^{2-}]}\dfrac{[Mg^{2+}]}{K_3}\left(\dfrac{K_p}{K_{ip}'} + \dfrac{K_p}{[Cr]}\right) + \dfrac{K_6}{[MgATP^{2-}]}\dfrac{[ATP^{4-}]}{K_1}\left(\dfrac{K_p}{K_p'} + \dfrac{K_p}{[Cr]}\right) + 1 + \dfrac{K_p}{[Cr]}} \qquad (1)$$

Because of the rapid equilibrium assumption, the concentration of E–MgATP will be independent of the pathway by which it is formed, so that

$$K_1K_2 = K_3K_4 = K_5K_6 \qquad (2)$$

and since $[MgATP^{2-}] = ([Mg^{2+}]\,[ATP^{4-}])/K_5$, Eqn. 1 may be expressed in the alternative form:

$$\frac{1}{v} = \frac{1}{V}\left\{\frac{K_1K_2}{[Mg^{2+}]\,[ATP^{4-}]}\left(\frac{K_p}{K_{ip}} + \frac{K_p}{[Cr]}\right) + \frac{K_4}{[ATP^{4-}]}\left(\frac{K_p}{K_{ip}'} + \frac{K_p}{[Cr]}\right) + \frac{K_2}{[Mg^{2+}]}\left(\frac{K_p}{K_p'} + \frac{K_p}{[Cr]}\right) + 1 + \frac{K_p}{[Cr]}\right\} \qquad (3)$$

Inspection of a rearrangement of this equation, *viz.*

$$\frac{1}{v} = \frac{K_2}{V}\left\{\frac{K_1}{[ATP^{4-}]}\left(\frac{K_p}{K_{ip}} + \frac{K_p}{[Cr]}\right) + \frac{K_p}{K_{p'}} + \frac{K_p}{[Cr]}\right\}\frac{1}{[Mg^{2+}]} + \\ + \frac{1}{V}\left\{\frac{K_4}{[ATP^{4-}]}\left(\frac{K_p}{K_{ip'}} + \frac{K_p}{[Cr]}\right) + 1 + \frac{K_p}{[Cr]}\right\} \quad (4)$$

shows that plots of $1/v$ against $1/[Mg^{2+}]$ at different nonsaturating concentrations of ATP^{4-} and a fixed concentration of creatine should yield a series of straight lines with both slope and intercept varying with the concentration of ATP^{4-}. Secondary plots of slopes and vertical intercepts from such a primary plot against $1/[ATP^{4-}]$ would be linear and yield apparent values of K_1, equal to

$$K_1\left\{\frac{1 + \frac{[Cr]}{K_{ip}}}{1 + \frac{[Cr]}{K_{p'}}}\right\} \text{ and } K_4, \text{ equal to } K_4\left\{\frac{1 + \frac{[Cr]}{K_{ip'}}}{1 + \frac{[Cr]}{K_p}}\right\},$$

respectively. Because of the symmetry of Eqn. 3, a similar series of lines would be expected from a plot of $1/v$ against $1/[ATP^{4-}]$ at different nonsaturating concentrations of Mg^{2+} and a fixed concentration of creatine. If these slopes and vertical intercepts were replotted against $1/[Mg^{2+}]$, apparent values would be obtained for K_3, equal to

$$K_3\left\{\frac{1 + \frac{[Cr]}{K_{ip}}}{1 + \frac{[Cr]}{K_{ip'}}}\right\} \text{ and } K_2, \text{ equal to } K_2\left\{\frac{1 + \frac{[Cr]}{K_{p'}}}{1 + \frac{[Cr]}{K_p}}\right\}, \text{ respectively.}$$

It is theoretically possible to calculate the true values for K_1, K_2, K_3 and K_4 from their apparent values, as obtained from experiments at a number of fixed nonsaturating concentrations of creatine, by solving sets of simultaneous equations. Values might also be obtained from the same data for the dissociation constants associated with the reaction of creatine. By using Eqn. 2, values for K_1, K_2, K_3 and K_4, and an independently determined value for K_5, K_6 could be calculated, and thus all 6 of the proposed dissociation constants might be evaluated.

However, results which are consistent with the above theory can only indicate the presence of kinetically significant concentrations of E–Mg and E–ATP, as well as E–MgATP, and the meanings of the dissociation constants determined from this treatment rest heavily on the equilibrium assumption. If the 3 enzyme–reactant complexes form in all the ways envisaged (Scheme I), then all the constants have the meanings attributed to them previously. On the other hand, if E–ATP and E–Mg form only because each is in equilibrium with E–MgATP, then only K_2 and K_4 (as well as K_5 and K_6) are real. It is also possible that E–ATP and E–Mg may not be in equilibrium with E–MgATP, but form only by the interaction of ATP^{4-} and Mg^{2+} with E. Thus any two links in Scheme I may be missing, provided that one pathway leading from E to E–MgATP remains intact and that both the missing links are not on the same pathway.

In order to elucidate further the reactions by which the E–Mg and E–ATP complexes may be formed, the reaction of Mg^{2+} and ATP^{4-} with the enzyme could

be studied under conditions where one of these components was virtually absent. The rationale of this approach is similar to that of MORRISON AND O'SULLIVAN[5]. Thus, if reaction occurred under conditions where either Mg^{2+} or ATP^{4-} was in excess of $MgATP^{2-}$, it would be expected that the enzyme–Mg or enzyme–ATP complex would function as a dead-end complex.

Inhibition by excess Mg^{2+}

(1) If Mg^{2+} reacts with the enzyme only at the $MgATP^{2-}$ binding site, the complex being designated E–Mg, and is present in high enough concentration to cause the ATP^{4-} concentration to become insignificant, Eqn. 1 simplifies to Eqn. 5 which can be written in double reciprocal form as

$$\frac{1}{v} = \frac{K_6}{V}\left\{\frac{K_p}{K_{ip}} + \frac{K_p}{[Cr]} + \frac{[Mg^{2+}]}{K_3}\left(\frac{K_p}{K_{ip'}} + \frac{K_p}{[Cr]}\right)\right\}\frac{1}{[MgATP^{2-}]} + \frac{1}{V}\left\{1 + \frac{K_p}{[Cr]}\right\} \qquad (5)$$

Thus Mg^{2+} would be expected to function as a competitive inhibitor with respect to $MgATP^{2-}$.

(2) If Mg^{2+} is considered to react also with the enzyme at a site distinct from the active site, so as to form an inactive complex which is designated Mg–E, and in such a way that its binding is influenced by the previous combination of ATP (but not of creatine or of Mg^{2+}) at the active site, account must also be taken of the following reactions with the same dissociation constant, K_i:

$$\begin{aligned} E + Mg^{2+} &\rightleftharpoons Mg\text{–}E \\ E\text{–}Mg + Mg^{2+} &\rightleftharpoons Mg\text{–}E\text{–}Mg \\ E\text{–creatine} + Mg^{2+} &\rightleftharpoons Mg\text{–}E\text{–creatine} \\ E\text{–}Mg\text{–creatine} + Mg^{2+} &\rightleftharpoons Mg\text{–}E\text{–}Mg\text{–creatine} \end{aligned}$$

In addition there are, of course, 4 analogous reactions in which ATP is always combined with the enzyme. The dissociation constant for each of these reactions can be represented by K_I.

Taking into account the reaction of Mg^{2+} at both sites, the initial velocity of the inhibited reaction with $MgATP^{2-}$ as the variable substrate may be written:

$$\frac{1}{v} = \frac{K_6}{V}\left\{\left(\frac{K_p}{K_{ip}} + \frac{K_p}{[Cr]} + \frac{[Mg^{2+}]}{K_3}\left[\frac{K_p}{K_{ip'}} + \frac{K_p}{[Cr]}\right]\right)\left(1 + \frac{[Mg^{2+}]}{K_i}\right)\right\}\frac{1}{[MgATP^{2-}]} + \frac{1}{V}\left\{\left(1 + \frac{K_p}{[Cr]}\right)\left(1 + \frac{[Mg^{2+}]}{K_I}\right)\right\} \qquad (6)$$

When creatine is the variable substrate, the equation may be expressed as

$$\frac{1}{v} = \frac{K_p}{V}\left\{\frac{K_6}{[MgATP^{2-}]}\left(1 + \frac{[Mg^{2+}]}{K_3}\right)\left(1 + \frac{[Mg^{2+}]}{K_i}\right) + 1 + \frac{[Mg^{2+}]}{K_I}\right\}\frac{1}{[Cr]} + \\ + \frac{1}{V}\left\{\frac{K_6}{[MgATP^{2-}]}\left(\frac{K_p}{K_{ip}} + \frac{K_p[Mg^{2+}]}{K_{ip'}K_3}\right)\left(1 + \frac{[Mg^{2+}]}{K_i}\right) + 1 + \frac{[Mg^{2+}]}{K_I}\right\} \qquad (7)$$

Thus Mg^{2+} would be expected to function as a noncompetitive inhibitor of the reaction with respect to both $MgATP^{2-}$ and creatine. While a secondary plot of vertical intercepts from inhibition with respect to $MgATP^{2-}$ (Eqn. 6) against Mg^{2+} concentration

would be linear, all of the other secondary plots of slopes and vertical intercepts (Eqns. 6 and 7) would be expected to appear parabolic.

Inhibition by excess ATP^{4-}

Because of the symmetry of Eqn. 1, analogous equations would be obtained for inhibition of the forward reaction by excess ATP^{4-}.

EXPERIMENTAL

Materials

Reagents and the preparation of creatine kinase were as previously described[1]. Aqueous solutions of magnesium acetate (Analytical reagent, British Drug Houses) were standardised in the same manner as those of $MgCl_2$ (ref. 1).

Methods

Measurement of creatine kinase activity. Reaction mixtures contained, in a volume of 1.0 ml, triethanolamine–HCl buffer (0.1 M at pH 8.0), EDTA, 0.01 mM, substrates at the concentrations indicated in the figures, and sufficient $MgCl_2$ or magnesium acetate to give the required concentration of Mg^{2+}. The amount of creatine kinase added per assay was 1.08 μg, and reactions were run for two time periods (0.5 and 1 min) to ensure that initial velocities were being measured. The reaction was stopped by the addition of 0.10 ml of 1 M HCl, and after 2 min, an equivalent amount of NaOH was added to each tube. The ADP produced was estimated enzymically[1] for all velocity determinations except those concerned with the inhibition by excess ATP^{4-}. For the latter, phosphocreatine production was followed using the procedure previously described[1].

Calculation of substrate concentrations. It was necessary to allow for the chelation of Mg^{2+} by ATP^{4-} in order to determine the amounts of total ATP and total magnesium needed for the required concentrations of Mg^{2+} and ATP^{4-}. This was done as previously described,[6] using the value of 70 000 M^{-1} for the apparent stability constant of $MgATP^{2-}$. Creatine does not complex with Mg^{2+}, and although acetate ion does so, it was calculated, on the basis of a dissociation constant of 0.14 M[7], that under the experimental conditions such complexing would alter the Mg^{2+} concentration by less than 1% and could therefore be neglected.

Analysis of results. The kinetic data were analysed using the computer programmes of CLELAND[8] in conjunction with an IBM 1620 computer. The lines of the illustrated double reciprocal plots were drawn using the constants obtained from analysis of the data for each line by means of the Hyper programme (Eqn. 1 of ref. 8) while the Line programme (Eqn. 3 of ref. 8) was used in connection with the fitting of the lines for the secondary plots. All kinetic constants together with their standard errors, were obtained by analysis of the primary data using the Sequen or Noncomp programmes (Eqns. 7 or 9 of ref. 8). Weighted mean values and their standard errors were calculated as described previously[1].

RESULTS

Initial velocity studies in the presence of Mg^{2+} and ATP^{4-}

Initial velocity data obtained at pH 8.0 and plotted with Mg^{2+} as the variable reactant at a number of nonsaturating concentrations of ATP^{4-} and at different fixed concentrations of creatine are illustrated in Fig. 1. The variation of the vertical intercepts in all figures with the concentration of ATP^{4-} is in accord with the formation of E–Mg, which is capable of reacting with ATP^{4-}. Replots of the slopes and vertical intercepts of the graphs in Fig. 1 against the reciprocal of the concentration of ATP^{4-} appeared linear and are shown in Fig. 2. Similar primary and secondary plots were obtained when the same data were analysed with ATP^{4-} as the variable reactant.

The horizontal intercepts of secondary plots of the vertical intercepts (Fig. 2b) are large, so that very small apparent values are obtained for K_4. On the other hand, the horizontal intercepts from the slope replots (Fig. 2a) are very small and have standard errors such that in some cases the lines could have passed through the origin. If this were so, it would indicate that K_1 was infinite and hence that ATP^{4-} did not combine with free creatine kinase. The analogous replots of slopes against the reciprocal

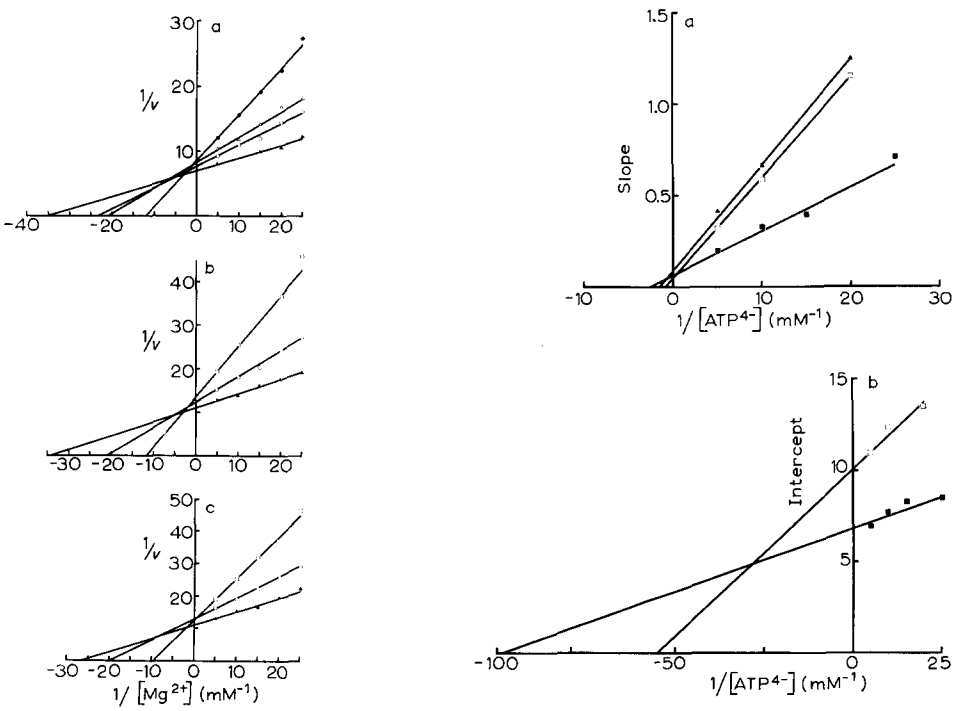


Fig. 1. The effect of the concentration of Mg^{2+} on the initial velocity of the forward reaction at pH 8.0 with various fixed concentrations of ATP^{4-} (●, 0.04 mM; □, 0.05 mM; △, 0.066 mM; ○, 0.10 mM; ▲, 0.20 mM) and with the creatine concentration held constant at (a) 30 mM, (b) 15 mM or (c) 10 mM. v is expressed as μmoles of ADP produced per μg of creatine kinase per min.

Fig. 2. Secondary plots of (a) slopes and (b) vertical intercepts from Fig. 1 against the reciprocal of the concentration of ATP^{4-}. The concentrations of creatine were: ▲, 10 mM; □, 15 mM and ■, 30 mM.

TABLE I

APPARENT KINETIC CONSTANTS FOR THE INTERACTION OF VARIOUS FORMS OF CREATINE KINASE WITH Mg^{2+}, ATP^{4-} AND $MgATP^{2-}$

Each value for K_1, K_2, K_3 and K_4 is the weighted mean* of the values obtained from the data of 2 experiments, including those shown in Fig. 1, by means of computer analysis using the SEQUEN programme of CLELAND[8]. In each case, K_6 was calculated from the relationships K_1K_2/K_5 or K_3K_4/K_5 (Eqn. 2), using K_1 and K_2, or K_3 and K_4 from this table, and 0.014 mM for K_5 (the inverse of the stability constant for $MgATP^{2-}$).

Creatine (mM)	*Dissociation constant (mM)*					
	K_1 $(E + ATP^{4-})$	K_2 $(E\text{–}ATP + Mg^{2+})$	K_3 $(E + Mg^{2+})$	K_4 $(E\text{–}Mg + ATP^{4-})$	K_6 $(E + MgATP^{2-})$	
					K_1K_2/K_5	K_3K_4/K_5
10	0.60 ± 0.40	0.007 ± 0.004	0.44 ± 0.33	0.005 ± 0.005	0.30 ± 0.26	0.16 ± 0.20
15	0.27 ± 0.17	0.006 ± 0.003	0.30 ± 0.10	0.017 ± 0.005	0.12 ± 0.09	0.17 ± 0.17
30	0.16 ± 0.05	0.014 ± 0.004	0.15 ± 0.04	0.017 ± 0.004	0.16 ± 0.07	0.18 ± 0.07

* The consequence of taking weighted mean values for each of the constants is that the relationship $K_1K_2 = K_3K_4$ does not necessarily hold.

of the Mg^{2+} concentration also pass close to the origin, with similar implications for K_3. However, computer analysis of the primary data yielded a positive value for each of the apparent constants (K_1 and K_3) in every experiment, and for most experiments the standard error of the constant was less than the value itself. Weighted mean apparent values for the constants obtained from the data of Fig. 1 and other similar data are shown in Table I. The apparent values obtained for K_1 and K_3 are similar as are those for K_2 and K_4.

The true value of each of these constants, assuming that each dissociation does occur, could theoretically have been calculated from apparent values obtained at 3 concentrations of creatine (see THEORY). However, the data were not sufficiently good for such calculations to yield meaningful values for the true constants K_1, K_2, K_3 and K_4 (or for the dissociation constants associated with the reactions of creatine), either by solving a set of simultaneous equations for each one, or by fitting a hyperbola to each set of three apparent values by means of the Hyper replot computer programme (Eqn. 5 of ref. 8). Nevertheless, some indication of the true values can be obtained from the trends in the apparent values shown in Table I. Since the apparent values of K_1 and K_3 increase with a decrease in creatine concentration, the true values at zero concentration of creatine, will be somewhat greater than any of the apparent values listed for these two constants. Moreover, apparent K_1 will increase as the creatine concentration decreases only if $K_{ip} > K_p$; similarly, the condition for the same effect on apparent K_3 is that $K_{ip} > K_{ip'}$ (see THEORY). Thus creatine appears to be bound more weakly to free enzyme than to E–Mg or to E–ATP, or alternatively, because of the equilibrium condition, Mg^{2+} and ATP^{4-} would appear to combine more weakly with free enzyme than with E–creatine. This effect resembles the earlier finding[1] that the combination of creatine is enhanced by the previous combination of $MgATP^{2-}$, and *vice versa*. The apparent values of K_2 and K_4 are too low and variable for trends with the concentration of creatine to be considered significant.

The apparent values for K_6 recorded in Table I are lower than the corresponding true value of 1.2 mM determined by MORRISON AND JAMES[1] with the Mg^{2+} concentration held constant at 1 mM. There is reasonable agreement between the apparent values for K_3 and that of approx. 0.2 mM, which is the apparent value determined for this constant in the reverse reaction[6].

Inhibition by excess Mg^{2+}

The inhibition of the reaction by excess Mg^{2+}, added as the chloride salt, was considerably weaker than that expected on the basis of the above results. To demonstrate inhibition it was necessary to use a range of concentrations of $MgCl_2$ which was an order of magnitude greater than that employed in the previous experiments. The inhibition so obtained was noncompetitive with respect to both $MgATP^{2-}$ and creatine (Fig. 3) as predicted by Eqns. 6 and 7. The simple noncompetitive inhibition with respect to creatine may be compared with the simple noncompetitive inhibition

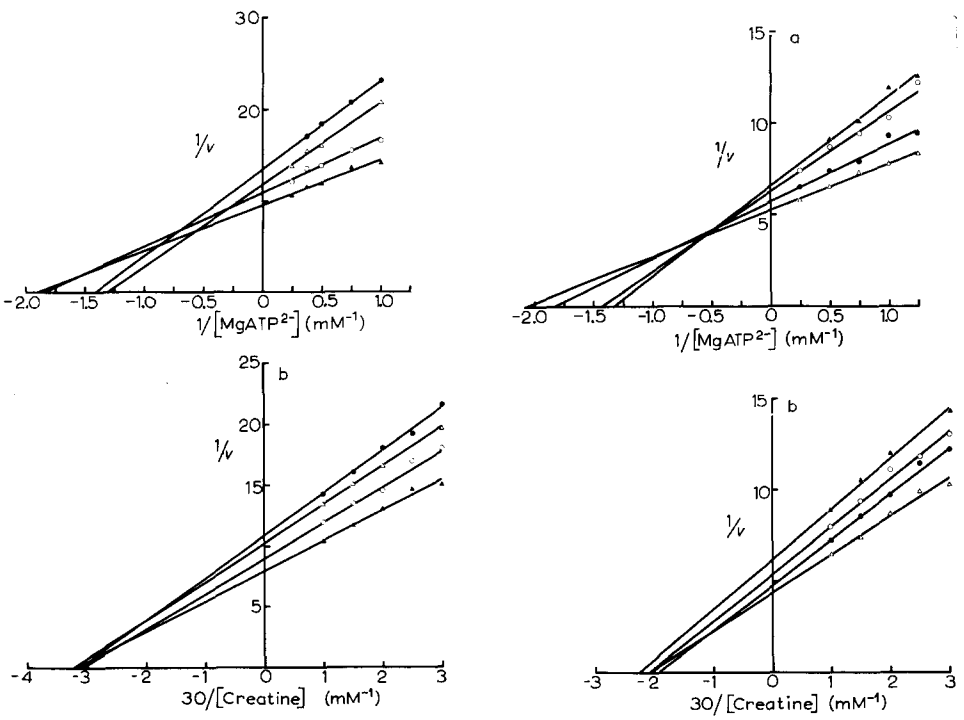


Fig. 3. Inhibition of the forward reaction by $MgCl_2$ at pH 8.0 (a) with $MgATP^{2-}$ as the variable substrate and the creatine concentration held constant at 30 mM, and (b) with creatine as the variable substrate and the $MgATP^{2-}$ concentration held constant at 4 mM. The concentrations of $MgCl_2$ added were; ●, 15 mM; △, 10 mM; ○, 5 mM and ▲, 1 mM. v is expressed as μmoles of ADP produced per μg of creatine kinase per min.

Fig. 4. Inhibition of the forward reaction by Mg^{2+}, added as acetate, in triethanolamine–acetate buffer at pH 8.0 (a) with $MgATP^{2-}$ as the variable substrate and the creatine concentration held constant at 30 mM, and (b) with creatine as the variable substrate and the $MgATP^{2-}$ concentration held constant at 2 mM. The concentrations of Mg^{2+} were: ▲, 45 mM; ○, 30 mM; ●, 15 mM and △, 1 mM. v is expressed as μmoles of ADP produced per μg of creatine kinase per min.

previously observed with respect to $MgADP^-$ (ref. 5). This similarity may be related to the absence of a transferable phosphate group in both $MgADP^-$ and creatine.

The inhibition of the reaction by excess Mg^{2+}, added as magnesium acetate, is illustrated in Fig. 4. Acetate ion was chosen because sodium acetate did not cause significant inhibition under the conditions used in these experiments, whereas sodium chloride did[7]. However, the inhibition pattern is the same whichever anion is added.

Replots of slopes and vertical intercepts from both Figs. 3a and 4a against concentration of Mg^{2+} (Fig. 5) would be considered linear, and the inhibition constants obtained by computer analysis of the primary data of these and other experiments are recorded in Table II. Qualitatively the results are similar in that for all three cases the values of $K_{i\ (slope)}$ and $K_{i\ (intercept)}$ are virtually equal when creatine is the variable substrate, whereas when $MgATP^{2-}$ is varied, the values for $K_{i\ (slope)}$ are less than for $K_{i\ (intercept)}$. However, it is apparent that the Cl^- of $MgCl_2$ makes a contribution to the inhibition obtained with this compound.

The detectable interactions of Mg^{2+} with the enzyme were in each case weaker by an order of magnitude than that indicated by the appropriate apparent value for K_3 (Table I).

Inhibition by excess ATP^{4-}

On the basis of the dissociation constants listed in Table I, and because of the ability of ADP^{3-} to cause strong inhibition of the reverse reaction with respect to

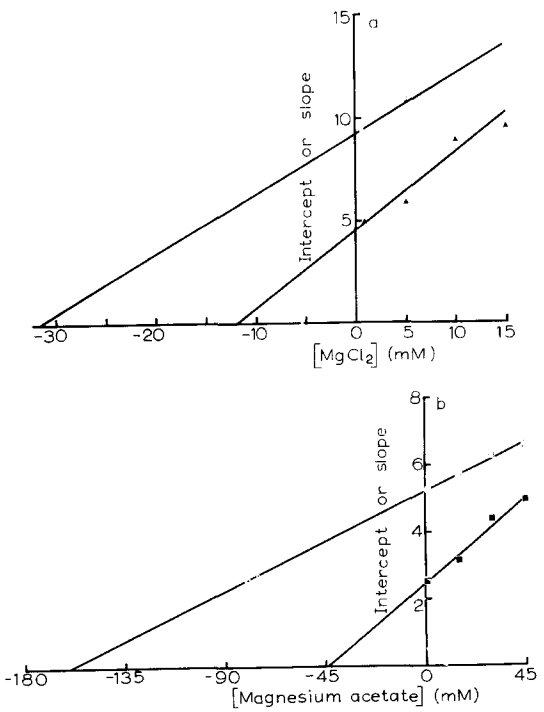


Fig. 5. Secondary plots of slopes (▲, ■) and vertical intercepts (○, □) from (a) Fig. 3a and (b) Fig. 4a against the concentrations of $MgCl_2$ and $Mg(Ac)_2$, respectively.

$MgADP^-$ (ref. 5), it was expected that ATP^{4-} when present in excess of $MgATP^{2-}$ would cause marked inhibition of the forward reaction. This was not observed; there was only slight inhibition by 2 mM ATP^{4-} with $MgATP^{2-}$ varying in concentration from 0.4 to 2.0 mM. The assay procedure precluded the use of higher concentrations of ATP^{4-} over this range of $MgATP^{2-}$ concentration, so that it was not possible to determine with certainty the nature of the inhibition. However, by assuming that ATP^{4-} is a competitive inhibitor with respect to $MgATP^{2-}$, an apparent inhibition constant of approx. 5 mM was calculated. This value may be considered similar to those of approx. 1 mM, from inactivation kinetics with iodoacetate[9], and approx. 2 mM as determined by KUBY AND NOLTMANN[10] from the kinetic data of NODA, NIHEI AND MORALES[7]. It is nevertheless considerably greater than the appropriate apparent value of K_1 in Table I (0.16 mM) and the thermodynamic values of 0.3 mM and 0.5 mM as reported by KUBY, MAHOWALD AND NOLTMANN[3] at a different temperature and ionic strength. Furthermore, the present results do not support a stronger competition between ATP^{4-} and $MgATP^{2-}$ than between ADP^{3-} and $MgADP^-$, and are thus not in agreement with the interpretation of magnetic resonance studies by COHN[4].

DISCUSSION

Both Mg^{2+} and ATP^{4-}, when each is present in excess of $MgATP^{2-}$, are less effective inhibitors of the forward reaction than might be expected from the results obtained when all three species Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ are present in significant concentration. These findings cast doubt on the reality of all the steps shown in Scheme I, and raise the possibility that Mg^{2+} and ATP^{4-} cannot interact directly with the free enzyme at the active site.

There is, however, reasonable evidence for the presence of kinetically significant concentrations of E–Mg and E–ATP when Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ are all present in significant concentration. It should be noted that the standard errors of the apparent values for K_1, K_2, K_3 and K_4, as calculated on the basis of Scheme I, are high (Table I). Since the slope replots in Fig. 2a and the corresponding replots of slopes against the

TABLE II

VALUES OF THE APPARENT KINETIC CONSTANTS OBTAINED FROM INHIBITION BY EXCESS Mg^{2+} OF THE FORWARD REACTION CATALYSED BY CREATINE KINASE

Each value is the weighted mean of the values obtained from the data of a number of experiments, including those of Figs. 3 and 4, by means of analysis with the NONCOMP computer programme of CLELAND[8]. When $MgATP^{2-}$ was the variable substrate the creatine concentration was held constant at 30 mM; when creatine was varied the $MgATP^{2-}$ concentration was 2 mM, except that when Cl^- was used exclusively, it was fixed at a concentration of 4 mM.

Mg^{2+} anion	*Buffer anion*	*Variable substrate*			
		$MgATP^{2-}$		*Creatine*	
		K_i (slope) (mM)	*K_I (intercept) (mM)*	*K_i (slope) (mM)*	*K_i (intercept) (mM)*
Chloride	Chloride	14 ± 3	28 ± 3	33 ± 13	31 ± 7
Acetate	Chloride	24 ± 5	90 ± 19	66 ± 21	62 ± 14
Acetate	Acetate	33 ± 7	162 ± 29	125 ± 43	108 ± 26

reciprocal of Mg^{2+} concentration (which are not shown) pass very close to the origin, it might be concluded that the values for K_1 and K_3, respectively, are not real and hence that E–Mg and E–ATP are not present in kinetically significant concentrations. Nevertheless, over a series of 10 experiments all values determined for each dissociation constant were positive, and in most cases the standard errors were less than the values of the constants. The use of the sign test as a simple statistical test of significance indicates that it is highly significant to obtain 10 positive values. Greater precision might have been expected if it had been possible to extend the ranges of concentration of ATP^{4-} and Mg^{2+} well above the approximate values of apparent K_1 and apparent K_3, but this was not feasible because of the high stability constant for $MgATP^{2-}$. However, the results are in accord with those obtained previously from studies on the reverse reaction[5,6].

In view of this evidence for the presence of kinetically significant concentrations of E–Mg under conditions where all reactants are present, the relatively weak inhibition observed with excess Mg^{2+} is of major importance. Moreover, Mg^{2+} is not a competitive inhibitor with respect to $MgATP^{2-}$, and so cannot interact with the enzyme only at the $MgATP^{2-}$ binding site; inhibitory reaction at this as well as at another site is possible, but is not in accord with the results. Thus, the replot of the slopes of lines from the inhibition by Mg^{2+} with respect to $MgATP^{2-}$ (Fig. 5) is not parabolic, and hence not in accord with Eqn. 6, which takes into account the proposed inhibitory reaction of Mg^{2+} with the free enzyme at both sites. The discrepancy between the observed slope replot and the approximate shape of the parabola calculated from Eqn. 6 on the basis of values of 0.15 mM for K_3, 24 mM for K_i (Table II) and 15.6 mM for K_{ip} and $K_{ip'}$ (ref. 1) (Table I), is so marked that the theoretical formulation must be modified. While there is no doubt that Mg^{2+} does interact as an inhibitor at a site distinct from the active site, it would appear that if the formation of the E–Mg complex with Mg^{2+} at the active site takes place, it occurs to a constant extent under the conditions of the inhibition experiments.

If a direct interaction of Mg^{2+} with the free enzyme does not occur, and hence E–Mg is formed only by the release of ATP^{4-} from E–MgATP, it would be expected that the concentration of E–Mg would be dependent on that of E–MgATP, which in turn would be dependent on the concentration of $MgATP^{2-}$. Because of the high apparent stability constant for $MgATP^{2-}$, under the present conditions, ATP would be present as 99% $MgATP^{2-}$ when the concentration of free Mg^{2+} is 1 mM. Therefore, any increase in the Mg^{2+} concentration above 1 mM, without a simultaneous increase in the total ATP concentration, would not significantly increase the concentration of $MgATP^{2-}$ and hence that of E–MgATP. It follows then that the concentration of E–Mg would not be increased by virtue of an increase in the concentration of E–MgATP.

But an increase in the Mg^{2+} concentration might, however, be expected to displace the equilibrium of the reaction

$$E\text{–}MgATP \overset{K_4}{\rightleftharpoons} E\text{–}Mg + ATP^{4-} \qquad \text{(i)}$$

to the right by removal of ATP^{4-} through chelation by Mg^{2+}. This would result in an increase in the concentration of E–Mg, which would be expected to function as a dead-end complex. In this connection it should be pointed out that the inhibition caused,

at a particular nonsaturating concentration of ATP, by increasing the free Mg^{2+} concentration from close to 0 mM, when Mg^{2+} and ATP were present in equimolar concentrations, up to 1 mM, was barely detectable. Thus the concentration of the E–Mg complex is probably very low compared with that of E–MgATP, which implies that the equilibrium of Reaction (i) may be well to the left even when the ATP^{4-} concentration is low. This conclusion is consistent with the low values for apparent K_4 which are given in Table I. Consequently, any change in the ATP^{4-} concentration caused by further increases in the Mg^{2+} concentration, in the range from 1 to 45 mM (Fig. 4), need have only an insignificant effect on the equilibrium. Under these conditions the concentration of E–Mg could be considered low and constant.

The possibility that $MgATP^{2-}$, as well as ATP^{4-}, could react with E–Mg should also be considered as a counteraction to the tendency to form higher concentrations of E–Mg. Such a reaction might occur under any conditions, and perhaps involve the initial combination of the ATP moiety of $MgATP^{2-}$ with E–Mg, followed by an exchange of the 2 magnesium ions associated with the ATP, *i.e.*,

$$E\text{–Mg} + \underline{\text{Mg}}\text{ATP}^{2-} \rightleftharpoons E\text{–}\underline{\text{Mg}}\text{ATP} + \text{Mg}^{2+} \qquad \text{(ii)}$$

Provided that the equilibrium of this reaction lies well to the right, the concentration of E–Mg would not be changed significantly by increasing the Mg^{2+} concentration alone. Moreover, the concentration of E–MgATP, and hence the velocity of the reaction, would not be significantly decreased. In the special case where the equilibrium constants of the Reactions (ii) and (iii)

$$E + \text{MgATP}^{2-} \overset{K_6}{\rightleftharpoons} E\text{–MgATP} \qquad \text{(iii)}$$

were of similar magnitude, E–Mg could be considered kinetically equivalent to E. This would not be so unless ATP^{4-} were virtually absent. Similarly, E–Mg–creatine might be considered equivalent to E–creatine.

If the concentrations of E–Mg and E–Mg–creatine are considered constant and low compared with those of E–MgATP and E–MgATP–creatine, respectively, or if the former two complexes are considered kinetically equivalent to the latter two, it becomes possible to modify Eqns. 6 and 7 to fit the results. The terms associated with E–Mg and E–Mg–creatine, and therefore containing $[Mg^{2+}]/K_3$, might, in the first case, be replaced by constants, and in the second case removed completely. Such modified equations are consistent with the linear relationships which are experimentally observed for plots of slopes and intercepts as a function of the concentration of Mg^{2+} (Fig. 4). The meanings of the inhibition constants determined from inhibition of the reaction by excess magnesium now become clear. At a fixed creatine concentration $K_{i\,(slope)}$ and $K_{i(intercept)}$ from inhibition with respect to $MgATP^{2-}$ (Fig. 4) represent, respectively, the dissociations of Mg^{2+} from the inhibitory site, distinct from the active site, in the absence (K_i) and presence (K_I) of ATP on the enzyme. Since K_I is greater than K_i, the presence of ATP makes this inhibitory reaction more difficult. Each value obtained from inhibition with respect to creatine is a complex of K_i and K_I.

While the weak inhibition by excess ATP^{4-} may be considered consistent with the kinetic data of other workers[9,10], it is apparently not in accord with the indication of a strong interaction with the enzyme given by the appropriate apparent value of K_1

(Table I) and the results of thermodynamic investigations[3,4]. The strong inhibition by ADP^{3-} with respect to $MgADP^-$ in the reverse reaction[5] contrasts with the present results, and is in agreement with the thermodynamic results of KUBY, MAHOWALD AND NOLTMANN[3]. If the *E*–ATP complex can only form from *E*–MgATP, and the equilibria analogous to those outlined above for the magnesium experiments are considered, the weak inhibition by excess ATP^{4-} can be explained in a manner similar to that for the inhibition caused by excess Mg^{2+}. However, if a direct interaction between ATP^{4-} and the active site of the enzyme does not occur, one is led to the conclusion that the strong interaction detected thermodynamically does not occur at the active site. A further possibility is that preparations of ATP used for the thermodynamic studies were contaminated by ADP which is a potent inhibitor.

It appears, then, that under conditions where Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ are all present, kinetically significant concentrations of *E*–Mg and *E*–ATP as well as of *E*–MgATP can be detected. Nevertheless, under conditions where either Mg^{2+} or ATP^{4-} is present in excess of $MgATP^{2-}$, the degree of inhibition is not consistent with the formation of such *E*–Mg or *E*–ATP complexes by direct interaction of Mg^{2+} or ATP^{4-} with the enzyme. Thus it seems plausible that the steps represented in Scheme I by K_3 and K_1 do not occur, and that *E*–Mg and *E*–ATP are formed only by the breakdown of *E*–MgATP.

The conclusion that *E*–Mg can be present is at variance with the interpretation of the proton relaxation rate and electron spin resonance measurements of COHN[4], which indicated that manganese was bound only to the nucleotide and not to the protein. It is not certain whether the same qualitative result might be expected with another metal ion; however, the difference may be quantitative rather than qualitative if the concentration of the enzyme–metal ion complex is low. There is the further possibility that the form of enzyme represented as *E*–Mg is, in fact, an *E*–Mg–creatine complex which is derived from an *E*–MgATP–creatine complex. The formation of such a complex would be detected only in kinetic experiments where, of necessity, creatine is also present.

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