Kinetic Studies of the Reverse Reaction Catalysed by Adenosine Triphosphate-Creatine Phosphotransferase

THE INHIBITION BY MAGNESIUM IONS AND ADENOSINE DIPHOSPHATE

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1. Kinetic investigations of the reaction catalysed by ATP-creatine phosphotransferase have been carried out. 2. No firm conclusions could be reached about the reaction of Mg^{2+} at the nucleotide-binding site of the enzyme. The value of the kinetic constant for this reaction depends on the value used for the apparent stability constant of the metal ion-nucleotide complex and, to a smaller extent, on the method of plotting the results. 3. At higher concentrations Mg^{2+} is a non-competitive inhibitor of the enzyme with respect to both $MgADP^-$ and phosphocreatine. 4. ADP^{3-} is a competitive inhibitor of the enzyme with respect to $MgADP^-$ and a non-competitive inhibitor with respect to phosphocreatine. 5. The concentration of phosphocreatine has little, if any, effect on the kinetic constants for the nucleotide reactants.

Kinetic investigations of enzymic reactions that involve the reversible transfer of the terminal phosphoryl group of ATP to an acceptor molecule to give ADP and a phosphorylated acceptor have been complicated by the fact that the essential bivalent metal ion undergoes non-enzymic reaction with both ADP and ATP. Thus, to determine or fix the concentrations of the various ionic species of metal and nucleotide, it is necessary to know the stability constants of the metal ion-nucleotide complexes. Since the apparent stability constants required for this purpose vary according to pH, ionic strength and temperature, and are influenced by the nature of the buffer and acceptor or donor, it is important that the values be determined or calculated for a particular set of conditions.

The interpretation of kinetic results is simplified if studies are carried out at pH 8.0 or above. At these pH values, the nucleotides may be considered to exist in solution completely as ATP^{4-} and ADP^{3-} , and so it is necessary to know only the apparent stability constants for $MgATP^{2-}$ and $MgADP^-$. Values for these constants under particular sets of conditions have been reported by O'Sullivan & Perrin (1964).

In their investigations of the activation of ATP-creatine phosphotransferase (EC 2.7.3.2; creatine kinase) by Mg^{2+} , Morrison, O'Sullivan & Ogston (1961) used an apparent stability constant for $MgADP^-$ to calculate the total amount of Mg^{2+} and ADP required to maintain the concentration of

free Mg^{2+} constant while varying the concentration of ADP^{3-} . It was concluded that the reaction may proceed via the formation of an active enzyme-Mg-ADP complex that could be formed by reaction of the enzyme with $MgADP^-$, with ADP^{3-} and then Mg^{2+} or with Mg^{2+} and then ADP^{3-} . Insofar as reaction of the enzyme with $MgADP^-$ and ADP^{3-} is concerned, the results agreed with those of Kuby, Noda & Lardy (1954) and Nihei, Noda & Morales (1961). However, whereas Nihei *et al.* (1961) considered ADP^{3-} simply as a competitive inhibitor of the reaction, Morrison *et al.* (1961) regarded the formation of an enzyme-ADP complex as a possible first step in the formation of an enzyme-Mg-ADP complex.

It is with respect to the reaction of Mg^{2+} with the enzyme that the results of Morrison *et al.* (1961) differed from those obtained by the other groups, although subsequent reassessment of earlier work has led Kuby & Noltmann (1962) to conclude that there appears to be some reaction of Mg^{2+} with the active site of creatine kinase.

A reinvestigation of the reverse reaction catalysed by creatine kinase by the same approach as that described by Morrison *et al.* (1961) has yielded results similar to those reported by these authors in that they indicated reaction of the enzyme with $MgADP^-$ and ADP^{3-} . However, the results on the interaction of Mg^{2+} and the enzyme were less conclusive. A kinetic study has been made of the reaction of Mg^{2+} and ADP^{3-} with the enzyme under

conditions where one of these components is virtually absent. It was considered that, if an enzyme-Mg-ADP complex could ultimately be formed as a result of an initial reaction of the enzyme with Mg^{2+} or ADP^{3-} , then each of the species must react at the same site on the enzyme as does MgADP^- . Thus, under conditions of excess of Mg^{2+} or excess of ADP^{3-} , when only MgADP^- and either Mg^{2+} or ADP^{3-} are present in appreciable concentrations, the 'dead-end' complexes enzyme- Mg^{2+} and enzyme-ADP should form, so that Mg^{2+} and ADP^{3-} should function as competitive inhibitors of the reaction with respect to MgADP^- . The results of such studies at a number of non-saturating concentrations of phosphocreatine have shown that, whereas ADP^{3-} is a competitive inhibitor, Mg^{2+} acts as a non-competitive inhibitor of the reaction.

THEORY

The reverse reaction catalysed by creatine kinase is assumed to occur via an enzyme-Mg-ADP-phosphocreatine complex that could be formed by the following sets of binary reactions, all of which are considered to be in rapid equilibrium (cf. Kuby & Noltmann, 1962):

where E, A, B and M represent enzyme, ADP^{3-} , phosphocreatine and Mg^{2+} respectively, and K_1 etc. are dissociation constants.

Further, in deriving the initial velocity equation, it has been supposed that: (i) A, M and MA do not affect the binding of B and vice versa; (ii) A and MA, as well as M and MA, are bound at the same site and thus compete with each other; (iii) the same active EMAB complex is formed irrespective of the pathway and that the rate of breakdown of this complex limits the reaction velocity; (iv) the reaction does not occur in the absence of M; (v) the steady-state concentrations of the various enzyme complexes are not sufficient to have any appreciable effect on the concentrations of M, A, MA or B.

The initial-velocity equation may be written in reciprocal form as:

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \left(1 + \frac{K_6}{[MA]} + \frac{K_6[A]}{K_1[MA]} + \frac{K_6[M]}{K_3[MA]} \right) \quad (1)$$

Because of the rapid equilibrium assumption, it follows that:

$$K_1 K_2 = K_3 K_4 = K_5 K_6$$

and since:

$$[MA] = \frac{[M][A]}{K_5}$$

the equation may be expressed in the alternative form:

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \left(1 + \frac{K_1 K_2}{[M][A]} + \frac{K_2}{[M]} + \frac{K_4}{[A]} \right) \quad (2)$$

This may be rearranged as:

$$\frac{1}{v} = \frac{K_2}{V} \cdot \frac{1}{[M]} \left(1 + \frac{K_7}{[B]} \right) \left(\frac{K_1}{[A]} + 1 \right) + \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \left(\frac{K_4}{[A]} + 1 \right) \quad (3)$$

Thus plots of $1/v$ against $1/[M]$ at different non-saturating concentrations of A and a particular concentration of B should yield a family of straight lines with both slopes and intercepts varying with the concentration of A. The value of K_1 may be determined from a secondary plot of slopes against $1/[A]$, and a secondary plot of vertical intercepts against $1/[A]$ gives a value for K_4 as well as the apparent maximum velocity, V , at a particular concentration of phosphocreatine. From a plot of $1/V'$ against $1/[\text{phosphocreatine}]$, the true maximum velocity and K_7 may be obtained. The reciprocals of the horizontal intercepts of the primary plot, K_2' , would equal:

$$K_2' = \frac{\left(\frac{K_1}{[A]} + 1 \right)}{\left(\frac{K_4}{[A]} + 1 \right)}$$

so that a plot of $K_2' \{ (K_4/[A]) + 1 \}$ against $1/[A]$ would yield values for K_2 as well as for K_1 . Values for K_3 and K_6 may then be obtained from the relationship:

$$K_1 K_2 = K_3 K_4 = K_5 K_6$$

If M does not react with the enzyme, then $K_3 = \infty$ and $K_4 = 0$. This would make the vertical intercepts of the primary plot independent of the concentration of A and K_2' equal to $K_2 \{ (K_1/[A]) + 1 \}$. The slopes of the lines would still be dependent on the concentration of A.

Since the initial-velocity expression is symmetrical, a similar expression:

$$\frac{1}{v} = \frac{K_4}{V} \cdot \frac{1}{[A]} \left(1 + \frac{K_7}{[B]} \right) \left(\frac{K_3}{[M]} + 1 \right) + \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \left(\frac{K_2}{[M]} + 1 \right)$$

is obtained with the concentration of A as the variable. The reciprocals of the horizontal intercepts, K'_4 , would equal:

$$K_4 \left(\frac{\frac{K_3}{[M]} + 1}{\frac{K_2}{[M]} + 1} \right)$$

and values for K_3 and K_2 may be determined from secondary plots of slopes and intercepts respectively against $1/[M]$ (see also Morrison *et al.* 1961). When the concentration of M rather than that of A is varied, it is at least theoretically possible to determine immediately from the primary plot if an enzyme-metal ion complex is formed. However, difficulty could be encountered if the steady-state concentration of this complex were low. Both procedures have been used to plot the results and to determine the kinetic constants.

Inhibition by Mg^{2+} . (a) If M reacts with the enzyme only at the nucleotide-binding site and is present at concentrations such that $A \rightarrow 0$, then the initial-velocity eqn. (1) may be written:

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \left(1 + \frac{K_6}{[MA]} + \frac{K_6[M]}{K_3[MA]} \right) \quad (4)$$

$$\text{or } \frac{1}{v} = \frac{K_6}{V} \cdot \frac{1}{[MA]} \left(1 + \frac{K_7}{[B]} \right) \left(1 + \frac{[M]}{K_3} \right) + \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \quad (5)$$

Thus M would function as a competitive inhibitor with respect to MA.

(b) If it is considered that M may also combine with the enzyme to give an inactive complex and in such a way that B influences its binding, then the following reactions (and inhibitor constants) must also be taken into account:

(An inactive complex is signified when M is written to the left of E.)

The initial velocity of the inhibited reaction may be expressed as:

$$v = \frac{V}{\left\{ \frac{K_6}{[MA]} \left(1 + \frac{[A]}{K_1} + \frac{[M]}{K_3} \right) \left(1 + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} \right) + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} + 1 \right\}} \quad (6)$$

Inhibition by Mg^{2+} with respect to $MgADP^-$. The inhibition eqn. (6) may be arranged in reciprocal form as:

$$\frac{1}{v} = \frac{K_6}{V} \cdot \frac{1}{[MA]} \left(1 + \frac{[A]}{K_1} + \frac{[M]}{K_3} \right) \left(1 + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} \right) + \frac{1}{V} \left(1 + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} \right) \quad (7)$$

so that plots of $1/v$ against $1/[MA]$ at various concentrations of M and a fixed concentration of B should be linear. If $[A]/K_1 \rightarrow 0$ and $[M]/K_3 \rightarrow 0$, then the equation becomes:

$$\frac{1}{v} = \frac{K_6}{V} \cdot \frac{1}{[MA]} \left(1 + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} \right) + \frac{1}{V} \left(1 + \frac{[M]}{K_I} + \frac{K_7}{[B]} + \frac{K_7[M]}{K_4[B]} \right) \quad (8)$$

and the inhibition by M would be simple non-competitive with respect to MA, with the lines cutting the abscissa at the same point. In this case, secondary plots of both slopes and intercepts against M at a particular concentration of B would yield the same value for apparent K_I , equal to:

$$K_I \left(\frac{1 + \frac{K_7}{[B]}}{1 + \frac{K_7}{[B]} \cdot \frac{K_I}{K_4}} \right)$$

Tertiary plots of apparent K_I against $[B]$ or $1/[B]$ would be expected to be non-linear, so that values could not be obtained readily for either inhibitor constant.

The value for K_7 may be obtained by plotting the intercepts of the primary plot against the concentration of M for each concentration of B, extrapolating to $[M] = 0$ and reploting the results so obtained against $1/[B]$.

Inhibition by Mg^{2+} with respect to phosphocreatine. Under conditions where $[A]/K_1 \rightarrow 0$, eqn. (7) may be rearranged as:

$$\frac{1}{v} = \frac{K_7}{V} \cdot \frac{1}{[B]} \left(1 + \frac{[M]}{K_4} \right) \left(1 + \frac{K_6}{[MA]} \left(1 + \frac{[M]}{K_3} \right) \right) + \frac{1}{V} \left(1 + \frac{[M]}{K_I} \right) \left(1 + \frac{K_6}{[MA]} \left(1 + \frac{[M]}{K_3} \right) \right) \quad (9)$$

so that plots of $1/v$ against $1/[B]$ at various fixed concentrations of M and a particular concentration of MA would yield a family of straight lines intersecting above, below or on the abscissa, depending on the values for K_I and K_4 . Values for K_4 and K_I , which are independent of the concentration of MA, may be determined from secondary plots of slopes and intercepts respectively against the concentration of M when K_3 is large compared with M.

Inhibition of the reaction by higher concentrations of ADP^{3-} . When relatively high concentrations of A are present, $[M] \rightarrow 0$ and the initial-velocity eqn. (6) takes the form:

$$v = \frac{V}{\left(1 + \frac{K_7}{[B]} \right) \left(\frac{K_6}{[MA]} + \frac{K_6[A]}{K_1[MA]} + 1 \right)} \quad (10)$$

This may be arranged in reciprocal form:

$$\frac{1}{v} = \frac{K_6}{V} \cdot \frac{1}{[MA]} \left(1 + \frac{K_7}{[B]} \right) \left(1 + \frac{[A]}{K_1} \right) + \frac{1}{V} \left(1 + \frac{K_7}{[B]} \right) \quad (11)$$

so that plots of $1/v$ against $1/[MA]$ at various concentrations of A and a fixed concentration of B should yield a family of

straight lines that cut the ordinate at the same point. [Eqn. (11) also follows from eqn. (1) with $[M] \rightarrow 0$, and eqn. (11) is analogous to eqn. (5).] The value of K_1 , which would be independent of the concentration of B, may be determined from a secondary plot of slopes against the concentration of A. The value of K_7 can be obtained from plots of the intercepts at various concentrations of B against $1/[B]$.

With the concentration of B as the variable, the equation can be written in reciprocal form as:

$$\frac{1}{v} = \frac{K_7}{V} \cdot \frac{1}{[B]} \left(1 + \frac{K_8}{[MA]} \left(1 + \frac{[A]}{K_1} \right) \right) + \frac{1}{V} \left(1 + \frac{K_6}{[MA]} \left(1 + \frac{[A]}{K_1} \right) \right) \quad (12)$$

so that primary plots of $1/v$ against $1/[B]$ at various concentrations of A and a fixed concentration of MA should give a family of straight lines that intersect at the same point on the abscissa, equal to $1/K_7$. Secondary plots of slopes and intercepts against the concentration of A should yield the same apparent value for K_1 equal to $K_1 \{ 1 + ([MA]/K_6) \}$. The true K_1 value may then be obtained by plotting the apparent K_1 values against the concentration of MA, or by calculation.

EXPERIMENTAL

The materials and methods were as described by Morrison *et al.* (1961), except for the following modifications that were introduced to ensure that there was no inhibition of the enzyme by heavy-metal ions (cf. O'Sullivan & Morrison, 1963). A solution of crystalline sodium phosphocreatine (1 g./50 ml.) was stirred for 3 hr. at 2° with 240 mg. dry wt. of Chelex-100 (Na⁺ form; 200–400 mesh) obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and the resin removed by filtration. The phosphocreatine was then recrystallized by the addition of 6 vol. of 95% (v/v) ethanol according to the procedure of Ennor & Stocken (1948) as modified by Peanasky, Kuby & Lardy (1957). A solution of sodium ADP (pH 7.4) at a concentration of 0.02 M was treated in a similar manner and, after standardization at 259 mμ (Bock, Ling, Morell & Lipton, 1956), stored at -10°. Reaction mixtures contained 0.005–0.01 mM-EDTA, which concentrations were considered to have only a negligible effect on the concentration of Mg²⁺. Further, in the experiments designed to determine the nature of the inhibition by Mg²⁺, the reaction was stopped by the addition of 0.6 ml. of 3 N-NaOH containing EDTA (0.14 M). The increased concentration of EDTA prevented the precipitation of Mg(OH)₂ without affecting the creatine estimation.

Since phosphocreatine reacts with Mg²⁺ (Smith & Alberty, 1956a) and was used at concentrations up to 20 mM, the effect of this compound on the apparent stability constant of MgADP⁻ at pH 8.0 and 30° was determined by the method of Burton (1959). At phosphocreatine concentrations of 10 and 20 mM, values of 3600 and 3300 M⁻¹ respectively were obtained. From these results, values of 3800 and 12 M⁻¹ were calculated for the apparent stability constants of MgADP⁻, in the presence of 5 mM-phosphocreatine, and Mg-phosphocreatine respectively. The value for Mg-phosphocreatine may be compared with directly determined values of 40 ± 16 M⁻¹ (O'Sullivan & Perrin, 1964) and 20 M⁻¹ (Smith & Alberty, 1956a). The appropriate apparent stability constant was used to calculate the total Mg²⁺ (as MgCl₂) and total ADP concentrations required to give various non-saturating con-

Fig. 1. Effect of the concentration of Mg²⁺ on the initial velocity of the reverse reaction catalysed by creatine kinase at various fixed concentrations of ADP³⁻ and phosphocreatine. The reaction mixtures (final vol. 1.0 ml.) contained: *N*-ethylmorpholine (pH 8.0), 0.1 M; EDTA, 0.005 mM; creatine kinase, 0.58 μg.; as well as the indicated concentrations of Mg²⁺, ADP³⁻ and phosphocreatine. The concentrations of ADP³⁻ were: ★, 0.066 mM; ●, 0.1 mM; ○, 0.2 mM; ▲, 0.4 mM. The concentrations of phosphocreatine were: (a) 20 mM; (b) 10 mM; (c) 5 mM. The incubation temperature was 30°. Velocity is expressed as μmoles of creatine/min./μg. of creatine kinase.

centrations of MgADP^- at different fixed concentrations of either Mg^{2+} or ADP^{3-} (see Morrison *et al.* 1961). No allowance was made for the small changes in the concentration of free phosphocreatine that occur as a result of increasing the Mg^{2+} concentration.

All experiments were run for two time periods (usually 45 and 90 sec.) to ensure that initial velocities were being measured.

The terminology suggested by Cleland (1963a) has been used to classify the various types of inhibition. For the primary double-reciprocal plots, the lines were fitted and values of the kinetic constants determined by the weighted regression analysis of Wilkinson (1961). The calculation of the kinetic constants from secondary and tertiary plots, as well as the fitting of the lines, was done by the method of least squares. When the standard errors of the kinetic constants are given, these were determined with the aid of an IBM computer.

RESULTS

Activation of creatine kinase by Mg^{2+} with respect to ADP^{3-} . The results illustrated in Fig. 1 and Table 1 indicate that, with the phosphocreatine concentration held constant at 10.0 or 20.0 mM, there is only a small variation in the vertical intercepts with the concentration of ADP^{3-} . It was concluded that there is little or no reaction of Mg^{2+} with the enzyme. The greater variation in the vertical intercepts with phosphocreatine at a concentration of 5.0 mM could be taken to indicate that there is a small interaction between enzyme and Mg^{2+} , although the experimental error at this concentration of phosphocreatine is greater than at higher concentrations. If, however, the variation in the vertical intercepts with phosphocreatine at a concentration of 5.0 mM is regarded as real, then K_3 and K_4 may be calculated to be 1.0 and 0.02 mM respectively. The former value is considerably higher than that reported by Morrison *et al.* (1961).

In contrast with the small changes in the vertical intercepts with the concentration of ADP^{3-} , there is a marked variation in the slopes of the lines and

the horizontal intercepts ($1/K_2'$) of Fig. 1. These findings are consistent with a reaction between ADP^{3-} and the enzyme. The values of the kinetic constant for this reaction (K_1) are listed in Table 2, together with those for K_2 and K_6 representing the interaction of the enzyme-ADP complex with Mg^{2+} and the enzyme with MgADP^- respectively (see also Fig. 2). The values for K_1 and K_6 are in fair agreement with those reported by Morrison *et al.* (1961) and those listed in Table 3 of the article by Kuby & Noltmann (1962). Further, they are not significantly affected by the concentration of phosphocreatine.

The value for K_7 for the reaction of phosphocreatine with the enzyme was determined as outlined in the Theory section and found to be 4.0 mM. From the same graph, the maximum velocity of the reaction was determined to be 0.20 $\mu\text{mole/min.}/\mu\text{g.}$ of creatine kinase.

For comparison with the results of Morrison *et al.* (1961), the data of Fig. 1 were replotted as $1/v$ against $1/[\text{ADP}^{3-}]$ at different fixed concentrations of Mg^{2+} . The kinetic constants determined from secondary and tertiary plots are given in Table 3, which shows that the values for K_1 , K_2 and K_6 are of similar magnitude to those listed in Table 2. However, values for K_3 are now obtained from the plots at each concentration of phosphocreatine and hence, on the basis of the equilibrium assumption, values may be determined for K_4 . The values for both constants exhibit a large standard error.

The graphs from which the above kinetic constants were determined are not shown, but the types

Table 1. Summary of the kinetic constants determined from the data of Fig. 1

The apparent maximum velocity, V' , is expressed as μmole of creatine/min./ $\mu\text{g.}$ of creatine kinase, and K_2' as mM.

Concn. of ADP^{3-} (mM)	Concn. of phosphocreatine (mM)...					
	5.0		10.0		20.0	
	K_2'	V'	K_2'	V'	K_2'	V'
0.4	0.09	0.11	0.08	0.14	—	—
0.2	0.13	0.11	0.14	0.15	0.12	0.16
0.1	0.17	0.10	0.18	0.14	0.20	0.15
0.066	0.20	0.09	0.23	0.14	0.27	0.15

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Table 2. Kinetic constants for the interaction of various forms of creatine kinase with Mg^{2+} , ADP^{3-} and MgADP^-

(A) Values for K_1 and K_2 were calculated from the plots shown in Fig. 2 for which any interaction of Mg^{2+} with the enzyme was neglected. K_6 was determined from the relationship $K_1 K_2 = K_5 K_6$. (B) Values for K_1 and K_2 were obtained from a plot of $K_2' \{ (K_4/[A]) + 1 \}$ against $1/[A]$, by using a K_4 value of 0.02 mM obtained from a plot of the vertical intercepts of Fig. 1(c) against $1/[A]$. The K_2' and V' values used were those listed in Table 1 for 5.0 mM-phosphocreatine. K_6 was determined as for A. All values are expressed as mM.

Kinetic constant	Concn. of phosphocreatine (mM)...	(A)			(B)
		5.0	10.0	20.0	5.0
K_1		0.17	0.28	0.31	0.25
		0.23*	0.16*	0.26*	0.23*
K_2		0.06	0.05	0.04	0.06
K_6		0.04	0.05	0.05	0.07

* These values were obtained by plotting the slopes of the lines of Fig. 1 against $1/[A]$.

Fig. 2. Determination of K_1 and K_2 from plots of K'_2 against $1/[ADP^{3-}]$. The concentrations of phosphocreatine were: (a) 20 mm; (b) 10 mm; (c) 5 mm. The K'_2 values used were those listed in Table 1 and any possible interaction of the enzyme with Mg^{2+} was neglected.

Table 3. Kinetic constants for the reaction of various forms of creatine kinase with Mg^{2+} , ADP^{3-} and $MgADP^-$

The data of Fig. 1 were replotted as $1/v$ against $1/[ADP^{3-}]$ at various fixed concentrations of Mg^{2+} , and the constants determined as described in the Theory section. All values are expressed as mm.

Kinetic constant	Concn. of phospho-creatine (mm) ...	5.0	10.0	20.0
K_1		0.23	0.16	0.19
K_2		0.07	0.08	0.09
K_3		0.55	0.95	0.62
		(± 0.49)	(± 0.49)	(± 0.36)
K_4		0.03	0.013	0.026
		(± 0.02)	(± 0.006)	(± 0.013)
K_6		0.06	0.04	0.05

of graph obtained were similar to those of Figs. 3 and 4 for which the concentration of Mg^{2+} was varied over a wider range with that of phosphocreatine held constant at 10.0 mm. The primary plot (Fig. 3) indicates that the horizontal intercepts decrease with decreasing concentrations of Mg^{2+} , and thus Morrison *et al.* (1961) were wrong in taking the average value for K_μ when a stability constant of 4150 M⁻¹ was used for $MgADP^-$ (cf. Kuby &

Fig. 3. Effect of the concentration of ADP^{3-} on the initial velocity of the reverse reaction catalysed by creatine kinase at various fixed concentrations of Mg^{2+} and with phosphocreatine at a concentration of 10 mm. The concentrations of Mg^{2+} were: Δ , 0.05 mm; $\bullet$, 0.066 mm; $\star$, 0.1 mm; $\blacktriangle$, 0.13 mm; $\circ$, 0.2 mm; $\blacksquare$, 0.4 mm. The conditions were similar to those of Fig. 1. Velocity is expressed as μ moles of creatine/min./ μ g. of creatine kinase.

Fig. 4. (a) Determination of K_2 from a plot of the vertical intercepts of Fig. 3 (see also Table 4) against $1/[Mg^{2+}]$. V' is expressed as μ moles of creatine/min./ μ g. of creatine kinase. (b) Determination of the values for K_4 and K_3 from a plot of $K'_4((K_2/[M]) + 1)$ against $1/[Mg^{2+}]$. For further details see the Theory section.

Table 4. Summary of the kinetic constants obtained from the data of Fig. 3

The apparent maximum velocity, V' , is expressed as $\mu\text{mole of creatine/min.}/\mu\text{g. of creatine kinase}$, and K'_4 as mM .

Concn. of Mg^{2+} (mM)	V'	K'_4
0.4	0.153 ± 0.009	0.059 ± 0.010
0.2	0.126 ± 0.015	0.069 ± 0.026
0.13	0.108 ± 0.035	0.084 ± 0.008
0.1	0.097 ± 0.004	0.087 ± 0.011
0.066	0.084 ± 0.004	0.111 ± 0.015
0.05	0.077 ± 0.003	0.133 ± 0.011

Table 5. Kinetic constants obtained from the results of Fig. 3 and Table 4

Values were determined by computer analysis with the sequential programme of Cleland (1963b) and are expressed as mM . (A) Variable component, ADP^{3-} concn. (B) Variable component, Mg^{2+} concn. Values for which standard errors are not given were obtained by calculation by using the assumed equilibrium relationships for the kinetic constants.

Kinetic constant	(A)	(B)
K_1	0.194 ± 0.030	0.22
K_2	0.068 ± 0.008	0.061 ± 0.008
K_3	0.50	0.75 ± 0.27
K_4	0.026 ± 0.006	0.018 ± 0.006
K_6	0.046	0.048

Noltmann, 1962). The kinetic constants from the data of Fig. 3 are collected in Table 4, and these values have been used to obtain the secondary and tertiary plots (Fig. 4). The line of Fig. 4(b) passes close to, but does not go through, the origin, as would be required if $K_3 = \infty$ and $K_4 = 0$. The data of Fig. 3 have also been analysed by using the sequential computer programme of Cleland (1963b). The values for K_1 , K_2 , K_3 , K_4 and K_6 (Table 5) for when Mg^{2+} or ADP^{3-} concentration is considered as the variable component are in reasonable agreement and in accordance with the reaction of Mg^{2+} at the nucleotide-binding site of creatine kinase. The effect of using the individual values for K'_4 in the tertiary plot (Fig. 4b) is to increase the value for K_3 and decrease the value for K_4 as compared with the values reported by Morrison *et al.* (1961). These results, together with the demonstration by Morrison *et al.* (1961) that K_μ is constant when an incorrect value of 1000 m^{-1} is used for the calculations, emphasize the need for using the correct stability constant for a particular set of conditions. A similar conclusion has been reached on theoretical grounds by Kuby & Noltmann (1962).

Fig. 5. Inhibition of creatine kinase by Mg^{2+} with respect to MgADP^- at different concentrations of phosphocreatine. The reaction conditions were as described in Fig. 1, except for the indicated concentrations of Mg^{2+} , MgADP^- and phosphocreatine, and the concentration of EDTA, which was 0.01 mM . The concentrations of Mg^{2+} were: $\star$, 15 mM ; $\bullet$, 10 mM ; $\circ$, 5 mM ; $\blacktriangle$, 1 mM . The concentrations of phosphocreatine were: (a) 20 mM ; (b) 10 mM ; (c) 5 mM . Velocity is expressed as $\mu\text{moles of creatine/min.}/\mu\text{g. of enzyme}$.

Inhibition of creatine kinase by Mg^{2+} . Plots of $1/v$ against $1/[\text{MgADP}^-]$ with Mg^{2+} at concentrations of 1, 5, 10 and 15 mM are shown (Fig. 5) for three concentrations of phosphocreatine. It is clear that the inhibition is non-competitive (eqn. 8) rather than competitive (eqn. 5).

Table 6. *Kinetic constants obtained from a study of the inhibition of creatine kinase by Mg^{2+} with respect to MgADP^-*

Values were calculated from the results shown in Fig. 5. V' is expressed as μmole of creatine released/min./ $\mu\text{g.}$ of creatine kinase, and K_6 as mM. The values for slope (K/V) were determined by using the average and individual values of K_6 . The slopes obtained with the latter value are given in parentheses.

Concn. of Mg^{2+} (mM)	Concn. of phospho-creatine (mM)...5.0			10.0			20.0		
	V'	K_6	$10 \times \text{Slope}$	V'	K_6	$10 \times \text{Slope}$	V'	K_6	$10 \times \text{Slope}$
1.0	0.128	0.099	8.67 (7.73)	0.154	0.093	6.82 (6.04)	0.188	0.095	5.16 (5.05)
5.0	0.103	0.120	10.78 (11.65)	0.120	0.092	8.75 (7.67)	0.152	0.099	6.38 (6.51)
10.0	0.078	0.105	14.23 (13.46)	0.099	0.121	10.61 (12.22)	0.134	0.103	7.24 (7.69)
15.0	0.067	0.119	16.57 (17.76)	0.083	0.113	12.65 (13.61)	0.107	0.088	9.07 (8.22)
Average value of K_6 ...	0.111			0.105			0.097

Fig. 6. Determination of apparent K_I for Mg^{2+} , at different concentrations of phosphocreatine, from plots of (a) slopes and (b) intercepts ($1/V'$) against Mg^{2+} concentration. The concentrations of phosphocreatine used were: $\blacktriangle$, 5 mM; $\circ$, 10 mM; $\blacksquare$, 20 mM. Slope and V' values were those listed in Table 6.

Table 7. *Effect of phosphocreatine concentration on K_I for Mg^{2+}*

Values were obtained from secondary plots of slope and intercept against Mg^{2+} concentration (Fig. 6). The apparent maximum velocity, V' , is expressed as μmole of creatine released/min./ $\mu\text{g.}$ of creatine kinase, and K_I as mM.

Concn. of phosphocreatine (mM)	Apparent K_I (intercept)	Apparent K_I (slope)	V'
5.0	14.0	14.0	0.137
10.0	16.0	15.9	0.161
20.0	18.4	18.2	0.188

The calculated values of K_6 and the apparent maximum velocities at saturating concentrations of MgADP^- from these plots have been collected in Table 6, together with the values for the slopes (K/V) of the lines. At each concentration of phosphocreatine the value for K_6 at different concentrations of Mg^{2+} shows random variation, so that it would appear that the presence of higher concentrations of ADP^{3-} with Mg^{2+} at 1 mM (0.5–0.1 of the K_I value) has little or no effect on the results. However, the average values for K_6 at each concentration of phosphocreatine suggest that K_6 may increase slightly with decreasing concentrations of phosphocreatine. Subsequent studies (J. F. Morrison & T. E. James, unpublished work) with Mg^{2+} at a concentration of 1 mM have confirmed that K_m for MgADP^- does vary to a small extent with the concentration of phosphocreatine.

As predicted by eqn. (8), secondary plots of the slopes and intercepts of Fig. 5 against the concentration of Mg^{2+} (Fig. 6 and Table 7) are linear

and give similar apparent K_i values, which are dependent on the concentration of phosphocreatine. This equation also predicts that tertiary

Fig. 7. Determination of K_i for Mg^{2+} at zero concentration of phosphocreatine (a) and of K_i at infinite concentration (b) of phosphocreatine. The apparent K_i values used were those given in Table 7.

Fig. 8. Determination of K_7 for phosphocreatine and the maximum velocity of the reaction at zero concentration of Mg^{2+} from a plot of the intercepts of Fig. 6(b) ($1/V'$) against $1/[\text{phosphocreatine}]$. The V' values are listed in Table 7.

plots of the apparent K_i values against the concentration of phosphocreatine and the corresponding double-reciprocal plot would be non-linear. Though straight lines have been drawn (Fig. 7), the points may well lie on hyperbolas, and thus the values of 12.8 mM at zero and 20.3 mM at infinite concentration of phosphocreatine cannot be taken as being reliable values for K_i and K_i respectively.

A plot of the reciprocal of the maximum velocity at saturating concentrations of MgADP^- and zero concentration of Mg^{2+} against the reciprocal of the phosphocreatine concentration (Fig. 8) yielded a

Fig. 9. Inhibition of creatine kinase by Mg^{2+} with respect to phosphocreatine at three concentrations of MgADP^- . The reaction conditions were as described in Fig. 5, except for the indicated amounts of Mg^{2+} , MgADP^- and phosphocreatine. The concentrations of Mg^{2+} ions were: $\star$, 15 mM; $\blacktriangle$, 10 mM; $\circ$, 5 mM; $\blacksquare$, 1 mM. The concentrations of MgADP^- were: (a) 0.2 mM; (b) 0.13 mM; (c) 0.1 mM. Velocity is expressed as $\mu\text{moles of creatine/min.}/\mu\text{g. of enzyme}$.

Table 8. *Kinetic constants obtained from a study of the inhibition of creatine kinase by Mg^{2+} with respect to phosphocreatine*

Values were calculated from the results of Fig. 9. The apparent maximum velocity, V' , is expressed as $\mu\text{mole of creatine/min.}/\mu\text{g. of creatine kinase}$, and the apparent K_7 for phosphocreatine as mM. Extrapolation to zero Mg^{2+} concentration gave K_7 values of 4.8, 5.3 and 4.5 mM (average 4.9 mM) at $MgADP^-$ concentrations of 0.1, 0.13 and 0.2 mM respectively.

Concn. of Mg^{2+} (mM)	Concn. of $MgADP^-$ (mM)...0.1			0.13			0.2		
	V'	K_7	Slope	V'	K_7	Slope	V'	K_7	Slope
1	0.105	5.0	47.6	0.125	5.3	42.6	0.128	4.4	34.6
5	0.091	5.9	64.8	0.109	6.2	56.6	0.105	5.0	47.6
10	0.078	6.8	86.8	0.091	6.9	75.8	0.095	5.2	54.6
15	0.065	8.0	124	0.077	7.6	98.2	0.077	5.7	74.2

value of 3.6 mM for K_7 and a maximum velocity of $0.23 \mu\text{mole of creatine/min.}/\mu\text{g. of creatine kinase}$.

The above findings argue against the interaction of Mg^{2+} at the active site of the enzyme as the first step in one of the possible pathways for the formation of an active enzyme-metal ion-substrate complex (cf. Morrison *et al.* 1961). If this were so, then a simple non-competitive inhibition, with the lines of the primary plot (Fig. 5) cutting at the same point on the abscissa, would not have been obtained. Further, this type of inhibition is consistent with rapid equilibrium kinetics (Morales, 1955).

Inhibition of creatine kinase by Mg^{2+} with respect to phosphocreatine concentration. The results of these experiments, which are consistent with eqn. (9), are shown in the form of double-reciprocal plots (Fig. 9), and the apparent values for K_7 , apparent maximum velocities and the slopes of the lines are recorded in Table 8. Secondary plots of the intercepts and slopes at each concentration of $MgADP^-$ against the concentration of Mg^{2+} gave a series of straight lines (Fig. 10), from which values of 21.1 mM for K_i and 10.5 mM for K_t were obtained. The values are independent of the $MgADP^-$ concentration, as required by eqn. (9). The K_7 values obtained by plotting the apparent K_7 values at each fixed concentration of $MgADP^-$ against the Mg^{2+} concentration and extrapolating to zero are given in Table 8. It appears that K_7 for phosphocreatine is independent of the concentration of $MgADP^-$, and thus the results are similar to those of Nihei *et al.* (1961). However, the magnitude of K_7 is considerably higher than that reported by these authors (cf. Kuby *et al.*, 1954).

The linear non-competitive inhibition demonstrated above is in accordance with the conclusions reached from the study of the inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$. However, in reaching these conclusions no cognizance was taken of the other, more indirect, effects that relatively high concentrations of magnesium

Fig. 10. Determination of K_i and K_t for Mg^{2+} from plots of slopes (a) and intercepts (b), obtained from Fig. 9, against Mg^{2+} concentration. The concentrations of $MgADP^-$ were: ●, 0.1 mM; ○, 0.13 mM; ■, 0.2 mM.

chloride could have in causing or contributing to lower reaction velocities. The agreement between a kinetic value of 10.5 mM, when the concentration of phosphocreatine is zero, and a thermodynamic value of $pK \approx 2$ (Kuby, Mahowald & Noltmann, 1962) for the interaction of Mg^{2+} with creatine kinase, may make it unnecessary to take into account other effects such as (a) the decrease in the concentration of $MgADP^-$ by virtue of the formation of Mg_2ADP^+ , (b) the decrease in the

concentration of phosphocreatine as a result of the formation of its Mg^{2+} complex, (c) the non-specific effect of ionic strength and (d) the effect of the Cl^- component of magnesium chloride. Nevertheless, some consideration has been given to these effects, as indicated below.

In the presence of high concentrations of Mg^{2+} it is conceivable that a second ADP complex, namely Mg_2ADP^+ , could be formed. Kuby *et al.* (1954) postulated the formation of this compound to explain the inhibition of creatine kinase by high concentrations of Mg^{2+} . However, there has been little direct evidence for its formation, although Burton (1959) has concluded that it could form and suggested an upper limit of 70 M^{-1} for K^* , where:

$$K^* = \frac{[\text{Mg}_2\text{ADP}^+]}{[\text{Mg}^{2+}][\text{MgADP}^-]}$$

If the inhibition by Mg^{2+} were due simply to the decrease of the MgADP^- concentration, then double-reciprocal plots that use the apparent concentration of MgADP^- should indicate that Mg^{2+} is a competitive inhibitor, and this is not the case. If, in addition, Mg_2ADP^+ acted as a competitive inhibitor with respect to MgADP^- (which seems unlikely), then the overall inhibition would be non-competitive, and only under the special circumstance that K_6 for MgADP^- was equal to the dissociation constant of the enzyme- Mg_2ADP^+ complex would the same inhibitor constant value be obtained from the secondary plots of slopes and intercepts. Further, if Mg_2ADP^+ reacted with the enzyme, but underwent reaction at a lower rate, then both secondary plots would not be linear, although it is possible that any non-linearity could escape detection. It would appear, therefore, that the inhibition by Mg^{2+} cannot be accounted for simply on the basis of the possible formation of Mg_2ADP^+ .

Similarly, the inhibition by Mg^{2+} cannot be ascribed to the formation of the Mg -phosphocreatine complex, and hence a decrease of the concentration of phosphocreatine, which functions as the substrate of the reaction (Kuby *et al.* 1954). Moreover, when Mg -phosphocreatine is considered to be inert and a stability constant of 12 M^{-1} for this complex is used to calculate the concentrations of Mg^{2+} and free phosphocreatine, replots of the results (Fig. 10) indicated that K_s and K_i for Mg^{2+} were virtually unaffected. Neither can the inhibition be accounted for simply on the basis of the increase in ionic strength that occurs when the magnesium chloride concentration is increased (cf. Kistiakowsky & Shaw, 1953). The addition of potassium chloride or sodium chloride at the same ionic strength caused much less inhibition, which could be due, at least partly, to the formation of KADP^{2-} or NaADP^{2-} (Smith & Alberty, 1956b).

Fig. 11. Inhibition of creatine kinase by ADP^{3-} with respect to MgADP^- at different concentrations of phosphocreatine. The reaction conditions were as described in Fig. 5, except for the indicated concentrations of ADP^{3-} , MgADP^- and phosphocreatine. The concentrations of ADP^{3-} were: Δ , 2.5 mm; $\circ$, 2.0 mm; $\blacksquare$, 1.0 mm; $*$, 0.5 mm; $\bullet$, 0.125 mm. The concentrations of phosphocreatine were: (a) 20 mm; (b) 10 mm; (c) 5 mm. Velocity is expressed as $\mu\text{moles of creatine/min.}/\mu\text{g. of enzyme}$.

Table 9. *Kinetic constants obtained from a study of the inhibition of creatine kinase by ADP³⁻ with respect to MgADP⁻*

Values were calculated from the results shown in Fig. 11. V' is expressed as $\mu\text{mole of creatine/min./}\mu\text{g. of creatine kinase}$, and apparent K_6 as mm. The average values for V' at phosphocreatine concentrations of 5.0, 10.0 and 20.0 mm were 0.116, 0.136 and 0.167 respectively.

Concn. of ADP ³⁻ (mm)	Concn. of phosphocreatine (mm)...5.0			10.0			20.0		
	V'	K_6	Slope	V'	K_6	Slope	V'	K_6	Slope
0.125	0.118	0.091	0.77	0.136	0.065	0.47	0.163	0.072	0.44
0.5	0.121	0.179	1.46	0.129	0.105	0.81	0.170	0.135	0.80
1.0	0.116	0.234	2.0	0.143	0.222	1.56	0.170	0.187	1.09
1.5	—	—	—	0.145	0.308	2.16	—	—	—
2.0	0.110	0.395	3.6	0.134	0.336	2.47	0.164	0.258	1.57
2.5	—	—	—	0.127	0.476	3.48	—	—	—

Attempts to determine the effect of ionic strength by using tetraethylammonium bromide, which does not interact with the nucleotide, were unsuccessful, since this compound caused marked inhibition of creatine kinase.

The contribution to the inhibition that might be made by the Cl^- component of magnesium chloride cannot be determined with certainty. Although Nihei *et al.* (1961) have reported K_i 0.11–0.12 M for Cl^- as a competitive inhibitor of the enzyme with respect to phosphocreatine, it cannot be considered reliable as Cl^- was added as sodium chloride (cf. above). Further, when allowance is made for the presence of 29 mm- Cl^- in the *N*-ethylmorpholine buffer (Dawson, Elliott, Elliott & Jones, 1959), replots of the slopes and intercepts of Figs. 5 and 9 against the Cl^- concentration give negative values for the dissociation constant of an enzyme- Cl^- complex. Hence it was concluded that the active inhibitory ion of magnesium chloride is Mg^{2+} . However, with both sodium chloride and magnesium chloride the inhibition is a function of the concentration of phosphocreatine.

Although no single effect can explain the observed inhibition of creatine kinase by Mg^{2+} , it is possible that the combined effects could make some contribution to the inhibition.

Inhibition of creatine kinase by excess of ADP³⁻ with respect to MgADP⁻. The study of the inhibition of creatine kinase by excess of ADP³⁻ was facilitated by the fact that within the range of concentrations used, 0.125–2.5 mm, and with the MgADP⁻ concentration in the range 1.0–0.033 mm, the concentration of Mg^{2+} was negligible compared with its K_i and K_i values. (With ADP³⁻ at a concentration of 0.125 mm, the concentration of Mg^{2+} varied from 2.0 to 0.066 mm.) In addition, the changes in ionic strength were less than those with magnesium chloride as an inhibitor. Plots of $1/v$ against

Fig. 12. Determination of K_1 for ADP³⁻ from plots of slopes, obtained from Fig. 11, against ADP³⁻ concentration at different concentrations of phosphocreatine. The concentrations of phosphocreatine were: ●, 5 mm; ○, 10 mm; ■, 20 mm.

$1/[\text{MgADP}^-]$ at different concentrations of ADP³⁻ and with phosphocreatine at three fixed non-saturating concentrations (Fig. 11) show that ADP³⁻ inhibits the enzyme competitively with respect to MgADP⁻, as required by eqn. (11). Calculated apparent values for K_6 , apparent maximum velocities and slopes are given in Table 9. Secondary plots (Fig. 12) show that the slopes are a linear function of the concentration of ADP³⁻ and vary with the concentration of phosphocreatine.

The concentration of phosphocreatine does not appear to affect the dissociation constant of the enzyme-ADP complex (Table 10), and the values are in reasonable agreement with the value of 0.3 mm

Table 10. *Effect of the concentration of phosphocreatine on K_1 for ADP^{3-} and K_6 for MgADP^-*

The kinetic constants were calculated from the data of Fig. 11, and are expressed as mm.

Concn. of phosphocreatine (mm)	K_1	K_6
5.0	0.4	0.08
10.0	0.2	0.04
20.0	0.4	0.06

Fig. 13. Inhibition of creatine kinase by ADP^{3-} with respect to phosphocreatine, with MgADP^- at a fixed concentration of 0.2 mm. The reaction conditions were as described in Fig. 1, except for the indicated concentrations of ADP^{3-} and phosphocreatine. The concentrations of ADP^{3-} were: $\circ$, 2.0 mm; $\blacksquare$, 1.0 mm; $\star$, 0.5 mm; $\bullet$, 0.125 mm. Velocity is expressed as $\mu\text{moles of creatine/min.}/\mu\text{g. of enzyme}$.

that was calculated by Kuby & Noltmann (1962) from the data of Morrison *et al.* (1961) and with those given in Tables 2, 3 and 5. The latter values were obtained under conditions where the reaction of ADP^{3-} with the enzyme was considered as a step in the reaction pathway. The average K_6 value of 0.06 mm obtained in these experiments is less than that obtained under conditions of excess of Mg^{2+} but similar to those reported in Tables 2, 3 and 5.

The average values of the intercepts, for each concentration of phosphocreatine (Table 9), were plotted against $1/[\text{phosphocreatine}]$ to give a value of 3.6 mm for K_7 and a maximum velocity, with the enzyme saturated with substrates and with the ADP^{3-} at zero concentration, of 0.193 $\mu\text{mole of creatine formed/min.}/\mu\text{g. of creatine kinase}$.

The above results show that reaction occurs in the virtual absence of ADP^{3-} or Mg^{2+} , and thus confirm that MgADP^- functions as a substrate for creatine kinase.

Inhibition of creatine kinase by excess of ADP^{3-} with respect to phosphocreatine. The inhibition by ADP^{3-} with respect to phosphocreatine at a fixed concentration of MgADP^- is illustrated in Fig. 13. It is difficult to reach any firm conclusions about the mechanism of the inhibition for, in a series of five experiments, though consistent values could be obtained for the slopes of the lines, this was not so for the vertical intercepts. However, the variation in the latter values was relatively small. Further investigations of the effect of phosphocreatine on the inhibition by ADP^{3-} must await the development of a more sensitive method for the estimation of the creatine released from phosphocreatine.

Since the slopes of the lines were a linear function of the concentration of ADP^{3-} , it was possible to determine an apparent value of 0.65 mm for K_1 (eqn. 12). By taking into account the concentration of MgADP^- and using a value of 0.06 mm for K_6 , a true value for K_1 of 0.15 mm could be calculated.

DISCUSSION

Kinetic investigations that rely solely on the measurement of initial velocities in the absence of products suffer from certain disadvantages. Thus a true picture of the reaction mechanism can be obtained only when the steady-state concentrations of each of the enzyme species are kinetically significant. The above results, as well as those of other authors as summarized by Kuby & Noltmann (1962), indicate that the steady-state concentrations of the complexes of creatine kinase with ADP^{3-} and MgADP^- are sufficiently high to permit of their detection. But it seems that this is not so for an enzyme-Mg complex, which could participate in the formation of an enzyme-Mg-ADP-phosphocreatine complex, and thus it is difficult to conclude whether or not it is formed. Different methods of plotting the results can give rise to different interpretations. Though an infinite value for K_3 could be taken as indicating that the reactions:

do not lead to the formation of an enzyme-Mg-ADP complex, a high value does not necessarily lead to the same interpretation, for it could be compensated for by a correspondingly low value for K_4 if equilibrium conditions apply. Reaction of Mg^{2+} with creatine kinase to a small extent would agree with the results of Cohn & Leigh (1962) and Cohn (1963), whose nuclear-magnetic-resonance studies showed that there is at the most only a slight reaction of Mn^{2+} with this enzyme.

The non-competitive inhibition of the reaction by magnesium chloride has been attributed to Mg^{2+} and interpreted to indicate that Mg^{2+} does not react

at the nucleotide-binding site of the enzyme, but at some other site and in such a way that it is influenced by the concentration of phosphocreatine. The K_i value for Mg^{2+} of 10.5 mM is similar to the thermodynamic value reported by Kuby *et al.* (1962). In view of the above results, it is necessary to enquire further into the reasons why the general approach adopted by Morrison *et al.* (1961) does not give a less ambiguous answer to the problem of the interaction of Mg^{2+} with creatine kinase. One factor is certainly the low or negligible steady-state concentration of the enzyme-Mg complex, but the apparent concentration of this complex can be influenced by the magnitude of the apparent stability constant that is used in the calculations. Comparison of the present results with those of Morrison *et al.* (1961) shows that the higher the stability constant used, the higher is the value obtained for K_3 (cf. Kuby & Noltmann, 1962). Thus the finding of a finite rather than an infinite value for K_3 could be due to the use of a stability constant that was too low for the experimental conditions. Application of the general approach demands the use of the correct apparent stability constant for the metal ion-nucleotide complex. Since the need to use the correct value is of less importance when relatively high concentrations of the metal ion are present, it seems that there is an advantage in studying the inhibition by the metal ion in relation to the metal ion-nucleotide substrate as a means of determining if both species react at the same site on an enzyme.

The linear competitive inhibition by ADP^{3-} with respect to $MgADP^-$ indicates that these species do react at the same site on the enzyme and that the enzyme-ADP complex so formed is a 'dead-end' complex, which, under the experimental conditions, does not undergo further reaction with Mg^{2+} . If the hyperbolic inhibition by ADP^{3-} with respect to phosphocreatine at a non-saturating concentration of $MgADP^-$ is regarded as real, it may be concluded that, when the enzyme-ADP complex is present in high concentrations, it can undergo conversion into an active central complex and that the rate of the overall reaction is less than that involving $MgADP^-$. But the reproducibility of these results was not sufficiently good to permit a definite conclusion, and no direct method is available to determine if the enzyme-ADP complex can undergo further reaction with Mg^{2+} .

From a comparison of the values presented above for the kinetic constants for the enzyme-MgADP (K_6) and enzyme-ADP (K_1) complexes with the kinetic and thermodynamic values listed in Tables 3 and 7 of the article by Kuby & Noltmann (1962), it seems that these species are in rapid equilibrium with their respective reactants under the conditions used for their determination. The linear reaction kinetics are also consistent with the establishment

of rapid equilibrium at each of the steps or with the predominance of one reaction pathway. The finding that the value for K_6 is lower than that for K_1 indicates that the affinity of the enzyme for $MgADP^-$ is greater than that for ADP^{3-} . This difference is apparently not due to the binding of the Mg^{2+} moiety of $MgADP^-$ to the enzyme, since the work of Cohn (1963) and Cohn & Leigh (1962) does not support the idea of the interaction of the metal ion with the enzyme. However, there is a correlation between the degree of binding and the charge on the various nucleotide species that react with the enzyme (Kuby *et al.* 1962).

Although the reaction catalysed by creatine kinase has been studied extensively by a number of workers, it is not yet possible to reach definite conclusions about the relative importance of the reactions that could lead to the formation of a creatine kinase-Mg-ADP-phosphocreatine complex. However, such studies suggest that the reaction of the enzyme with $MgADP^-$ may constitute the main reaction pathway and that, if reaction does occur via an enzyme-ADP complex, it is of less importance. Since the formation of an enzyme-Mg complex must still be considered uncertain, it is perhaps unlikely that the pathway involving this intermediate is of importance.

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