BBA 66148

STUDIES ON THE INHIBITION OF ATP: CREATINE PHOSPHOTRANSFERASE BY NaCl

ELIZABETH HEYDE AND J. F. MORRISON

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. (Australia)

(Received April 21st, 1970)

SUMMARY

Kinetic experiments have been undertaken to determine the apparent inhibition constants for the reaction of Cl^- with various forms of creatine kinase. The conditions were similar to those previously used for initial-velocity and isotope-exchange studies of the reaction.

It has been shown that NaCl is a non-competitive inhibitor with respect to both substrates of the forward and reverse reactions. With the lower concentrations of the inhibitor used in studying the forward reaction, both the slopes and vertical intercepts of double-reciprocal plots appeared to be linear functions of the concentration of NaCl. On the other hand, with the higher concentrations of NaCl used to inhibit the reverse reaction, the non-competitive inhibition was of the slope-parabolic, intercept-linear type.

From the values obtained for the apparent inhibition constants, it appears that Cl^- may well react at sites on the enzyme at which substrate normally combines, and that the presence of a substrate on the enzyme affects the combination of Cl^-. Whereas the interaction of Cl^- with the ternary enzyme complex is very weak, two Cl^- can react with free enzyme.

The inhibition of the reaction by sodium acetate is considerably less than that by NaCl.

INTRODUCTION

Isotope exchange studies with ATP: creatine phosphotransferase (EC 2.7.3.2; creatine kinase) have shown that there is inhibition of the initial exchange rate at higher concentrations of the $MgATP^{2-}/MgADP^-$ pair of reactants[1]. This result was not in accord with kinetic data[2] which indicated that the reaction mechanism for this enzyme was of the rapid equilibrium, random type[3]. Because the Mg–nucleotide complexes were formed as a result of the non-enzymic reaction between $MgCl_2$ and the sodium salts of ATP and ADP, it appeared possible that the inhibition might be accounted for by the concomitant increase in the concentration of NaCl. This was especially so since it had been found by NODA *et al.*[4] and NIHEI *et al.*[5] that NaCl does

inhibit the reaction. These authors reported that NaCl is a non-competitive inhibitor with respect to $MgADP^-$ and a competitive inhibitor in relation to $MgATP^{2-}$ and phosphocreatine. They also reported values for the inhibition constants, but as these values were for complex constants and no allowance had been made for the complexing between Na^+ and the nucleotides, they could not be used in connection with the testing of the above hypothesis.

This paper reports the results of kinetic experiments which were undertaken to determine apparent inhibition constants* for the reaction of Cl^- with various forms of enzyme under conditions similar to those used for initial velocity and isotope exchange studies[1,2]. It has been found that NaCl is a non-competitive inhibitor of the reaction with respect to all four substrates, and from the results, it has been concluded that Cl^- combines more strongly with the binary enzyme forms than it does with the ternary enzyme complexes and in addition, can react twice with the free form of enzyme.

THEORY

The experimental conditions were similar to those used previously in that the concentration of free Mg^{2+} was held constant and reactions were carried out at pH 8.0. Thus the reaction could be considered to be bireactant *viz.*

$$MgATP^{2-} + \text{creatine}^0 \rightleftharpoons MgADP^- + \text{phosphocreatine}^{2-}$$

and to have a rapid equilibrium, random mechanism[2]. The initial velocity equations for the forward and reverse reactions can be expressed, respectively, as

$$v = \frac{V_1 PQ}{K_{ip}K_q + K_p Q + K_q P + PQ} \tag{1a}$$

$$v = \frac{V_2 AB}{K_{ia}K_b + K_a B + K_b A + AB} \tag{1b}$$

where A, B, P and Q represent the concentrations of $MgADP^-$, phosphocreatine, creatine and $MgATP^{2-}$, respectively; K_{ia} and K_{ip} are dissociation constants for the reaction of A and P with free enzyme; K_a, K_b, K_p and K_q are Michaelis constants (which are also dissociation constants for a rapid equilibrium, random mechanism) for the reaction of A, B, P and Q with EB, EA, EQ and EP, respectively. Because of the reaction mechanism, it also follows that $K_{ia}K_b = K_a K_{ib}$ and $K_{ip}K_q = K_p K_{iq}$ where K_{ib} and K_{iq} are dissociation constants for the reaction of B and Q with free enzyme.

A wider range of concentration of NaCl was used in studies of the reverse reaction than in investigations of the inhibition of the forward reaction. Since the slope replots appear parabolic (see RESULTS), it is necessary to consider the reaction of 2 moles of Cl^- with the free form of the enzyme, in addition to the reaction of

* Apparent inhibition constants are those which were calculated from data obtained in the presence of 0.1 triethanolamine–HCl buffer (pH 8.0) by neglecting the presence of 40 mM Cl^-.

1 mole with each of the other forms of enzyme. When this is done, the velocity Eqn. 1b becomes

$$v = \frac{V_2 AB}{K_{ia}K_b\left(1 + \frac{[Cl^-]}{K_1} + \frac{[Cl^-]^2}{K_1K_1'}\right) + K_aB\left(1 + \frac{[Cl^-]}{K_2}\right) + K_bA\left(1 + \frac{[Cl^-]}{K_3}\right) + AB\left(1 + \frac{[Cl^-]}{K_4}\right)} \quad (2)$$

where K_1–K_4 represent dissociation constants for the reaction of Cl^- with E, EB, EA and EAB, respectively. K_1' represents the dissociation constant of the reaction:

$$\text{Enzyme–Cl} + Cl^- \rightleftharpoons \text{Enzyme–Cl}_2$$

Rearrangement of Eqn. 2 in reciprocal form with $MgADP^-$ (A) and phosphocreatine (B) as the variable substrates gives Eqns. 3 and 4, respectively.

$$\frac{1}{v} = \frac{K_a}{V_2}\left(1 + \frac{K_{ib}}{B}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_{ib} + B}{\frac{K_{ib}}{K_1} + \frac{B}{K_2}}\right]} + \frac{[Cl^-]^2}{K_1K_1'\left(1 + \frac{B}{K_{ib}}\right)}\right\}\frac{1}{A} + \frac{1}{V_2}\left(1 + \frac{K_b}{B}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_b + B}{\frac{K_b}{K_3} + \frac{B}{K_4}}\right]}\right\} \quad (3)$$

$$\frac{1}{v} = \frac{K_b}{V_2}\left(1 + \frac{K_{ia}}{A}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_{ia} + A}{\frac{K_{ia}}{K_1} + \frac{A}{K_3}}\right]} + \frac{[Cl^-]^2}{K_1K_1'\left(1 + \frac{A}{K_{ia}}\right)}\right\}\frac{1}{B} + \frac{1}{V_2}\left(1 + \frac{K_a}{A}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_a + A}{\frac{K_a}{K_2} + \frac{A}{K_4}}\right]}\right\} \quad (4)$$

These equations predict that the inhibition by NaCl would be non-competitive with respect to both $MgADP^-$ and phosphocreatine, with the slopes of the lines varying as a parabolic function and the vertical intercepts varying as a linear function of the NaCl concentration.

By substitution of the appropriate values for K_b, K_{ib} and B or K_a, K_{ia} and A into the expressions for the apparent kinetic constants associated with $[Cl^-]$ in Eqns. 3 or 4, respectively, and the use of the values for the apparent kinetic constants, there would be obtained four simultaneous equations in four unknowns. Thus it would be expected that the equations could be solved to obtain values for K_1, K_2, K_3, and K_4. However, the four equations are not in fact independent, because of the rapid equilibrium random mechanism which makes $K_{ia}K_b = K_aK_{ib}$, and therefore such a calculation is precluded. The method used to determine values for K_1–K_4 was to study the inhibition by NaCl with respect to $MgADP^-$ (A) at two different

fixed concentrations of phosphocreatine so as to obtain two values for each of the apparent kinetic constants associated with $[Cl^-]$ in the slope and intercept terms of Eqn. 3. After substitution of the appropriate values for K_{ib} and B in the slope expression (or K_b and B in the intercept expression), the set of two simultaneous equations was solved for K_1 and K_2 (or K_3 and K_4). K_1' was determined by substitution into the $[Cl^-]^2$ terms of Eqns. 3 and 4 for the appropriate values of the other constants.

A narrower range of concentration of NaCl was used for investigations of the forward reaction. Since the slope and intercept replots appeared linear (see RESULTS), it was assumed that under these conditions only 1 mole of Cl^- reacts with the free enzyme (K_1) as well as with each of the other forms of enzyme. Eqn. 1a then becomes

$$v = \frac{V_1 PQ}{K_p K_{iq}\left(1 + \frac{[Cl^-]}{K_1}\right) + K_p Q\left(1 + \frac{[Cl^-]}{K_5}\right) + K_q P\left(1 + \frac{[Cl^-]}{K_6}\right) + PQ\left(1 + \frac{[Cl^-]}{K_7}\right)} \quad (5)$$

where K_5, K_6 and K_7 are dissociation constants for the interaction of Cl^- with EQ, EP and EPQ, respectively. Rearrangement of Eqn. 5 in reciprocal form gives

$$\frac{1}{v} = \frac{K_q}{V_1}\left(1 + \frac{K_{ip}}{P}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_{ip} + P}{\frac{K_{ip}}{K_1} + \frac{P}{K_6}}\right]}\right\}\frac{1}{Q} + \frac{1}{V_1}\left(1 + \frac{K_p}{P}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_p + P}{\frac{K_p}{K_5} + \frac{P}{K_7}}\right]}\right\} \quad (6)$$

when $MgATP^{2-}$ (Q) is the variable substrate, and

$$\frac{1}{v} = \frac{K_p}{V_1}\left(1 + \frac{K_{iq}}{Q}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_{iq} + Q}{\frac{K_{iq}}{K_1} + \frac{Q}{K_5}}\right]}\right\}\frac{1}{P} + \frac{1}{V_1}\left(1 + \frac{K_q}{Q}\right)\left\{1 + \frac{[Cl^-]}{\left[\frac{K_q + Q}{\frac{K_q}{K_6} + \frac{Q}{K_7}}\right]}\right\} \quad (7)$$

when creatine (P) is varied. Secondary plots of slopes and vertical intercepts would be linear and yield apparent values for the inhibition constants associated with $[Cl^-]$.

By substitution of the appropriate values for K_{ip}, K_p, K_{iq} and K_q, as well as the concentrations of P and Q, four simultaneous equations in K_1, K_5, K_6 and K_7 are obtained. Again the equations are not independent, because of the relationship $K_{ip}K_q = K_{iq}K_p$, and therefore cannot be solved simultaneously. However, K_5, K_6 and K_7 can be detremined by using the value for K_1 obtained from studies of the reverse recation.

MATERIALS AND METHODS

Materials

These were as previously described[2], except that NaCl and sodium acetate were purchased from British Drug Houses.

Methods

Reaction mixtures contained, in a total volume of 1.0 ml, 0.1 M triethanol-

amine–HCl or triethanolamine–acetate buffer (pH 8.0), 0.01 mM EDTA; substrates (and NaCl) at the concentrations indicated in the figures and sufficient $MgCl_2$ to give the required concentrations of $MgATP^{2-}$ or $MgADP^{-}$ while maintaining the concentration of free Mg^{2+} constant at 1.0 mM. Allowance was made for the ability of phosphocreatine to complex with Mg^{2+} and for the reaction of Na^{+} with the adenine nucleotides (see below). The amount of creatine kinase added corresponded to 1.08 and 0.54 μg of protein for the reactions in the forward and reverse directions, respectively. The temperature was 30°. Reactions were stopped and determinations of the release of ADP from ATP or creatine from phosphocreatine were made as described by MORRISON AND JAMES[2]. Neither NaCl nor sodium acetate was found to interfere with the estimations of ADP and creatine. Each reaction was run for at least two time periods (between 0.5 and 2.5 min) to ensure that initial velocities were being measured.

Calculation of substrate concentrations

The general procedure was similar to that outlined by MORRISON *et al.*[6], but because the reactions involved in this study were of greater complexity than those of ref. 6, further elaboration of the calculations are given. The most complex system was that concerning the inhibition of the reverse reaction by sodium acetate when it was necessary to take into account the following reactions:

(a) Mg^{2+} + phosphocreatine^{2-} $\rightleftharpoons$ Mg–phosphocreatine
(b) Mg^{2+} + acetate $\rightleftharpoons$ Mg–acetate^{+}
(c) Mg^{2+} + ADP^{3-} $\rightleftharpoons$ $MgADP^{-}$
(d) Na^{+} + ADP^{3-} $\rightleftharpoons$ $NaADP^{2-}$

It was assumed that the Mg–acetate^{+}, Mg–phosphocreatine and $NaADP^{2-}$ complexes were inert and the total concentrations of reactants which were necessary to give fixed concentrations of $MgADP^{-}$ and phosphocreatine in the presence of sodium acetate while maintaining free Mg^{2+} constant were calculated as described below.

The total concentration of phosphocreatine, $[PC]_t$ required to give a particular concentration of free phosphocreatine, [free PC], at a fixed concentration of free Mg^{2+} was determined from the relationships

$$[PC]_t = [\text{free PC}] + [Mg^{2+}]_{PC} \tag{8}$$

$$[Mg^{2+}]_{PC} = \frac{[Mg^{2+}]\,[\text{free PC}]}{K_{MgPC}} \tag{9}$$

where $[Mg^{2+}]_{PC}$ represents the amount of phosphocreatine (or Mg^{2+}) present as a Mg–phosphocreatine complex and K_{MgPC} represents the dissociation constant for this complex which was taken to be 25 mM (ref. 7). The amount of Mg^{2+} bound in the Mg–acetate^{+} complex, $[Mg^{2+}]_{Ac}$, at a fixed concentration of free Mg^{2+} was calculated from the relationship

$$[Mg^{2+}]_{Ac} = \frac{[Mg^{2+}]\,[\text{acetate}]}{K_{MgAc}} \tag{10}$$

where K_{MgAc}, the dissociation constant for the Mg–acetate^{+} complex, was taken as being 313 mM (ref. 8). Calculation showed that there was only an insignificant reduction (less than 1%) in the acetate concentration with the relatively high

concentrations of sodium acetate used. Thus the free acetate concentration was considered to be equal to the concentration of sodium acetate added.

Because of the reaction of ADP^{3-} with the Na^+ ion of sodium acetate, the amount available for reaction with Mg^{2+} to form $MgADP^-$ is reduced. Allowance was made for this effect by using an apparent dissociation constant* for $MgADP^-$ which was determined from the relationship

$$\text{Apparent } K_{MgADP^-} = K_{MgADP^-}\left(1 + \frac{[Na]}{K_{NaADP^-}}\right) \quad (11)$$

The resulting value was then used to calculate the total concentration of ADP' $[ADP]_t$, to give the required concentration of $MgADP^-$ at a fixed concentration of free Mg^{2+} (Eqn. 12)

$$[ADP]_t = [MgADP^-]\left(1 + \frac{\text{Apparent } K_{MgADP^-}}{[Mg^{2+}]}\right) \quad (12)$$

Dissociation constants for the $NaADP^{2-}$ and $MgADP^-$ complexes were taken to be 167 and 0.25 mM, respectively. While the above procedure allows for the reduction in the nucleotide concentration as a result of the formation of an Na–nucleotide complex, it does not allow for the reduction in the Na^+ concentration. However, this was negligible compared with the concentration of sodium acetate used in the inhibition studies.

The total concentration of $MgCl_2$, $[Mg^{2+}]_t$, which was necessary to give the required concentrations of free phosphocreatine and $MgADP^-$ at a fixed concentration of free Mg^{2+} was calculated using the relationship

$$[Mg^{2+}]_t = [Mg^{2+}]_{PC} + [MgADP^-] + [Mg^{2+}]_{Ac} + [Mg^{2+}] \quad (13)$$

Similar calculations were made when NaCl was used as an inhibitor except that any complexing between Mg^{2+} and Cl^- was considered to be negligible[8]. Further, the amount of NaCl formed as a result of the formation of an Mg–nucleotide complex from $MgCl_2$ and the sodium salt of the nucleotide was neglected. This was small in relation to the concentrations of NaCl that were used. Calculations relating to the forward reaction were as described above, except that Mg^{2+} was considered not to complex with creatine. The values used for the dissociation constants of the $NaATP^{3-}$ and $MgATP^{2-}$ complexes were 167 and 0.014 mM, respectively[7].

Analysis of data

The velocity data were plotted graphically in double-reciprocal form to check the linearity of the lines and to determine the pattern of the secondary plots. Analysis was then made by means of the appropriate computer programme of CLELAND[9], in conjunction with an IBM 360 computer, to obtain values for the apparent kinetic constants and their standard errors. Data conforming to linear non-competitive inhibition and slope-parabolic, intercept-linear non-competitive inhibition were fitted

* All the stability and dissociation constants used should be regarded strictly as being apparent.

to Eqns. 14 and 15, respectively. The constants determined from overall analysis of data were used to draw the lines of the figures.

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{iS}}\right) + A\left(1 + \frac{I}{K_{il}}\right)} \tag{14}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{iSA}} + \frac{I^2}{K_{iSB}}\right) + A\left(1 + \frac{I}{K_{il}}\right)} \tag{15}$$

RESULTS

Inhibition of the reverse reaction by NaCl

The non-competitive inhibition of the reverse reaction by NaCl with respect to both $MgADP^-$ and creatine, in the presence of triethanolamine–HCl buffer, is illustrated in Fig. 1. Secondary plots of the slopes of Figs. 1a and 1b against the concentration of NaCl were parabolic, while similar replots of the vertical intercepts were linear. Both sets of data gave good fits to Eqn. 15 and thus the inhibition can

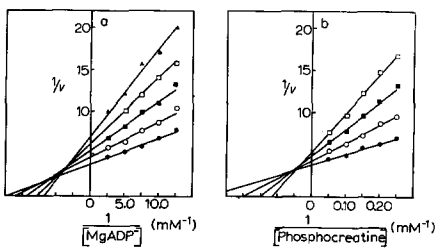

Fig. 1. Inhibition of the reverse reaction by NaCl at pH 8.0 in the presence of triethanolamine–HCl buffer with (a) $MgADP^-$ as the variable substrate and phosphocreatine held constant at 10 mM and (b) phosphocreatine as the variable substrate and $MgADP^-$ held constant at 0.4 mM. The lines were drawn using constants obtained by fitting the data to Eqn. 15. The concentrations of NaCl added were: ●, none; ○, 40 mM; ■, 80 mM; □, 120 mM; ▲, 160 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.

be described as slope-parabolic, intercept-linear non-competitive. With plots of this type the family of straight lines do not have a common point of intersection to the left of the ordinate[10]. The values of the apparent inhibition constants obtained by analysis of the data of Fig. 1, and similar data obtained with $MgADP^-$ as variable substrate and the phosphocreatine concentration fixed at 5 mM, are given in Table I.

Using the calculations outlined in THEORY, values of 86 ± 73 mM for K_1, 1,900 ± 63,000 mM for K_2, 44 ± 15 mM for K_3 and 880 ± 1860 mM for K_4 were obtained. The standard errors are high, because of the nature of the calculations. The value of K_2 indicates that the formation of an enzyme–phosphocreatine–chloride complex is not significant, and therefore the dissociation constants were recalculated after setting $K_2 = \infty$ in the various equations. A value for K_1 comes from the linear term in the slope expression of Eqn. 3, and can be used to calculate K_3 from the linear term in the slope expression of Eqn. 4. K_4 can be calculated from the intercept

TABLE I

INHIBITION CONSTANTS FOR THE REACTION OF Cl^- WITH CREATINE KINASE IN THE REVERSE REACTION

The values recorded are the weighted mean values from two to four experiments, and were obtained by fitting data, including that of Fig. 1, to Eqn. 15 using the S PARA NONCOMP computer programme. All values are expressed as mM and are apparent in that the presence of 40 mM Cl^- in the triethanolamine–HCl buffer was neglected.

Inhibition constant	*Variable substrate*		
	MgADP⁻		*Phosphocreatine MgADP⁻ (0.4 mM)*
	Phosphocreatine (5 mM)	*Phosphocreatine (10 mM)*	
K_i (slope)			
$[Cl^-]$ term	132 ± 19	176 ± 39	63 ± 8
$[Cl^-]^2$ term	17 000 ± 2 000	16 000 ± 2 000	15 000 ± 3 000
K_i (intercept)	111 ± 5	167 ± 10	302 ± 34

term of Eqn. 4. The values so obtained are listed in Table III. When the values for K_3 and K_4 are substituted into the intercept term of Eqn. 3, values of 146 ± 19 and 114 ± 18 mM are obtained at phosphocreatine concentrations of 10 and 5 mM, respectively. These values are in good agreement with the experimental results in Table I. Values for $K_{1'}$, the dissociation constant for the interaction of Cl^- with the enzyme–chloride complex, were calculated to be 134 ± 35 and 320 ± 83 mM with $MgADP^-$ varied and phosphocreatine at 5 and 10 mM, respectively, and 108 ± 32 mM with phosphocreatine as the varied substrate.

The data in Fig. 2 were obtained varying the concentrations of $MgADP^-$ and phosphocreatine in constant ratio. The variation of the intercept on the ordinate with the concentration of NaCl indicates that Cl^- can combine with the enzyme–MgADP–phosphocreatine complex, and hence that the dissociation constant K_4 has physical significance.

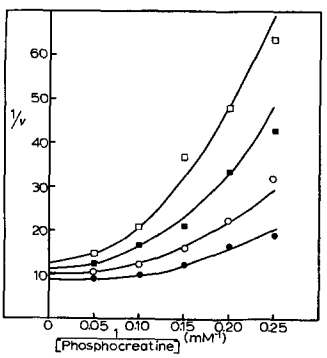

Fig. 2. Inhibition of the reverse reaction by NaCl at pH 8.0 in the presence of triethanolamine–HCl buffer with the ratio of the concentrations of phosphocreatine to $MgADP^-$ held constant at 50, and the velocity data plotted with respect to the phosphocreatine concentration. The lines were drawn using the fit of the data to the equation $y = VX^2/(B + CI + DI^2 + EX + GXI + X^2 + RX^2I)$ by means of a computer programme written by Dr. W. W. Cleland. The concentrations of NaCl added were: ●, none; ○, 50 mM; ■, 100 mM; □, 150 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.

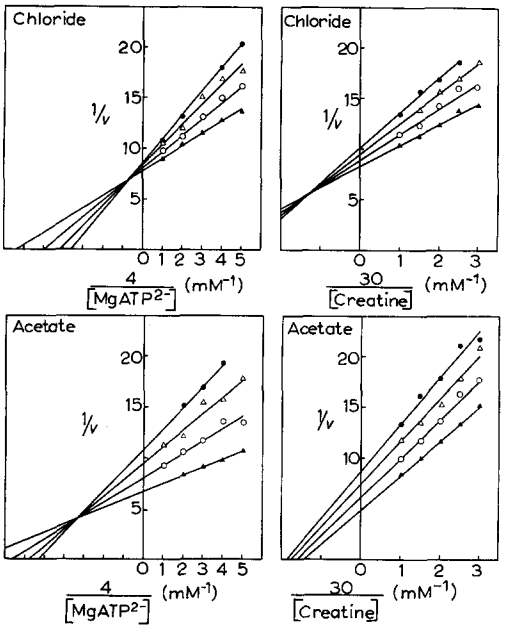

Fig. 3. Inhibition of the forward reaction by NaCl at pH 8.0 in the presence of triethanolamine–HCl or triethanolamine–acetate buffer, with creatine held constant at 30 mM when $MgATP^{2-}$ is the variable substrate, and $MgATP^{2-}$ held constant at 2 mM when creatine is varied. The lines were drawn using constants obtained by fitting the data to Eqn. 14. The concentrations of NaCl added were: ▲, none; ○, 30 mM; △, 60 mM; ●, 90 mM. v is expressed as μmoles of ADP per μg of creatine kinase per min.

Inhibition of the forward reaction by NaCl

The inhibition of the forward reaction by NaCl, in the presence of triethanolamine–HCl buffer, was also non-competitive with respect to both $MgATP^{2-}$ and creatine, and similar results were obtained when triethanolamine–acetate was used as the buffer (Fig. 3). In contrast to the results shown in Fig. 1, secondary plots of the slopes from Fig. 3 were linear. Similar replots of vertical intercepts were also linear. Thus the results are in accord with Eqns. 6 and 7. The apparent inhibition

TABLE II

INHIBITION CONSTANTS FOR THE REACTION OF Cl^- WITH CREATINE KINASE IN THE FORWARD REACTION

The values recorded are the weighted mean values from two or three experiments, and were obtained by fitting data, including those of Fig. 3, to Eqn. 14 using the NONCOMP computer programme. All constants are expressed as mM, and the values obtained in triethanolamine–HCl buffer are apparent in that the presence of 40 mM Cl^- in this buffer was neglected.

Buffer anion	*Cl⁻*		*Acetate*	
Variable substrate	*$MgATP^{2-}$*	*Creatine*	*$MgATP^{2-}$*	*Creatine*
K_i (slope)	102 ± 10	137 ± 23	55 ± 8	222 ± 44
K_i (intercept)	339 ± 43	329 ± 51	188 ± 24	125 ± 15

TABLE III

APPARENT DISSOCIATION CONSTANTS FOR THE REACTION OF Cl^- WITH VARIOUS FORMS OF CREATINE KINASE

The dissociation constants were calculated as outlined in THEORY, after setting $K_2 = \infty$, using the inhibition constants of Tables I and II. The following values were used for kinetic constants: 0.05 ± 0.01, 0.17 ± 0.02, 2.9 ± 0.3 and 8.6 ± 1.3 mM for K_a, K_{ia}, K_b and K_{ib}, respectively; and 6.1 ± 1.0, 15.6 ± 4.9, 0.48 ± 0.10 and 1.2 ± 0.3 mM for K_p, K_{ip}, K_q and K_{iq}, respectively[2]. All values are apparent in that the presence of 40 mM Cl^- in the triethanolamine–HCl buffer was neglected.

Reaction of Cl^- with	*Dissociation constant*	*Dissociation constant (mM)*
Free enzyme	K_1	82 ± 12
Enzyme–phosphocreatine	K_2	∞
Enzyme–MgADP	K_3	57 ± 10
Enzyme–MgADP–phosphocreatine	K_4	268 ± 31
Enzyme–MgATP	K_5	229 ± 162
Enzyme–creatine	K_6	117 ± 42
Enzyme–MgATP–creatine	K_7	581 ± 352

constants obtained directly from analysis of the data of Fig. 3 are recorded in Table II. Table III lists the values calculated for the dissociation constants for the interaction of Cl^- with the various enzyme forms.

Inhibition by sodium acetate

Sodium acetate also caused non-competitive inhibition of the reverse reaction with respect to $MgADP^-$ (Fig. 4). This inhibition is considerably less than that given by similar concentrations of NaCl (Fig. 1a). It was also found that the reaction velocity with $MgADP^-$ as the variable substrate was only slightly less in 0.2 M than in 0.1 M triethanolamine–acetate buffer. Moreover, the addition of up to 90 mM sodium acetate did not cause significant inhibition of the forward reaction when carried out in triethanolamine–acetate buffer. Thus it has been concluded that the inhibition by NaCl is primarily due to Cl^-.

While the inhibition constants obtained using triethanolamine–acetate buffer can be regarded as close to the true values, those obtained using triethanolamine–HCl

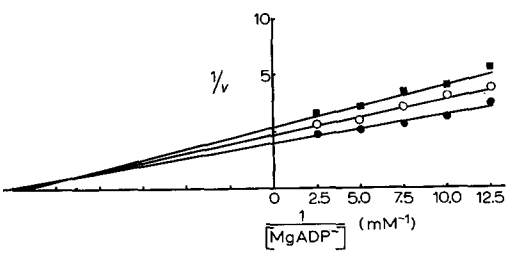

Fig. 4. Inhibition of the reverse reaction by sodium acetate at pH 8.0 in the presence of triethanolamine–HCl buffer with $MgADP^-$ as the variable substrate and phosphocreatine held constant at 10 mM. The lines were drawn using constants obtained by fitting the data to Eqn. 14. The concentrations of sodium acetate added were: ●, none; ○, 80 mM; ■, 160 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.

buffer (and neglecting the presence of 40 mM Cl^- in this buffer) must be considered apparent constants. Nevertheless, it is the latter values which are of importance when considering the effects that additions of Cl^-, above that present in the triethanolamine–HCl buffer, would have on the reaction (*cf.* ref. 1).

DISCUSSION

From a comparison of the effect of NaCl and sodium acetate on the reaction catalysed by creatine kinase, it is apparent that the chloride salt is far more inhibitory and this result is in accord with the findings of other authors[4,5]. Furthermore, it would appear that the small inhibition obtained with sodium acetate might well be due to the acetate ion, rather than to Na^+, since this salt and triethanolamine–acetate buffer are capable of inhibiting to about the same extent. This conclusion is confirmed by the results of NIHEI *et al.*[5], who reported that the apparent K_i for acetate was 350–400 mM. Thus the inhibition by NaCl has been attributed to the Cl^- and the calculations of the values for the various inhibition constants have been made on this basis.

The dissociation constants listed in Table III have rather high standard errors. This is due to the manner in which the constants must be derived; a division into a relatively large numerator by a denominator which is the difference between two similar values is often involved.

The non-competitive inhibition by NaCl with respect to each of the four reactants is consistent with the reaction of Cl^- at a site on the enzyme which is distinct from the active site. However, Cl^- resembles the substrates in being negatively charged, and may well react at sites on the enzyme at which the substrates combine. It is plausible that, for example, K_1 is genuinely different from K_3 (Table I), and hence that Cl^- reacts more readily with enzyme–$MgADP^-$ than with free enzyme. Such an effect resembles the enhancement by one substrate of the binding of the second substrate for the reaction in the same direction[2]. Moreover, Cl^- apparently reacts to a very small but significant extent with the ternary enzyme complexes (*cf.* Fig. 2), and yet seems to combine twice with the free enzyme when the Cl^- concentration is high. At lower Cl^- concentrations, such as those used in studying the forward reaction, the second combination of Cl^- with the free enzyme is not obvious; moreover, values obtained for K_1, K_2, K_3 and K_4 from analysis of data within the range of Cl^- concentration used in the forward reaction are similar to the values from analysis of the whole range of data.

It therefore appears likely that Cl^- in some respects can function as a substrate analogue, combining at the substrate sites and affecting the combination of other negatively charged molecules. At lower concentrations of Cl^- and non-saturating concentrations of the fixed substrate, the inhibition would be non-competitive (Eqns. 3, 4, 6 and 7). However, as the concentration of the fixed substrate tends to infinity, the inhibition could be almost competitive, irrespective of which substrate is varied, because of the relatively high values for K_4 and K_7. Thus the type of inhibition plot obtained could depend on the concentration of the fixed substrate. This, together with the fact that NODA *et al.*[4] and NIHEI *et al.*[5] did not make allowance for the reduction of nucleotide concentration in the presence of high Na^+ concentrations (*cf.* ref. 11), may account for their reports that NaCl was a competitive

inhibitor with respect to both $MgATP^{2-}$ and phosphocreatine. In any event, no valid comparison can be made between the estimates of the specific dissociation constants obtained in the present work and the apparent, complex K_i values quoted by the above authors.

In determining the inhibition constants reported here, it has been assumed that changes in ionic strength do not significantly affect the stability constants which have been used. There appears to be some justification for this assumption in the finding that relatively high concentrations of sodium acetate have little effect on the velocity of the reverse reaction.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. W. Cleland of The University of Wisconsin for his helpful comments, and to Mrs. M. Labutis for skilled technical assistance. E. H. (James) is the holder of a General Motors-Holden's Postgraduate Research Fellowship. The work was supported in part by Grant Tw-98-02 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 J. F. MORRISON AND W. W. CLELAND, *J. Biol. Chem.*, 241 (1966) 673.
2 J. F. MORRISON AND E. JAMES, *Biochem. J.*, 97 (1965) 37.
3 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
4 L. NODA, T. NIHEI AND M. F. MORALES, *J. Biol. Chem.*, 235 (1960) 2830.
5 T. NIHEI, L. NODA AND M. F. MORALES, *J. Biol. Chem.*, 236 (1961) 3203.
6 J. F. MORRISON, W. J. O'SULLIVAN AND A. G. OGSTON, *Biochim. Biophys. Acta*, 52 (1961) 82.
7 W. J. O'SULLIVAN AND D. D. PERRIN, *Biochemistry*, 3 (1964) 18.
8 R. K. CANNAN AND A. KIBRICK, *J. Am. Chem. Soc.*, 60 (1938) 2314.
9 W. W. CLELAND, *Nature*, 198 (1963) 463.
10 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 173.
11 S. A. KUBY AND E. NOLTMANN, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 6, Academic Press, New York, 2nd. ed., 1962, p. 515.