The Effect of Magnesium on the Reactivity of the Essential Sulfhydryl Groups in Creatine Kinase–Substrate Complexes*

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ABSTRACT: The divalent metal ion, Mg^{2+}, required for the enzymatic activity of creatine kinase has been shown to have a marked effect on the reactivity of the essential sulfhydryl groups of enzyme–substrate complexes toward alkylating agents. The metal ion alone has a negligible effect; the free nucleotide, ADP^{3-}, protected the enzyme against inactivation by iodoacetic acid, but the metal complex, $MgADP^-$, increased the rate of inactivation. Slight protection was seen with creatine alone and moderate protection with ADP^{3-} and creatine. With $MgADP^-$ and creatine, the data indicated complete protection against inactivation at saturating concentrations of both substrates. Unlike the reaction with iodoacetic acid, the reaction between iodoacetamide and creatine kinase was not affected by ADP^{3-}, $MgADP^-$, or creatine alone. However, as in the iodoacetic acid reaction, both of the combinations, ADP^{3-} plus creatine and $MgADP^-$ plus creatine, afforded considerable protection against inactivation by iodoacetamide. Again, only with $MgADP^-$ and creatine was complete protection indicated with saturating concentrations of both substrates. The results with the two alkylating agents have been interpreted to indicate different conformations for native creatine kinase, the ADP–enzyme complex, and the MgADP–enzyme complex; the dissociation constants of creatine for the three species were 50, 30, and 10 mM, respectively, as determined from the rate of inactivation as a function of creatine concentration for each species. It was concluded that creatine was bound in a different manner to the different forms of the enzyme and, in fact, was bound to the essential sulfhydryl group only in the case of the MgADP–enzyme complex since only in this quaternary complex are the SH groups completely protected against inactivation by both alkylating reagents.

Creatine kinase (ATP[1]:creatine phosphotransferase; EC 2.7.3.2)[1] is one of a group of phosphotransferases for which the bulk of the experimental evidence leads to the conclusion that the metal–nucleotide complex is the active substrate species (Kuby and Noltmann, 1962; Morrison and O'Sullivan, 1965) and the essential metal ion, for the enzymatic reaction, is bound only to the nucleotide in the ternary metal–nucleotide–enzyme complex (Cohn and Leigh, 1962; O'Sullivan and Cohn, 1966a). Since thermodynamic and kinetic studies have established that the association constants of the enzyme with the metal nucleotide and with the free nucleotide are of the same order of magnitude (Kuby and Noltmann, 1962) and kinetic studies have definitely established that the two compete for the same site on the enzyme (Morrison and O'Sullivan, 1965), the question arises as to what function the metal ion serves in the enzymatic reaction that accounts for the all-or-none effect on activity.

The only experimentally observed difference in the behavior of the metal–ADP–enzyme complex as compared to the ADP–enzyme complex has been reported by Watts and Rabin (1962), who found that the reactive sulfhydryl groups of creatine kinase could be protected against reaction with iodoacetic acid by ADP alone but ADP in the presence of magnesium chloride enhanced the rate of the reaction. Conflicting results on the same reaction were reported by Mahowald *et al.* (1962), who studied the kinetics of the inactivation of creatine kinase by iodoacetic acid in the presence of different substrates and inhibitors. Mahowald *et al.* (1962) concluded that ADP (and also ATP), both in the presence and absence of $MgCl_2$, protected the enzyme against attack by the alkylating agent. Since it might well be pertinent to the role of the metal ion in the enzymatic reaction, a comparison of the effect of ADP^{3-} and $MgADP^-$ on the iodoacetic acid reaction under different conditions has been reinvestigated in detail and is reported in this paper. The effect of other nucleotides and their metal complexes on the same reaction has been reported (O'Sullivan and Cohn, 1966b).

In addition, it appeared plausible that a differential effect of creatine on the SH reactivity of the enzyme

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; d(2′)ADP, deoxyadenosine 2′-diphosphate; IDP, inosine diphosphate; GDP, guanosine diphosphate; AMP, adenosine monophosphate; PP, and PPP, pyro- and tripolyphosphate. The designations E, enzyme, X, enzyme modifier, EX, enzyme–modifier complex, and the subscript T to denote total, have also been used.

toward carboxymethylating reagents might be detected in the metal–ADP–enzyme complex in contrast to the free nucleotide–enzyme complex. Previous investigators have all shown that creatine alone has little effect on the SH reactivity of creatine kinase (Watts and Rabin, 1962; Mahowald *et al.*, 1962; Lui and Cunningham, 1966). Similarly, previous work in this laboratory (O'Sullivan and Cohn, 1966a) had shown that creatine alone has no effect on the proton relaxation rate of water with manganese and enzyme. However, in the presence of MnADP and enzyme, addition of creatine lowers the proton relaxation rate of water. Furthermore, with the inactivated enzyme in which the two essential SH groups had stoichiometrically reacted with 2 moles of iodoacetic acid, the proton relaxation rate of water in the Mn–ADP–enzyme complex remained unchanged, but the creatine effect thereon had disappeared.[2] These observations led us to the conclusion that the SH groups are involved in the binding of creatine in the active enzyme, but the expression of this involvement is not observable with creatine alone and is observable in the presence of metal–ADP. Experimental confirmation of this conclusion is afforded by the recent finding of Lui and Cunningham (1966), who reported considerable protection against iodoacetamide by creatine in the presence of $MgADP^-$ but not in the absence of $MgADP^-$. In the investigation reported here, a comparison has been made of the effect of creatine on the SH reactivity of the enzyme toward iodoacetic acid and the uncharged reagent, iodoacetamide, in the case of the MgADP–enzyme complex, the ADP–enzyme complex, and the free enzyme in an attempt to assess the role of the metal ion.

Experimental Section

Materials. Iodoacetamide was obtained from Sigma Chemical Co. and recrystallized from hot water. Other reagents were as described in previous papers (O'Sullivan and Cohn, 1966a,b). The creatine kinase used had a specific activity of 60–65 Kuby units (Kuby *et al.*, 1954).

Inactivation of Creatine Kinase by Iodoacetic Acid and by Iodoacetamide. Except where stated otherwise, the reaction between iodoacetic acid (0.1 mM) and creatine kinase (1.37 μM) was carried out in a total volume of 2.0 ml in 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, at 25°. Samples (0.1 ml) were taken at zero time and at other time intervals as required and diluted to 0.4 ml in 10 mM cysteine, 5 mM *N*-ethylmorpholine–HCl, pH 8.0. Portions (50 μl) of the diluted enzyme were taken for assay. Assays were carried out on a Radiometer TTTlc pH-Stat (O'Sullivan and Cohn, 1966b). The velocity in the direction of ATP formation was measured. The conditions for the reaction between iodoacetamide and creatine kinase were the same as those for iodoacetic acid except that the experiments were carried out at 20°.

[2] W. J. O'Sullivan and M. Cohn (unpublished experiments).

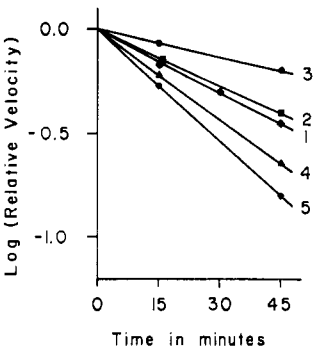

FIGURE 1: Kinetics of the reaction between iodoacetic acid (0.1 mM) and creatine kinase (1.37 μM) in 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, at 25° in the absence and presence of ADP^{3-} and $MgADP^-$, respectively. Samples (0.1 ml) were taken at zero time and at other time intervals as required and diluted to 0.4 ml in 10 mM cysteine, 5 mM *N*-ethylmorpholine–HCl, pH 8.0, for assay. The logarithm of the ratio of the enzymatic activity at time t, to the rate at zero time is plotted as a function of time. The pseudo-first-order rate constant (k_1) for creatine kinase alone (curve 1) was calculated to be 2.3×10^{-2} min^{-1}. Observed first-order rate constants (k') for creatine kinase in the presence of various modifiers were as follows: curve 2 (0.05 mM ADP) 1.84; curve 3 (0.2 mM ADP), 0.97; curve 4 (0.05 mM ADP, 0.5 mM $MgCl_2$), 3.22; curve 5 (0.2 mM ADP, 2 mM $MgCl_2$), 3.93. (All k' values are expressed as 10^{-2} min^{-1}.)

Determination of Rate Constants. In the presence of a large constant excess of inhibitor, the rate equation for the reaction between the inhibitor and the enzyme, E, in the presence and absence of an enzyme modifier, X, may be expressed as

$$k' = k_1[E]/[E]_T + k_2[EX]/[E]_T \qquad (1)$$

In this equation, k_1 and k_2 are the pseudo-first-order rate constants for the reaction between the inhibitor and the native enzyme and the enzyme–modifier complex, E–X, respectively, and k' is the observed, apparent first-order rate constant. Details of the methods of the determination of k_1 and k_2, and also for the determination of the dissociation constants of the E–X complex, are given in O'Sullivan and Cohn (1966b).

Results

The Inactivation of Creatine Kinase by Iodoacetic Acid. THE EFFECT OF ADP^{3-} AND $MgADP^-$. As previously observed by Mahowald *et al.* (1962), a pseudo-first-order reaction was obtained when creatine kinase reacted with a large excess of iodoacetic acid at 25°. The reaction was followed by measuring the enzymatic activity at various time intervals after the addition

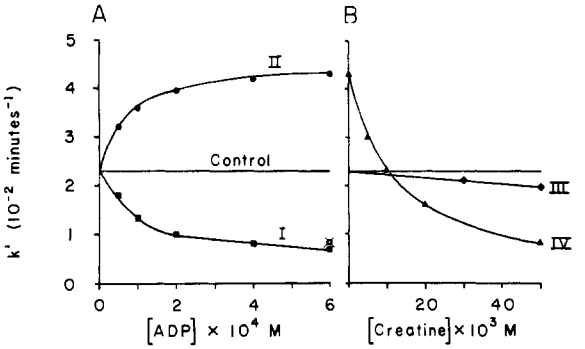

FIGURE 2: The rate of inactivation of creatine kinase by iodoacetic acid. (A) Plot of observed first-order rate constants, k', *vs.* concentration of ADP^{3-} (curve I) and $MgADP^-$ (curve II). The value of k' obtained with ADP^{3-} (no added metal ion) in the presence of 50 mM creatine is indicated by ☒. (B) Plot of k' *vs.* creatine concentration; curve III, creatine alone; curve IV, creatine plus 0.6 mM $MgADP^-$ ($[ADP]_T$ = 0.6 mM, $[MgCl_2]_T$ = 3 mM).

of iodoacetate. The results obtained are illustrated in curve 1 of Figure 1.

In accord with Mahowald *et al.* (1962) and Watts and Rabin (1962), ADP^{3-} was found to protect the enzyme against this inactivation (*cf.* curves 2 and 3 of Figure 1), and Mg^{2+} alone had a negligible effect on the rate of inactivation. However, contrary to the results of Mahowald *et al.* (1962) and consistent with the findings of Watts and Rabin (1962), $MgADP^-$ was found to accelerate the reaction between iodoacetic acid and creatine kinase (curves 4 and 5 of Figure 1) provided that a sufficient excess of magnesium was added to complex the ADP^{3-} completely. While ADP alone gave the rate shown in curve 3, the addition of increasing amounts of magnesium chloride to reaction mixtures containing the same concentration of ADP (0.2 mM) gave progressively increasing rates of inactivation until the "saturating" value of k' for $MgADP^-$, represented by curve 5, was reached; in effect, it was possible to "titrate" k' between ADP^{3-} and $MgADP^-$ with increasing amounts of Mg^{2+}.

Both the protective effect of ADP^{3-} and the accelerating effect of $MgADP^-$ exhibited saturation behavior, *i.e.*, the value of the apparent rate constant, k', approached a limiting value with increasing nucleotide concentration (see Figure 2A). A procedure similar to that of Scrutton and Utter (1965) was used to evaluate k_2, the pseudo-first-order rate constant for the reaction between iodoacetic acid and enzyme–modifier complex, and k_d, the dissociation constants of the enzyme–modifier complexes (see O'Sullivan and Cohn, 1966b). For ADP^{3-}, k_2 was found to be 0.4 × 10^{-2} min^{-1}, and K_d, 0.1 mM; for $MgADP^-$, k_2 was 4.9 × 10^{-2} min^{-1}, and K_d, 0.09 mM; the value, k_1, for the native enzyme, was 2.3 × 10^{-2} min^{-1} (second-order rate constant, 2.3 × 10^2 min^{-1} M^{-1}). The dissociation constants are in reasonable agreement with determinations by other methods (Kuby and Noltmann, 1962). The fact that k_2 was greater than 0, even in the case of ADP^{3-}, would suggest that ADP^{3-}, as well as $MgADP^-$, does not bind at the SH group of the enzyme.

Mahowald *et al.* (1962) reported that $MgADP^-$, as well as ADP^{3-}, protected creatine kinase against attack by iodoacetic acid. Inspection of their data (Figure 9A of Mahowald *et al.* (1962)) shows that only 10–30% of the ADP present would have been complexed with metal under their conditions.[3] At very low concentrations, the protection by ADP was slightly greater in the presence of equimolar $MgCl_2$ than in its absence. However, with increasing concentration of ADP, the protection in the presence of $MgCl_2$ decreased relative to that for ADP alone. It is probable that at still higher concentrations of $MgCl_2$ and ADP, where $MgADP^-$ would have been the predominant species, potentiation of the iodoacetic acid reaction might have resulted, as observed in our laboratory. From the experimental results of the current investigation, some decrease in protection relative to ADP alone would have been anticipated even at the lowest concentration of $MgCl_2$ and ADP tested, rather than the slight additional protection reported (Mahowald *et al.*, 1962). As the experiments of Mahowald *et al.* (1962) were carried out at 0° and those of Watts and Rabin (1962) and experiments reported above were carried out at 25°, it was possible that the observed differences at low concentrations of $MgCl_2$ and ADP could be ascribed to a difference in the iodoacetic acid–creatine kinase reaction at the two temperatures, a suggestion which was put forward by Watts and Rabin (1962). Therefore experiments were carried out at 0°.

At 0°, with creatine kinase at 27 μM and iodoacetic acid at 1.5 mM in 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, a pseudo-first-order rate constant, k_1, of 2.3 × 10^{-2} min^{-1} (second-order rate constant 15.3 min^{-1} M^{-1}) was obtained for the inactivation reaction. Addition of ADP^{3-} at 0.1 mM (*i.e.*, $[ADP^{3-}]$:[enzyme] equals 3.7), protected against this inactivation, the observed rate constant being lowered by 43% (k' equals 1.3 × 10^{-2} min^{-1}). Addition of equimolar $MgCl_2$ to the ADP^{3-} increased the rate of inactivation relative to ADP^{3-} alone, the observed value of k', 1.8 × 10^{-2} min^{-1}, being only 22% below the control. In comparable experiments, Mahowald *et al.* (1962) observed[4] k_1 as 1.48 × 10^{-2} min^{-1}, with creatine kinase 21 μM and iodoacetic acid 1.55 mM in 0.01 M Tris–0.15

[3] It should be noted that it is necessary to have at least fivefold excess of Mg over ADP, and for less concentrated solutions of the nucleotide, as much as a tenfold excess of Mg, to ensure that all the ADP is present as the magnesium complex. At the level of 0.1 mM of both ADP and $MgCl_2$, under the conditions used by Mahowald *et al.* (1962), less than 10% of the ADP would be present as $MgADP^-$; at concentrations of 0.25 mM, about 20% of the ADP would be complexed (see footnote 5).

[4] The symbols k_1 and k', as defined in the Experimental Section of this paper, are adhered to; they correspond to k' and k_3', respectively, as used by Mahowald *et al.* (1962).

M KCl, pH 7.9, at 0°. At an [ADP^{3-}]:[enzyme] ratio of 3.7, these investigators found that k' decreased by 31%, with k' equal to 1.04×10^{-2} min^{-1}. Thus, the results with enzyme alone and with enzyme plus ADP were quite similar in the earlier and current investigations. In the earlier investigation, addition of equimolar $MgCl_2$ with ADP further decreased the rate of inactivation slightly to a value 33% below that of the control, k' equal to 1.02×10^{-2} min^{-1} (*cf.* Figure 9A of Mahowald *et al.*, 1962). On the other hand, the results of the experiments described above with the same [Mg^{2+}]:[ADP^{3-}]:[enzyme] ratio showed an increased rate of inactivation of the enzyme compared to the rate in the absence of magnesium.

Apart from the difference in temperature, the difference between our conditions and those of Mahowald *et al.* (1962) was in the choice of buffers and ionic strength; *viz.*, 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, as compared to 0.01 M Tris–0.15 M KCl, pH 7.9. To ensure that these differences were not responsible for the difference in experimental findings, the above experiments were repeated in 0.01 M Tris–0.15 M KCl, pH 7.9 (measured at 0°). The results obtained were similar to those for the experiments in *N*-ethylmorpholine. The average of two experiments gave the value of the pseudo-first-order rate constant for enzyme alone as 1.8×10^{-2} min^{-1}. In the presence of ADP^{3-} at 0.1 mM, the observed rate constant was lowered by 36% ($k' = 1.17 \times 10^{-2}$ min^{-1}). Addition of equimolar $MgCl_2$ to the ADP^{3-} increased the rate of inactivation, the observed value of k', 1.38×10^{-2} min^{-1}, being only 25% below the control.

As the same value for the observed k_1 could be obtained at 0° as at 25° with appropriate changes in concentrations of enzyme and iodoacetic acid, it was possible to use the results from 25° to compare experimental and predicted values of k' for different concentrations of $MgCl_2$ and ADP at 0°. For mixtures of ADP and $MgCl_2$, k' values for the respective concentrations of ADP^{3-} and $MgADP^-$ in a given mixture can be obtained from the plots of k' *vs.* ADP^{3-} and $MgADP^-$ concentration (Figure 2A). The observed value of k' should be the weighted average of that for $k'(ADP^{3-})$ and $k'(MgADP^-)$. Such predicted values for k' are compared in Table I with the experimental values obtained at 0° for $MgCl_2$ and ADP concentrations at 0.1 and 0.25 mM. To obtain the predicted values, a stability constant of 1000 M^{-1} for $MgADP^-$ was used to calculate the relative concentrations of free ADP^{3-} and $MgADP^-$.[5] The k' values for these particular concentrations of ADP^{3-} and $MgADP^-$ were taken directly from Figure 2A, and a weighted average was calculated. The agreement between observed and predicted values appears to be reasonable.

TABLE I: Inactivation of Creatine Kinase by Iodoacetic Acid.[a]

Addn (mM)	k' Exptl (10^{-2} min^{-1})	k' Predicted (10^{-2} min^{-1})
—	2.3	—
ADP (0.1)	1.3	1.3
ADP (0.1), $MgCl_2$ (0.1)	1.8	1.9
ADP (0.25), $MgCl_2$ (0.25)	2.5	2.1

[a] Comparison of experimental rates of inactivation of creatine kinase (27 μM) by iodoacetic acid (1.5 mM) at 0°, with the values predicted from the experiments at 25° (Figure 2A), for various concentrations of ADP and ADP plus $MgCl_2$.

Other nucleoside diphosphates, d(2′)ADP, IDP, and GDP, also protected the enzyme against iodoacetic acid, and their metal complexes accelerated the reaction. Both free and metal nucleoside triphosphates protected the enzyme, though the effect was much greater with the free compounds. The apparent discrepancy between the diphosphates and the triphosphates has been discussed in detail elsewhere (O'Sullivan and Cohn, 1966b). The sodium salts of inorganic phosphate (P_i), pyrophosphate (PP), and tripolyphosphate (PPP) were all found to afford considerable protection to creatine kinase against attack by iodoacetic acid. From the experiments with the inorganic phosphates, approximate values for k_2 of 0.3, 0.5, and 0.5×10^{-2} min^{-1} and for K_d of 10, 4, and 4 mM were obtained for P_i, PP, and PPP, respectively. These values are approximate because they have not been corrected for the effect of ionic strength (Watts and Rabin, 1962).

Stoichiometry of the Iodoacetic Acid Reaction with Enzyme–Modifier Complexes. The interpretation of the inactivation experiments tacitly assumes that the same two SH groups of the eight in the enzyme are reactive in the native enzyme as in the ADP–enzyme and the MgADP–enzyme complexes. Direct proof of this assumption would require the identity of the amino acid sequence in the region of the cysteine residues after reaction with the alkylating reagent in the presence and absence of ADP^{3-} and $MgADP^-$ (*cf.* Thomson *et al.*, 1964). However, in lieu of such experiments, cogent evidence that the same SH groups are involved was obtained by studying the stoichiometry of the reaction between iodoacetic acid and creatine kinase.

The reaction was carried out with creatine kinase at 56 mg/ml (0.7 mM corresponding to 1.4 mM active sites) and molar ratios of enzyme to iodoacetic acid concentration of 1:1 and 1:2. End points of 47% and 95% loss of activity, respectively, were observed.

[5] The value of 1000 M^{-1} was based on the value of 4000 M^{-1} reported by O'Sullivan and Perrin (1964) extrapolated to 0° and 0.05 ionic strength as indicated from the data of Burton (1959). As no work on the stability constants of metal–nucleotide complexes has been done at low temperatures, the extrapolation should be viewed with some caution but was considered satisfactory (probably an upper limit) for the required purpose.

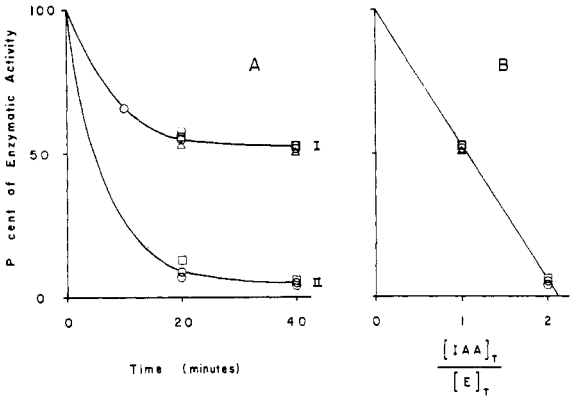

FIGURE 3: Time course of reaction between creatine kinase and iodoacetic acid, in 0.05 M *N*-ethylmorpholine–HCl, pH, 8.0 at 30° (A) and stoichiometry of the alkylation reaction (B). (A) The creatine kinase concentration was 56 mg/ml and the iodoacetic acid concentration was 0.7 mM in curve I and 1.4 mM in curve II. Samples (5 μl) were withdrawn at zero times and at other times as indicated, diluted to 1.0 ml in 10 mM cysteine, 5 mM *N*-ethylmorpholine–HCl, pH 8.0; 2-, 5-, or 50-μl samples, of the diluted enzyme were taken for assay. The activity of creatine kinase, relative to its activity at zero time, is plotted against time. The solid lines are drawn through the experimental points obtained with the unmodified enzyme (○). The per cent of enzymatic activity in the presence of 0.6 mM ADP is indicated by the symbol □; in the presence of 0.6 mM ADP and 6 mM $MgCl_2$ by the symbol ○; and in the presence of 10 mM ADP and 100 mM $MgCl_2$ by the symbol Δ. (B) The residual per cent enzymatic activity at the end point (40 min) from Figure 3A is plotted as a function of initial mole ratio [iodoacetic acid]:[creatine kinase]. The symbols have the same meaning as in Figure 3A.

The time course of these experiments is shown in Figure 3A, and a plot of loss of activity *vs.* the [iodoacetic acid]:[creatine kinase] mole ratio is shown in Figure 3B. The experiments were repeated in the presence of both ADP^{3-} and $MgADP^-$ at 0.6 mM, respectively, for each concentration of iodoacetic acid and also at 10 mM ADP^{3-} or $MgADP^-$ with iodoacetic acid at 0.7 mM. The results of these experiments as indicated in Figure 3A and B show that the same final enzymatic activity was obtained in each case within experimental error. Under these conditions, the reaction was very fast and was virtually complete after 10 min. Thus there was no significant difference in the extent of reaction observable with enzyme alone and with enzyme in the presence of ADP^{3-} or $MgADP^-$. These results strongly suggest that iodoacetic acid was reacting with the same SH groups of creatine kinase in the presence of ADP^{3-} or $MgADP^-$ as in their absence.

Effect of Creatine on the Iodoacetic Acid Inactivation of Creatine Kinase. The effect of creatine by itself, and in the presence of free ADP^{3-} and of $MgADP^-$, on the reaction between iodoacetic acid and creatine kinase is shown in Figure 2B. Creatine alone (curve III) had a very slight protective effect. A direct plot (*cf.* O'Sullivan and Cohn, 1966b) of k' *vs.* the reciprocal of creatine concentration gave a value for k_2 for the enzyme–creatine complex of 1.7×10^{-2} min^{-1}. The same plot was used to obtain a value of 50 mM for the dissociation constant of the enzyme–creatine complex. Similar experiments with phosphocreatine gave values of 1.4×10^{-2} min^{-1} and 3 mM for k_2 and K_d, respectively.

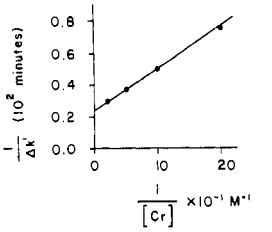

FIGURE 4: Double reciprocal plot of $\Delta k'$ *vs.* creatine concentration. $\Delta k'$ is the difference in k' values in the absence and presence of creatine; $MgADP^-$ concentration was 0.6 mM. Data were taken from Figure 2B.

The addition of creatine at a concentration of 50 mM to creatine kinase in the presence of ADP (0.6 mM) did not appear to affect the rate of inactivation by iodoacetic acid. However 50 mM creatine in the presence of 0.6 mM $MgADP^-$ reversed the effect of the metal-nucleotide; a decrease rather than an increase in the rate of inactivation relative to the enzyme alone was observed. The variation in k' as a function of creatine concentration with $MgADP^-$ at 0.6 mM is shown in Figure 2B (curve IV). In the absence of creatine, a value of 4.3×10^{-2} min^{-1} was obtained for k' with 0.6 mM $MgADP^-$ (curve II, Figure 2A). The addition of increasing concentrations of creatine at this concentration of $MgADP^-$ decreased the observed value of k' below the value of enzyme alone; at 50 mM creatine, with $MgADP^-$ equal to 0.6 mM, k' was equal to 0.85×10^{-2} min^{-1} compared to 2.3×10^{-2} min^{-1} for enzyme alone.

It is possible to use the data shown in Figure 2B to obtain an estimate of k' when the enzyme is saturated with both $MgADP^-$ and creatine. If $\Delta k'$ is defined as the difference between k' values in the absence and presence of creatine, as in the equation

$$\Delta k' = k'_{(MgADP-enzyme)} - k'_{(MgADP-enzyme-creatine)} \quad (2)$$

the value of $\Delta k'$ at infinite creatine concentration may be obtained from a double reciprocal plot of $\Delta k'$ *vs.* concentration of creatine (Figure 4). On the assumption that this plot is linear, a value of $\Delta k'$ at infinite creatine concentration of 4.2×10^{-2} min^{-1} was obtained by extrapolation. Since the value of $k'_{(MgADP-enzyme)}$ is 4.3×10^{-2} min^{-1}, $k'_{(MgADP-enzyme-creatine)}$ becomes 0.1×10^{-2} min^{-1} (see eq 2) at infinite creatine concentration, a value which would be experimentally

indistinguishable from 0. The result, therefore, is consistent with k' approaching 0 as the creatine concentration approaches infinity.

The data in Figure 4 gave a value of 10 mM for the dissociation constant of creatine from the MgADP–enzyme–creatine complex, based on the concentration of creatine required to give one-half of the saturation effect. This may be compared to the range of 8–17 mM, reported by Morrison and James (1965) on the basis of kinetic studies, for the dissociation constant of creatine from the same complex.

Inactivation of Creatine Kinase by Iodoacetamide. The reaction between creatine kinase (1.37 μM) and a large excess of iodoacetamide (0.1 mM) followed pseudo-first-order kinetics, with a rate constant, k_1, of approximately 7×10^{-2} min^{-1}. The reaction was not affected by saturating concentrations of ADP^{3-}, $MgADP^-$, ATP^{4-}, $MgATP^{2-}$ or by creatine at 50 mM (*cf.* Watts and Rabin, 1962). However, addition of creatine at a concentration of 50 mM in the presence of 0.6 mM $MgADP^-$, almost completely protected the enzyme against inactivation by iodoacetamide. A similar observation was recently reported by Lui and Cunningham (1966). Partial protection of the enzyme was also afforded by creatine (50 mM) in the presence of ADP^{3-} (0.6 mM), though the effect was less at any given concentration of creatine than that obtained in the presence of the metal–nucleotide. Some protection was also observed with the combinations, AMP (4 mM) and phosphocreatine (10 mM); ADP (0.6 mM) and phosphocreatine (10 mM); and ADP (0.6 mM), $MgCl_2$ (6 mM), and phosphocreatine (10 mM), *i.e.*, an equilibrium mixture.

The effect of various concentrations of creatine in the presence of saturating concentrations of $MgADP^-$ (curve I) and ADP^{3-} (curve II), respectively, on the observed pseudo-first-order rate constant, k', for the iodoacetamide–creatine kinase reaction is shown in Figure 5A. In Figure 5B, k' is plotted *vs.* the reciprocal of creatine concentration. It is seen that the results with $MgADP^-$ (curve I of Figure 5B) could reasonably be extrapolated to k' equals 0 at infinite concentration of creatine but that this is not the case with ADP^{3-} (curve II of Figure 5B). A double reciprocal plot of $\Delta k'$, the difference between the k' values without and with creatine at different concentrations, *vs.* creatine concentration is shown in Figure 5C. The points for both $MgADP^-$ (curve I) and ADP^{3-} (curve II) could be fitted to straight lines and the extrapolations shown give values of $\Delta k'$ of approximately 7.0 and 4.3×10^{-2} min^{-1}, respectively, at infinite creatine concentrations. Thus, k' equals zero for saturating concentrations of $MgADP^-$ and creatine but equals 2.7×10^{-2} min^{-1} for saturating concentrations of ADP^{3-} and creatine. In other words, while both the free nucleotide and the metal–nucleotide with creatine protect the SH groups against iodoacetamide, only in the case of the metal–nucleotide is the protection absolute. On the basis that saturation behavior was being observed in both cases, the results shown in Figure 5C were used to obtain approximate values of 10 and 30 mM for the dissociation constant of creatine from the complexes MgADP–enzyme–creatine and ADP–enzyme–creatine, respectively.

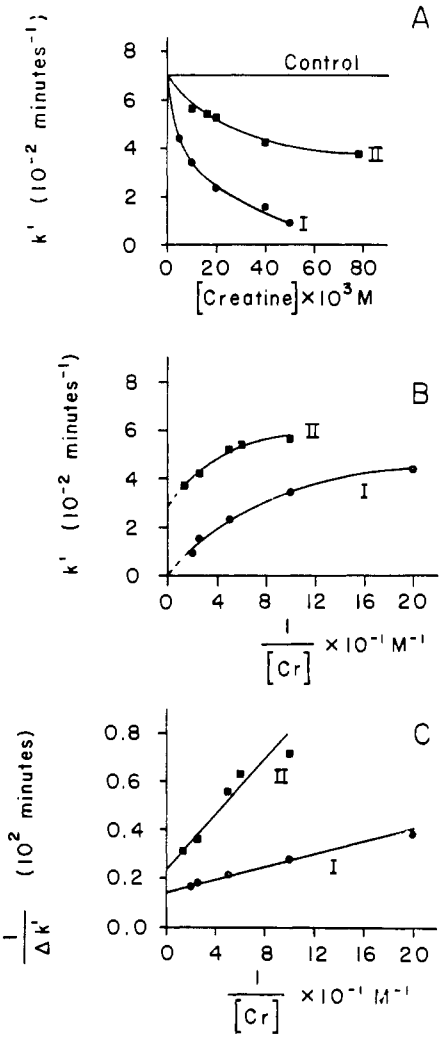

FIGURE 5: The inactivation of creatine kinase (1.37 μM) by iodoacetamide (0.1 mM), in 0.05 M *N*-ethylmorpholine–HCl, pH 8.0, at 20°. (A) Plot of observed rate constant, k', *vs.* creatine concentration; curve I, with $MgADP^-$ ($[ADP]_T$ = 1 mM, $[MgCl_2]_T$ = 10 mM), and curve II, with ADP^{3-} ($[ADP]_T$ = 1 mM). (B) Plot of k' *vs.* reciprocal of creatine concentration, curve I, with $MgADP^-$, and curve II, with ADP^{3-}. Data taken from Figure 5A. (C) Double reciprocal plot of $\Delta k'$ *vs.* creatine concentration; $\Delta k'$ is the difference between k' values in the absence and presence of creatine. Curve I, with $MgADP^-$, and curve II, with ADP^{3-}. Data taken from Figure 5A.

Discussion

The interpretation of the experiments described in this paper is dependent on the assumption that the reaction between iodoacetic acid and creatine kinase

is specific for the SH groups of the enzyme and that any reactions with other amino acid residues is negligible. The inactivation of creatine kinase by iodoacetic acid has been studied in some detail by previous investigators (Watts *et al.*, 1961; Watts and Rabin, 1962; Mahowald *et al.*, 1962). This work demonstrated clearly an exact correlation between loss of enzymatic activity and alkylation of the two reactive sulfhydryl groups. Mahowald *et al.* (1962) also demonstrated by analysis of the products formed, even at high molar ratios of iodoacetic acid:enzyme, that any side reactions were negligible up to 80% of the inactivation.

The results presented in this paper are in agreement with the earlier report by Watts and Rabin (1962) that qualitative differences in the reactivity of the two essential sulfhydryl groups of creatine kinase toward iodoacetic acid exist with respect to the free enzyme, the enzyme inhibitor (E–ADP) complex, and the enzyme substrate (E-MgADP) complex. We have previously proposed two types of conformational change of creatine kinase to explain the observed protection and activation of the SH groups in the presence of free nucleoside diphosphates and their metal complexes, respectively (O'Sullivan and Cohn, 1966b).

For the free nucleotide, ADP^{3-}, the protective effect might be attributed solely to charge repulsion of the iodoacetate ion by the bound negatively charged phosphate moiety. This is supported by the observed protective effect of the inorganic phosphate, pyrophosphate, and tripolyphosphate anions and also by the fact that increasing ionic strength decreased the rate of reaction (Watts and Rabin, 1962; O'Sullivan and Cohn, 1966b). However, if charge repulsion were the only factor, it would be expected that other nucleoside diphosphates such as $d(2')ADP^{3-}$, IDP^{3-}, and GDP^{3-} would (at saturation) give the same amount of protection as ADP^{3-}. This is not the case, and the ordered protection given by these compounds appears to reflect an ordered degree of conformational change (see O'Sullivan and Cohn, 1966b).

Binding of $MgADP^-$ might also be expected to exert a repulsive effect on the iodoacetate ion. From the fact that the SH groups of creatine kinase are more accessible to iodoacetic acid in the presence of $MgADP^-$ it is clear that an additional factor is introduced by the metal ion over and above simple electrostatic repulsion. This factor can more unreservedly be ascribed to a change in the protein conformation, though such a change may be localized at the active site. Again, a graded behavior is seen for the metal complexes of different nucleoside diphosphates, indicating a graded degree of a second type of conformational change (O'Sullivan and Cohn, 1966b).

The argument, that the effect of ADP^{3-} and $MgADP^-$ upon the SH reactivity with iodoacetic acid reflects a conformational change at the active site as well as a direct charge effect, needs to be reconciled with the finding that neither the free nor the metal complex form of the nucleotide causes any change in the reactivity of the SH groups toward iodoacetamide (*cf.* Watts and Rabin, 1962; Lui and Cunningham, 1966). It need only be postulated that the conformational changes, in particular for the metal–nucleotides, are of a kind which alter the charge distribution in the region of the active site. For example, the binding of $MgADP^-$ may "reveal" a positive moiety (or "bury" a negative moiety) in the vicinity of the SH group. The resulting increase in positive charge would facilitate the reaction with the iodoacetate ion but not that with the uncharged iodoacetamide. Such an explanation is consistent with the experimental finding that the rate of reaction between iodoacetamide and creatine kinase alone (second-order rate constant, 7×10^2 min^{-1} M^{-1} at 20°) exceeds the highest rate achieved with iodoacetic acid in its reaction with MgADP–enzyme (second-order rate constant, 4.9×10^2 min^{-1} M^{-1} at 25°).[6] In other words, even $MgADP^-$ does not completely overcome the retarding charge effect observed with iodoacetate ion which is nonexistent for the uncharged iodoacetamide. From their ionic strength experiments, Watts and Rabin (1962) also postulated the existence of positive groups on the enzyme near the SH moiety.

More direct experimental evidence that differing conformational changes occur on the binding of ADP^{3-} or $MgADP^-$ to creatine kinase, as revealed in the iodoacetic acid reaction though unexpressed in the iodoacetamide reaction, was obtained from the effect of creatine on the different complexes. Lui and Cunningham (1966) have shown that the SH group is protected against inactivation by iodoacetamide in the abortive quaternary complex, MgADP–enzyme–creatine. Evidence for the formation of this quaternary complex has also been presented by Morrison and James (1965) and Morrison and Cleland (1966) from steady-state and isotope-exchange kinetic studies, respectively.

In the current study, the difference between the enzyme complexes of ADP^{3-} and $MgADP^-$ is manifested, upon addition of creatine, in the reactivity toward both iodoacetic acid and iodoacetamide. Creatine alone affords very little protection against iodoacetic acid and none against iodoacetamide; partial protection was obtained with free ADP^{3-} and creatine for both alkylating agents. However, in the presence of saturating $MgADP^-$, complete protection of the SH group against both iodoacetic acid and iodoacetamide was found by extrapolation of the experimental results to infinite creatine concentration. The factor of 5 in the magnitude of the dissociation constant (10 mM) of creatine from the quaternary MgADP–enzyme–creatine complex compared to that (50 mM) from the enzyme–creatine complex is indicative that the creatine is bound in a different manner in each case. While neither ADP^{3-} nor creatine alone affected the

[6] It has been previously demonstrated (Watts and Rabin, 1962; Mahowald *et al.*, 1962) that the reaction between iodoacetic acid and creatine kinase is first order with respect to both reactants. Watts and Rabin (1962) found that the reaction between iodoacetamide and creatine kinase was first order with respect to the enzyme, and experiments in this laboratory have shown that this reaction is also first order with respect to iodoacetamide.

iodoacetamide reaction, partial protection was seen at saturating concentrations of both. The dissociation constant of 30 mM for creatine from the ADP–enzyme complex lies between the values obtained with free enzyme and MgADP–enzyme, and suggests a third type of binding for creatine to the enzyme.

A number of attempts to measure directly conformation changes in creatine kinase on addition of substrates have been made (Samuels *et al.*, 1961; Kägi and Li, 1965; Lui and Cunningham, 1966). While no gross differences in protein structure have been detected, Lui and Cunningham (1966) concluded from deuterium exchange experiments that a small decrease in the number of amide hydrogens which could exchange with deuterium was observed on the binding of $MgADP^-$; 11–18 amide groups/active site were affected. It should be emphasized that the method used to study conformational changes in this paper can only reveal changes in the highly localized region of the active site and gives no information concerning conformational changes in other regions of the protein molecule.

The fact that creatine in the presence of $MgADP^-$ appears to afford complete protection against inactivation by both iodoacetic acid and iodoacetamide supports the conclusion that creatine is bound to the reactive SH in the quaternary MgADP–enzyme–creatine complex and probably not in the ADP–enzyme–creatine or enzyme–creatine complexes. Evidence for the involvement of the metal ion in the "correct" binding of creatine has also come from magnetic resonance studies. Thus, the decrease in proton relaxation rate on the addition of creatine to the binary MnADP–enzyme (O'Sullivan and Cohn, 1966a) was not observed with creatine kinase that had been carboxymethylated at the two essential SH groups.[2] While the proposed existence of an SH binding site for creatine only in the presence of metal nucleotide gives an attractive model for the working enzyme insofar as it assigns a function to both the metal ion and the essential SH groups (*cf.* Watts and Rabin, 1962), such a model awaits direct verification.

Some reservations remain in applying the results obtained from a study of the abortive MgADP–enzyme–creatine complex to the catalytically active complex. However, the good agreement between the dissociation constant of creatine from the quaternary complex as found above and the kinetically determined values of Morrison and James (1965) is consistent with the creatine being bound to the enzyme in the same manner in each case. Interactions between the nucleotide and guanidine substrates, as reflected by differences in K_m, have been demonstrated from kinetic studies (Morrison and O'Sullivan, 1965; Morrison and James, 1965). The finding, that the initial complex must be bound before creatine can bind in the "correct" manner, necessitates the incorporation of the conformational change of the enzyme into the kinetic scheme.

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