Nucleotide Specificity and Conformation of the Active Site of Creatine Kinase

MAGNETIC RESONANCE AND SULFHYDRYL REACTIVITY STUDIES*

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SUMMARY

The manganese-enzyme-substrate complexes of a number of nucleoside diphosphate substrates of creatine kinase showed a range of enhanced values of the longitudinal proton relaxation rate of water with respect to the manganous aquocation, although the small enhancement factors, \( \epsilon_n \), of all the binary manganese-nucleotide complexes were the same, approximately 1.6. The order of the values of \( \epsilon_n \), the enhancement factor of the respective ternary enzyme-metal-substrate complexes, was: manganese-adenosine diphosphate (19.4) > Mn-3'-dADP (7.7) > Mn-2'-dADP (6.7) > manganese-inosine diphosphate (5) > manganese-guanosine diphosphate (4). This same order is observed for the maximum velocity of the enzymatic reaction with these substrates. There was no significant difference in the dissociation constants of the enzyme-metal-substrate complexes for the three manganese-adenine nucleotides. Values of \( \epsilon_t \) for the nucleoside triphosphates were: MnATP (9.8) > Mn-2'-dATP (4.5) > MnGTP (3.7) > MnITP (3.2). The order of relative velocities was found to be MnATP > Mn-2'-dATP > MnITP > MnGTP.

The relative effects of a number of substrates and inhibitors of creatine kinase on the rate of reaction between iodoacetic acid and the essential sulfhydryl groups of the enzyme was also investigated. Metal nucleoside diphosphates increased the \(-SH\) reactivity in the order M-ADP > M-2'-dADP > M-IDP > M-GDP, while the free nucleotides protected in the order ADP > 2'-dADP > IDP > GDP. Both the metal complexes and the free forms of nucleoside triphosphates protected creatine kinase against iodoacetic acid, although the effects were much greater for the free nucleotides. With both series of compounds, an order similar to that for the diphosphates was observed.

The parallel order of the values of \( \epsilon_t \), the maximum velocities of the enzymatic reaction, and the rate constants for the iodoacetic acid reaction with the iodoacetic acid reaction with the manganese-enzyme-substrate complexes may be ascribed to differing degrees of conformational change induced at the active site of creatine kinase. Thus the properties of water in the coordination sphere of the manganese in the enzyme-metal-substrate complex as manifested by the enhancement of proton relaxation rate may be used as a probe to reveal changes in the conformation of the enzyme at its active site in the same way as the modification of the reactivity of the essential cysteine residues is used. With the latter criterion, the effect of binding free nucleotides and metal-nucleotides could be investigated and the observed rate constants for the reaction between iodoacetic acid and creatine kinase could be explained by induced conformational changes, which increased the accessibility of the \(-SH\) groups upon binding of metal nucleotides and decreased the accessibility of the \(-SH\) groups upon binding of the free nucleotides.

The phenomenon of enhancement of the manganese contribution to the longitudinal relaxation rate of the proton spins water in solutions of manganese complexes with macromolecules has been amply documented (1-4). Since the magnetic interaction between manganese and the protons of water falls off inversely as the sixth power of the distance, the effect is essentially limited to the first coordination sphere of the manganese, and the magnitude of the enhancement, \( \epsilon_n \), is therefore determined by the immediate environment of the manganese at its binding site. Experimentally it has been demonstrated in the case of the bovine serum albumin complex (5) that the magnitude of its enhancement, \( \epsilon_n \), was altered by conditions which produced conformational changes in the protein, for example, denaturing agents such as urea and guanidinium chloride and changes in pH. It seemed possible, therefore, to use the enhancement parameter as a measure of conformational change at the site of manganese binding and, furthermore, to investigate whether the local changes reflected by changes in \( \epsilon_n \) could be correlated with changes in other portions of the protein molecule.

The series of ternary complexes formed by creatine kinase (adenosine triphosphate:creatine phosphotransferase, 2.7.3.2) with various metal nucleotide-substrates appeared...
provide an ideal system for this investigation. The differences in maximum velocity range over a factor of 25 from the most reactive substrate, ADP, to the least reactive, guanosine diphosphate. Kinetic and equilibrium studies have shown that the order of reactivity does not correspond to the order of binding constants of the metal-nucleotides to the enzyme, thus implicating a change in reactivity, induced by substrate binding, of some catalytic group on the enzyme distinct from the binding site. Thus, creatine kinase might be expected to exhibit substrate-induced conformational changes of the type envisaged in Koshland's induced fit theory (6).

The advantages of using the creatine kinase system for studies of the variation of PRR enhancement with substrate are numerous. From the experimental point of view, the magnitude of $\epsilon_i$ for the manganese-ADP-enzyme complex is fairly high, about 20 (see the preceding paper (7)), permitting the measurement of decreasing values for other substrates with considerable accuracy. From the theoretical point of view, the chosen system is favorable, since a comparison of the relative values of $\epsilon$ in this system avoids many of the difficulties inherent in the interpretation of the absolute magnitude of $\epsilon$ for a particular complex. For example, in ternary metal-nucleotide-enzyme complexes, it is highly probable that the number and type of metal ligands, factors which affect the value of the PRR enhancement, will remain invariant with variations in the base or ribose moieties of the substrate. Thus the change in magnitude of $\epsilon_i$ with various substrates may be expected to depend predominantly on the variables that are controlled by the conformation of the protein in the localized region of the binding of the metal nucleotide.

In addition, an independent criterion is available for the investigation of conformational changes at the active site induced by substrates for creatine kinase. It has been reported previously (8, 9) that binding of substrates to the enzyme changes the reactivity of the two essential sulfhydryl groups in this enzyme toward alkylating agents. This property of the enzyme permitted an investigation of the possible specificity of the bound metal-nucleotide substrates with respect to the -SH group reactivity.

This paper reports the results of an investigation of two criteria of conformational change in the region of the active site of creatine kinase induced by substrate or inhibitor, (a) the enhancement of the PRR of ternary complexes of manganese-nucleotide-enzyme and (b) the sulfhydryl reactivity with iodoacetic acid of the same ternary complexes and of the inhibitory binary complexes of enzyme and nucleotide.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**—Manganese and magnesium chlorides were spectrographically standardized reagents from Johnson, Matthey, and Company, Ltd., London. Other metal salts used were reagent grade.

$N$-Ethylmorpholine, tetramethylammonium chloride, and iodoacetic acid were purchased from Eastman. $N$-Ethyl-$p$-toluenesulfonate was made up at 0.5 molar concentration after adjustment to the required pH with concentrated HCl. The tetramethylammonium chloride was recrystallized from acetone-ether and stored in a desiccator. Iodoacetic acid was recrystallized from ether-hexane and stored in the cold (10).

$3'$-dADP was a gift from Dr. H. T. Shigeura. Other nucleotides were purchased from Sigma, Calbiochem, or Pabst; phosphocreatine was obtained from Sigma or Nutritional Biochemicals; creatine, from Fisher; and bovine serum albumin, from Armour.

Creatine kinase was prepared as described in the preceding paper (7). For the studies reported in this paper, preparations with activities of 60 to 65 Kuby units were used (11).

**Magnetic Resonance Measurements**—Free manganese was measured by the intensity of its EPR spectrum with a Varian model V-14500 A spectrometer at 9.5 G per sec. The bound manganese was studied by its effect on the PRR of water by a pulsed nuclear magnetic resonance method at 25 me per sec, as described previously (5).

The observed enhancement of the effect of manganese on the proton relaxation rate, $\epsilon^*$, is defined as (5)

$$\epsilon^* = \frac{1/T_1 - 1/T_{10}}{1/T_1 - 1/T_{10}} \tag{1}$$

where $T_1$ and $T_{10}$ are the observed longitudinal relaxation times of water in the buffer solutions with and without manganese, respectively. The symbols with asterisks represent the same parameters in the presence of a complex-forming agent.

Unless otherwise indicated, all experiments were carried out in 0.05 m $N$-ethylmorpholine-HCl buffer, pH 8.0, with a total MnCl$_2$ concentration of 0.1 mm and various concentrations of nucleotide and creatine kinase.

**Calculation of Enhancement and Binding Parameters**—The procedures for determining the values of the enhancement of the PRR of binary manganese-substrate complexes, $\epsilon_i$, and dissociation constants, $K_i$, where

$$K_i = \frac{[M][S]}{[MS]} \tag{2}$$

have been presented elsewhere (5, 12). A detailed analysis of the treatment of PRR and EPR data for an evaluation of the enhancement parameter, $\epsilon_i$, of ternary EMS complexes has been given for creatine kinase in the preceding paper (7). For the results reported in this paper, the dissociation constant for the reaction between enzyme and manganese substrate, $K_s$, where

$$K_s = \frac{[E][MS]}{[EMS]} \tag{3}$$

was usually obtained by one or both of two procedures, I and III, described in the preceding paper (7). Procedure III also allowed the determination of $K_s$ where

$$K_s = \frac{[E][S]}{[ES]} \tag{4}$$

**Inactivation of Creatine Kinase by Iodoacetic Acid**—The recrystallized iodoacetic acid was washed with hexane or carbon tetrachloride, and solutions of the required concentration were made up immediately before use. Solutions of creatine kinase in the range of 1 $\mu$L, in a total volume of 2.0 ml, were incubated...
in 0.05 M N-ethylmorpholine-HCl, pH 8.0, at 25°C for 3 min prior to addition of iodoacetic acid to a concentration of 1 mM. Samples (0.1 ml) were withdrawn at zero time and at other time intervals as required, and the reaction was stopped by dilution with 0.3 ml of a solution containing 10 mM cysteine and 5 mM N-ethylmorpholine-HCl, pH 8.0 (8). A 50-μl portion of the diluted enzyme solution was used for assay. For the studies on inactivation reported in this paper, the activity of the enzyme was determined from the rate of the reverse reaction, i.e. the rate of formation of ATP and creatine. The assay conditions were: ADP, 1 mM; MnCl₂, 1 mM; phosphocreatine, 10 mM; bovine serum albumin, 0.04%; and tetramethylammonium chloride, 0.05 M. The reaction was initiated by addition of enzyme at pH 7.5 and 25°C ± 1°C, and the rate of uptake of H⁺ was followed by the addition of either 1 mM or 0.5 mM HCl with a Radiometer TTT1c pH-stat (9).

**Calculation of First Order Rate Constants for Inactivation**—The reaction between an inactivator, I, and an enzyme, E, may be expressed as the bimolecular reaction

\[ E + I \rightarrow E \cdot I \]  

If the bimolecular rate constant is denoted as \( \theta \), the rate of inactivation becomes

\[ -\frac{d[E]}{dt} = \theta [E][I] \]  

and, under conditions in which there is a large excess of I and the reaction follows pseudo-first order kinetics, the pseudo-first order rate constant, \( k_1 \), is given by

\[ k_1 = \frac{1}{t} \ln \left( \frac{[E]_0}{[E]} \right) \]  

where \([E]_0\) and \([E]\) are the concentrations of active enzyme at zero time and at time, \( t \), respectively. The concentrations of active enzyme are determined by assaying the reaction mixture for creatine kinase activity at different times after the addition of iodoacetic acid, and \( k_1 \) is obtained from a plot of log (relative activity) against time.

**Calculation of First Order Rate Constants in Presence of Enzyme Modifier**—The reaction between an inactivator and the enzyme may be influenced by the presence of a modifier, X, which combines with the enzyme. In this case, it is possible to calculate the pseudo-first order rate constant, \( k_1 \), for the reaction between the inactivating agent and the E-X complex and also the dissociation constant for E-X. The following treatment is essentially similar to that used by Scrutton and Utter (13). Since the reaction in the presence and absence of modifier has been shown to be bimolecular (9), the rate equation may be expressed as

\[ -\frac{d[E]_T}{dt} = \theta [E][I] + \theta [E][EX][I] \]  

and, in the presence of a large excess of I, the observed, apparent first order rate constant, \( k' \), becomes

\[ k' = k_1[E]/[E]_T + k_2[EX]/[E]_T \]

Since

\[ [E]_T = [E] + [EX] \]  
\[ [X]_T = [X] + [EX] \]

and

\[ K = [E][X]/[EX] \]

then Equation 9 may be expressed as

\[ k' = \frac{k_1K}{(K + [X])} + \frac{k_2X}{(K + [X])} \]

where \([X] = [X]_T\) for the condition that \([X]_T \gg [E]\). When \( k' < k_1\), Equation 13 was used in the form

\[ k' = \frac{K}{[X]} \left( 1 - \frac{k'}{k_1} \right) + \frac{k_2}{k_1} \]

and a plot of \( k'/k_1\) as ordinate against \((1 - k'/k_2)/[X]\) as abscissa should have intercepts of \( k_2/k_1 \) and \(( -k_2/k_1)/K\) on the ordinate as abscissa, respectively (cf. Reference 13). On the other hand when \( k' > k_1\), Equation 13 was used in the form

\[ k' = \frac{k_2}{k_1} - \frac{K}{[X]} \left( \frac{k'}{k_1} - 1 \right) \]

so that a plot of \( k'/k_1\) as ordinate against \((k'/k_1 - 1)/[X]\) as abscissa has intercepts of \( k_2/k_1 \) and \((k_2/k_1)/K\), respectively.

**Velocities of Forward and Reverse Enzymatic Reactions with Various Nucleotide Substrates**—The relative velocities will differ for the forwad and reverse reactions. For the forward reaction, the conditions were as described above except that the relative velocities for ADP, 2'-dADP, and 3'-dADP were compared at concentrations of 0.2 mM with MnCl₂ also at 0.2 mM; there was insufficient 3'-dADP available for more than one experiment with this substrate. In the forward direction, the nucleoside triphosphates were compared at concentration of 0.5 mM with 30 mM creatine and the release of H⁺ was followed by the addition of 1 mM KOH.

The relative velocity of the forward reaction was also followed by measuring the phosphocreatine formed, determined as inorganic phosphate according to the method of Martin and Doty (14). The nucleoside triphosphates were compared as substrates in the range between 0.5 and 2 mM, in the presence of equal concentrations of MnCl₂ and of 30 mM creatine in 0.05 M Tris HCl, pH 8.0, at 25°C in a volume of 1.0 ml. The reaction was initiated by the addition of enzyme (4 μg) and stopped after min with 0.5 ml of acid molydate (14). The mixture was allowed to stand for 20 min at room temperature to assure complete hydrolysis of the phosphocreatine before the phosphate was determined. Duplicate assays, without enzyme, were carried out at the same time to correct for any phosphate in the reaction mixture.

**RESULTS**

**Enhancements and Association Constants for Binary Complex of Manganese and Nucleoside Di- and Triphosphates**—Values of manganese and nucleoside di- and triphosphates—Values of the enhancements, \( \epsilon_a \), for the manganese complexes of 2'-dADP, 3'-dADP, IDP, GDP, UDP, and CDP were found to be approximately the same as for ADP, viz. 1.6 (7). Similarly, values of \( \epsilon_a \) of 1.6 to 1.7 were obtained for 2'-dATP, ITP, and GTP. Further, the association constants for the manganese complex of all the nucleoside di- and triphosphates were, to a first approximation, equal to the values of 2.5 × 10⁴ M⁻¹ for MnADP⁻² and approximately 10⁴ M⁻¹ for MnATP⁻³, respectively, obtained previously under the conditions used (7).
Enhancements and Dissociation Constants of Ternary Complexes Formed by Manganese, Nucleoside Diphosphate, and Creatine Kinase.—The values of the enhancements, $\epsilon_t$, of the ternary complexes formed from a series of nucleoside diphosphates with manganese and creatine kinase are summarized in Table I. It was found that a large enhancement was observed with both ADP and 3'-dADP, although values were less than those obtained with ADP. These experiments were carried out under conditions identical with those used for ADP (7). In each case reactions were carried out at three different manganese concentrations; reciprocal plots of $\epsilon^*$ against [S]$_2$ were used to obtain $\epsilon^*_t$, the enhancement at infinite substrate concentration for each enzyme concentration, and $\epsilon_t$ was obtained from a double reciprocal plot of $\epsilon^*$ against [E]$_2$.

Under conditions comparable to those usually used for ternary complexes of ADP, the nucleoside diphosphates of other bases failed to reveal any detectable enhancement. However, when the experiments were carried out under conditions designed to produce greater concentrations of the ternary complex, namely higher concentrations of enzyme and nucleotide (creatine kinase, 0.31 mm; MnCl$_2$, 1.1 mm; nucleoside diphosphate, 0.4 to 1 mm; $N$-ethylmorpholine-HCl, pH 8.0, 0.05 m), a low enhancement was observed. The relative enhancements were in the order IDP > GDP > UDP and CDP, but only in the case of IDP was it possible to extract a reliable value for $\epsilon_t$. With the other nucleotides, although reasonable values were obtained for the extrapolated value, $\epsilon^*_t$, the actual measured enhancements were only slightly greater than for the binary complexes alone, and the values for $\epsilon_t$ should be regarded as subject to considerable error.

In Table I, the enhancements of the ternary complexes for the various nucleoside diphosphates are compared with the relative maximum velocities of the reaction with these nucleotides as substrates. In both cases the order ADP > 3'-dADP > 2'-dADP > IDP > GDP was observed for the purine nucleotides. The relative values of the velocity, with Mn$^{2+}$ as the activating ion, for ADP and 2'-dADP were in reasonable agreement with the maximum velocities obtained by James and Morrison (7) with Mg$^{2+}$ as the activating ion, and it is assumed that the order of velocities for the entire series is the same with either Mg$^{2+}$ or Mn$^{2+}$.

Values of the dissociation constant, $K_s$, for EMS to enzyme and MS, and values of $K_s$, for ES to enzyme and $S$, were also estimated. For the adenine nucleotides Procedures I and III were used (see preceding paper (7)); for the other nucleotides only Procedure I was used, so that $K_s$ but not $K_a$ was obtained. The values obtained are included in Table I.

In contrast to the variation of the enhancement, $\epsilon_t$, and the velocity with changes in the structure of the ribose moiety of the adenine nucleotides, such changes had little if any effect on the binding of the metal-nucleotide to the enzyme. The large change found in both $\epsilon_t$ and the enzyme velocity as a result of the seemingly minor structural modifications between ribose and deoxyribose substrates is not surprising in view of the major conformational differences reported from analysis of the nuclear magnetic resonance spectra of ribose and deoxyribose nucleosides (15, 16). There also seemed to be little difference in the binding of the three free adenine nucleotides to creatine kinase. Approximately values of $1 \times 10^{-4}$ and $1.2 \times 10^{-4}$ m were obtained for $K_s$ for 3'-dADP and 2'-dADP, respectively, and these values do not differ significantly from the value of $1.6 \times 10^{-4}$ m for ADP (7).

The 1:1 correspondence in the order of $\epsilon_t$ and the maximum velocity for the series of nucleotides in Table I does not hold strictly; guanosine diphosphate appears to be out of place. The predominant structural changes which occur upon binding of the substrate affect both the enhancement parameter and the enzymatic activity, so that low values of $\epsilon_t$ correlate with low values of the reaction velocity. At low enhancement values, however, an exact correspondence may no longer hold because steric factors other than protein conformation associated with the catalytic activity may make a relatively greater contribution to the enhancement than when the enhancement is high.

Comparison of enhancements (PRR), velocities, and dissociation constants of creatine kinase-metal-nucleoside diphosphate complexes

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$\epsilon_t$</th>
<th>Relative velocity</th>
<th>$K_s$ (E+MS) a</th>
<th>$K_s$ (MnS) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>19.4</td>
<td>100</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>3'-dADP</td>
<td>7.7</td>
<td>42</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>2'-dADP</td>
<td>6.7</td>
<td>31</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>IDP</td>
<td>5</td>
<td>29</td>
<td>~10</td>
<td>11</td>
</tr>
<tr>
<td>GDP</td>
<td>4</td>
<td>4</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>UDP</td>
<td>3.5</td>
<td>10</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>CDP</td>
<td>3.5</td>
<td>10</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

a Determined by James and Morrison (7).
b $K_s$ is the dissociation constant for EMS = E + MS. For MnS, $K_s$ was determined by PRR and EPR; for MgS, $K_s$ is the kinetically determined constant.

J. F. Morrison and M. L. Uhr (personal communication) obtained a value of $0.7 \times 10^{-4}$ m for $K_s$, determined kinetically with Mn$^{2+}$ as the activating ion.

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TABLE I
M-ITP to creatine kinase are greater by almost an order of magnitude than those of the corresponding nucleoside diphosphates, while MnADP - binds slightly more strongly than MnATP -.

Determination of the relative velocity, carried out with a pH-stat with the nucleoside triphosphates at a concentration of 5 × 10 -4 m, indicated that the reaction proceeded with 2'-dATP at approximately half the rate of ATP and that ITP and GTP were both less than 10% of this rate. The conclusions were confirmed by direct measurement of the phosphocreatine formed and the results shown in Table II are from these experiments.

The triphosphates also exhibit a correlation between e, the PRR enhancement of the ternary complexes of a given nucleotide, and its velocity in the enzymatic reaction; ATP and 2'-dATP, in which only the ribose moiety is altered, have higher values of e and velocity whereas GTP and ITP, in which the bases have been altered, have lower values of both e and reaction velocity. However, there is an inversion of the order of GTP and ITP in the enhancement with respect to their order in the reaction velocity. As already pointed out for the diphosphates, it may well be that the enhancement is due to more than one change in the steric environment of the metal ion; the particular contribution from the steric change which is related directly to the enzyme activity predominates when the enhancement is high, but at low enhancements effects not related to the activity of the configuration become an important contribution to the total observed enhancement. Such an explanation is consistent with the findings on the nucleoside diphosphate series, in which the parallel order of enhancement and velocity breaks down for the GDP complex, which has a lower enhancement (e = 4).

Kinetics of Reaction between Creatine Kinase and Iodoacetic Acid—The change in magnitude of e for the manganese complexes of different nucleotide substrates of creatine kinase, and the close correlation between e and the relative velocities (Table I), suggest that the variation in both of these properties might be explained by conformational changes at the active site of the enzyme induced by the substrate. To adduce supporting evidence for this explanation, it was considered desirable to investigate the existence of a correlation between substrate specificity and another independent property of the protein which reflects conformational change. The modification by substrate of the reactivity of a particular amino acid residue is often a most useful criterion for demonstrating changes in the steric environment at the active site. This approach has been used, for example, in studies on chymotrypsin (17), phosphoglucomutase (18), and ribonuclease (19).

The carboxymethylation of the two essential —SH groups of creatine kinase resulting from the reaction between iodoacetic acid and the enzyme offered a number of advantages for investigation of the effect of a series of nucleotide substrates. This reaction has been thoroughly investigated (8, 9) and provided that the conditions are rigorously controlled, can be made stoichiometric with respect to the two reactive sulfhydryl groups of the enzyme (9, 20). Furthermore, Mahowald, Nolmann, and Kuby (9) studied the kinetics of inactivation of creatine kinase by iodoacetic acid. These investigators found that nucleotide, with or without metal ion, protected the enzyme against inactivation by iodoacetic acid. The modifying effect of substrate could be due to a conformational change, or different substrates might show different degrees of conformational change by this criterion. Further, for such a study it was possible to use either Mg2+ or Mn2+ as the divalent ion and also to study the effect of the free nucleotides.

Effect of Nucleoside Diphosphates on Iodoacetic Acid Reaction The observed rate constants of the inactivation reaction obtain for the series of nucleoside diphosphates, ADP, 2'-dADP, IDP, and GDP, and for their respective metal complexes are shown in Fig. 1. In this plot, values of the apparent first order constant, k', for the reaction between creatine kinase and iodoacetic acid, in the presence of various concentrations of nucleotide or metal nucleotide, are plotted against nucleotide concentration. The horizontal line in Fig. 1 represents the pseudo-first order rate constant, k1, which is equal to 2.3 × 10 3 min -1 and a corresponds to the bimolecular rate constant, k1, of 2.3 × 10 3 min -1 for creatine kinase alone. In agreement with Mahowald et al. (9), the reaction between creatine kinase and a large excess of iodoacetic acid at 25° obeyed pseudo-first order kinetics.

As observed by previous investigators (8, 9), ADP - protects the enzyme against attack by iodoacetic acid. This is illustrated by decreasing values of k' with increasing ADP concentration. For the other nucleoside diphosphates, ADP, 2'-dADP, IDP, and GDP, the order of protection was ADP > 2'-dADP > IDP > GDP, this being the same order as for the PRR enhancement and the relative velocity of the enzymatic reaction with metal complexes of nucleotides.

The complexes MgADP -, Mg2'-dADP -, MnIDP -, MgGDP - were found to accelerate the reaction between creatine kinase and iodoacetic acid (Fig. 1) (cf. Reference 8). No effect of MgIDP on the reaction with the alkylating agent was observed. However, MnIDP - was found to activate the reaction and the curve obtained with this complex is included in Fig. 1. Moreover, protection was also afforded by 2'-dADP, IDP, and GDP. The order of protection was ADP > 2'-dADP > IDP > GDP, the same as for the ADP and GDP complexes. No significant difference in the results obtained for the manganese or the magnesium complexes explained for the anomalous behavior of MgIDP - was apparent.

The order of activation of the metal complexes was MgADP - > Mg2'-dADP - > MnIDP - > MgGDP -. These experiments were carried out in the presence of a 5- to 10-fold excess of metal ion to ensure that all the nucleotide present was complexed. The effect of metal ion alone, as indicated in the inset in Fig. 1, was negligible (cf. Reference 9). Values of k' of 2.2 and 2.1.

1 The possibility that the activation in the presence of MgADP - could be due to the presence of heavy metal impurities (cf. Reference 21) was checked by including relatively low concentrations of EDTA (10 -4 to 10 -6 M) in the reaction mixtures. Inclusion of the EDTA had a negligible effect on the rate of inactivation.
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10⁻² min⁻¹, relative to a control of k₁ of 2.3 × 10⁻² min⁻¹, were obtained with MgCl₂ at 10 and 30 mM, respectively. These small effects of MgCl₂ could be ascribed solely to ionic strength, since KCl and NaNO₃ at the same ionic strength as the highest concentration of MgCl₂ (30 mM) gave the same value of k₁. It was concluded that magnesium alone had a negligible effect and that, in agreement with Wuts and Rabin (8), increasing ionic strength slightly decreased the rate of alkylation. For the range of ionic strengths generally used in this investigation, the ionic strength differences could be neglected.

Some experiments were also carried out with molar ratios of enzyme to iodoacetic acid concentration of 1:2 (enzyme, 1.22 × 10⁻⁴ M; iodoacetic acid, 2.5 × 10⁻⁴ M) in 0.05 M N-ethylmorpholine, pH 8.0, at 25°C. Under these conditions the reaction was apparently second order with a rate constant of approximately 280 × 10⁻⁵ min⁻¹. In preliminary experiments the free nucleotides were found to inhibit in the order ADP > 2'-dADP > IDP > GDP, and the magnesium nucleotides were observed to activate the reaction. As this behavior appeared identical with that observed at lower concentrations of enzyme, these experiments were not pursued. A detailed study of the activating effect of MgADP⁻ (and the other metal nucleoside diphosphates), which differs in some respects from previously reported results (8, 9), will be presented elsewhere.

Calculation of k₂ and Dissociation Constant, K_d—The value of k₂, the rate constant for the reaction between iodoacetic acid and the enzyme-nucleotide complex, could be extracted from the observed rate constant, k′, which is a sum of the weighted rate constants of the free enzyme, k₁, and of the complexed enzyme, k₂, by the method described under “Experimental Procedure,” i.e., by plotting k′/k₁ against (1 - k'/k₁)/[S]. The dissociation constant can also be evaluated from such a plot (cf., “Experimental Procedure”). Plots of k′/k₁ against (1 - k'/k₁)/[S], where S is ADP and 2'-dADP, respectively, are shown in Fig. 2.

The same type of analysis was used to obtain k₂ and K_d for MgADP⁻ as the enzyme modifier (see Fig. 3). However, a similar graphical analysis for the other metal-nucleotides did not prove feasible for obtaining reliable values of k₂ and K_d since the parameter (k′/k₁ - 1) varied over too small a range to permit extrapolation of the experimental points. Therefore direct plots of k′ against 1/[MS] were used to obtain approximate values of k₂ and K_d for MgADP⁻ (and Fig. 4). Extrapolation of the curve for MgADP⁻ yielded a value of k₂, i.e., k' at infinite [MgADP⁻], of 5.0 × 10⁻² min⁻¹, compared to 4.9 × 10⁻² min⁻¹ obtained by the more exact formulation presented in Fig. 3. The direct plot of Fig. 4 yielded a value of K_d of 1.0 × 10⁻⁴ M, compared to 10⁻² M, relative to a control of k₁ of 2.3 × 10⁻² min⁻¹, were obtained with MgCl₂ at 10 and 30 mM, respectively. These small effects of MgCl₂ could be ascribed solely to ionic strength, since KCl and NaNO₃ at the same ionic strength as the highest concentration of MgCl₂ (30 mM) gave the same value of k₁. It was concluded that magnesium alone had a negligible effect and that, in agreement with Wuts and Rabin (8), increasing ionic strength slightly decreased the rate of alkylation. For the range of ionic strengths generally used in this investigation, the ionic strength differences could be neglected.

Some experiments were also carried out with molar ratios of enzyme to iodoacetic acid concentration of 1:2 (enzyme, 1.22 × 10⁻⁴ M; iodoacetic acid, 2.5 × 10⁻⁴ M) in 0.05 M N-ethylmorpholine, pH 8.0, at 25°C. Under these conditions the reaction was apparently second order with a rate constant of approximately 280 × 10⁻⁵ min⁻¹. In preliminary experiments the free nucleotides were found to inhibit in the order ADP > 2'-dADP > IDP > GDP, and the magnesium nucleotides were observed to activate the reaction. As this behavior appeared identical with that observed at lower concentrations of enzyme, these experiments were not pursued. A detailed study of the activating effect of MgADP⁻ (and the other metal nucleoside diphosphates), which differs in some respects from previously reported results (8, 9), will be presented elsewhere.

Calculation of k₂ and Dissociation Constant, K_d—The value of k₂, the rate constant for the reaction between iodoacetic acid and the enzyme-nucleotide complex, could be extracted from the observed rate constant, k′, which is a sum of the weighted rate constants of the free enzyme, k₁, and of the complexed enzyme, k₂, by the method described under “Experimental Procedure,” i.e., by plotting k′/k₁ against (1 - k'/k₁)/[S]. The dissociation constant can also be evaluated from such a plot (cf., “Experimental Procedure”). Plots of k′/k₁ against (1 - k'/k₁)/[S], where S is ADP and 2'-dADP, respectively, are shown in Fig. 2.

The same type of analysis was used to obtain k₂ and K_d for MgADP⁻ as the enzyme modifier (see Fig. 3). However, a similar graphical analysis for the other metal-nucleotides did not prove feasible for obtaining reliable values of k₂ and K_d since the parameter (k′/k₁ - 1) varied over too small a range to permit extrapolation of the experimental points. Therefore direct plots of k′ against 1/[MS] were used to obtain approximate values of k₂ and K_d.

* Such a plot may be justified from Equation 136, as |X| approaches infinity, K(k′/k₁ - 1)|X| → 0 and k′/k₁ → k₂/k₃, i.e., k' → k₂.
that the dissociation constants determined from the effect on the reactivity of the -SH groups by different concentrations of given modifier showed good agreement with the values obtained by the measurement of other independent parameters (cf. Table III).

**Effect of Nucleoside Triphosphates on Reaction between Creatine Kinase and Iodoacetic Acid**—Investigations of the effect of the nucleoside triphosphates, ATP, 2'-dATP, GTP, and ITDP, on the...

![Figure 2](image_url)

**Figure 2.** Plot of $k'/k_1$ against $(1 - k'/k_1)/[S]$ where $S$ represents ADP (Curve 1) or 2'-dADP (Curve 2). Data are taken from experiments of Fig. 1, plotted according to Equation 13a.

![Figure 3](image_url)

**Figure 3.** Plot of $k'/k_1$ against $(k'/k_1 - 1)/[MgADP^-]$. Data are taken from experiments of Fig. 1, plotted according to Equation 13b. The line drawn intercepts the abscissa at 2.1 and the ordinate at $2.2 \times 10^2$ M⁻¹; values of $4.9 \times 10^{-2}$ min⁻¹ for $k_2$ and of $0.9 \times 10^{-4}$ M for $K_d$ were calculated.

0.9 $\times 10^{-4}$ M obtained from Fig. 3. Thus, the use of such direct plots not only was satisfactory for comparison of the relative effectiveness of the different nucleotides as modifiers but also gave reasonably reliable values of $k_2$ and $K_d$. The values of $k_2$ and $K_d$ for the ES and EMS complexes of all the nucleoside diphosphates are summarized in Table III. It should be noted that the values in parentheses were obtained from a plot of $k'$ against $(k'/k_1 - 1)/[MgADP^-]$ (see the text).

<table>
<thead>
<tr>
<th>Modifier</th>
<th>$k_2$</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>MgADP⁻⁻</td>
<td>5.0 (4.9)⁺</td>
<td>1.0 (0.9)⁺</td>
</tr>
<tr>
<td>Mg-2'-dADP⁻⁻</td>
<td>3.6</td>
<td>2</td>
</tr>
<tr>
<td>MnADP⁻⁻</td>
<td>3.5</td>
<td>~20</td>
</tr>
<tr>
<td>MgGDP⁻⁻</td>
<td>3.1</td>
<td>~20</td>
</tr>
<tr>
<td>ADP⁻⁻</td>
<td>0.44</td>
<td>1.0</td>
</tr>
<tr>
<td>2'-dADP⁻⁻</td>
<td>0.58</td>
<td>1.3</td>
</tr>
<tr>
<td>IDP⁻⁻</td>
<td>0.62</td>
<td>11</td>
</tr>
<tr>
<td>GDP⁻⁻</td>
<td>0.69</td>
<td>7</td>
</tr>
</tbody>
</table>

Values in parentheses were obtained from a plot of $k'/k_1$ against $(k'/k_1 - 1)/[MgADP^-]$ (see the text).

*This paper; results for manganese complexes.

**Table III**

*Values of $K_d$ correspond to $K_1$ for the free nucleotides and to $K_2$ for the metal-nucleotide complexes.*

![Figure 4](image_url)

**Figure 4.** Plot of $k'$ against $1/[MgADP^-]$ (Curve 1). A similar plot for $[Mg-2'-dADP^-]$ (Curve 2) is included. Data are taken from experiments of Fig. 1.

![Graph](image_url)

**Graph 3.** Plot of $k'/k_1$ against $1/[MgADP^-]$ (Curve 1). The line drawn intercepts the abscissa at 2.1 and the ordinate at $2.2 \times 10^2$ M⁻¹; values of $4.9 \times 10^{-2}$ min⁻¹ for $k_2$ and of $0.9 \times 10^{-4}$ M for $K_d$ were calculated.
to explain the effects as in the usual template theory of substrate hindrance due to structural changes in the substrate suffices to

sequence, is nevertheless fraught with some danger. It is necessary that the changes in the steric environment at the active site are

by iodoacetic acid reflects changes in the steric environment of manganese, and the susceptibility of the enzyme to inactivation environment of the water in the first coordination sphere of the enzyme; the PRR enhancement reflects changes in the steric

properties induced by varying the nucleotide structure enzymatic reaction as illustrated in Fig. 5 for the metal-nucleoside triphosphates.

The order of protection of the free nucleotides at low concentrations was ATP > 2'-dATP > GTP > ITP. At higher concentrations, however, there was little difference in the limiting values of k'. Thus extrapolation of plots of k' against 1/[S] yielded values for k', the value of k' at infinite substrate concentration, of approximately 0.7 \times 10^{-3} \text{ min}^{-1} for all of the nucleo-
side triphosphates.

For the metal-nucleoside triphosphates, the order of decreasing k5 (increasing protection) was MgATP > Mg-2'-dATP > MgGTP > MgITP. It might be noted that this is the same order as observed for the enhancement; again there is an inversion of GTP and ITP compared to their velocities in the enzymatic reaction. Values of k5, obtained from plots of k' against 1/[MS], were 1.8, 1.6, 1.5, and 1.4 \times 10^{-4} \text{ min}^{-1} for MgATP\textsuperscript{2+}, Mg-2'-dATP\textsuperscript{2+}, MgGTP\textsuperscript{2+}, and MgITP\textsuperscript{2+}, respectively. These values should be regarded as being very approximate, as only a few concentrations were tested for each compound. Further, the differences in the results obtained for the enzyme in the presence and absence of the modifier were small and thus subject to considerable error.

DISCUSSION

The investigations with a series of nucleoside di- and triphosphate substrates of creatine kinase reported in this paper have revealed an ordered series of two properties of the metal-nucleo-
side-enzymes complexes, namely the enhancement of the proton relaxation rate of water due to manganese and the reactivity of the essential sulfhydryl groups of the enzyme toward iodoacetic acid; the order of the substrates with regard to these two properties correlates well with the order of their reactivities in the enzymatic reaction as illustrated in Fig. 5 for the metal-nucleoside diphosphate series. There is no doubt that the changes in these properties induced by varying the nucleotide structure reflect changes in the steric environment of the enzyme; the PRR enhancement reflects changes in the steric environment of the water in the first coordination sphere of the manganese, and the susceptibility of the enzyme to inactivation by iodoacetic acid reflects changes in the steric environment of the essential sulfhydryl groups. The most plausible explanation, that the changes in the steric environment at the active site are a consequence of a conformational change in the protein structure, is nevertheless fraught with some danger. It is necessary to eliminate the obvious alternative interpretation that the steric hindrance due to structural changes in the substrate suffices to explain the effects as in the usual template theory of substrate specificity. We shall attempt to prove that the latter explanation is inadequate to interpret the observed phenomena.

It should be emphasized that the two parameters, the PRR enhancement and the —SH reactivity, are properties of two different loci within the active site of the protein that are not mutually interdependent. The former is a manifestation of the environment of the binding site of the metal-nucleotide, and the —SH group is not that site of binding. The findings that the free nucleotides never afforded complete protection of the —SH group from attack by iodoacetate and that the metal-nucleoside diphosphates in fact increase the reactivity of the —SH group provided strong evidence that the nucleotides were not binding at the susceptible —SH groups. Direct proof was obtained by PRR measurement of the binding of MnADP\textsuperscript{2+} to a completely inactivated enzyme which had two alkylated sulfhydryl groups formed by a stoichiometric reaction of 2 moles of iodoacetic acid per mole of enzyme (4, 20). This inactive form of creatine kinase bound MnADP\textsuperscript{2+} with the same association constant as the native enzyme, and, furthermore, the PRR enhancement factor, \( \epsilon \), did not differ from that of the native enzyme. In the same experiment, excess ADP\textsuperscript{2+} showed binding to the inactive enzyme in a manner similar to its binding to native enzyme. Thus it may be concluded that the binding of metal-nucleotide substrates and free nucleotide inhibitors does not occur at the —SH groups which are essential for activity.\(^7\)

It might be argued that although the binding of the nucleotides does not occur directly on the relevant —SH groups, the binding site is located in a region very close to these —SH groups and a change in structure of the substrate is sufficient to affect two groups in close spatial contact. This reasoning is untenable in view of the finding that the binding of metal-nucleotides affects the reactivity of the —SH groups but that carboxymethyl-

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\(^7\) W. J. O'Sullivan and M. Cohn, unpublished results.
The PRR enhancement factor, $\epsilon$, is the ratio of $1/T_{1M}$ for a manganous complex to $1/T_{1P}$ for the manganous aquocation; $1/T_{1P} = P/(\tau_M + T_{1M})$ (see Equation 20 of the preceding paper), where $P$ is the ratio of the number of protons in the first coordination sphere of the manganese which can equilibrate with the bulk water to the total number of protons in the solution; $\tau_M$ is the lifetime of a water molecule in the coordination sphere, i.e. the reciprocal of the chemical exchange rate of water coordinated to the metal ion; and $T_{1M}$ is the longitudinal relaxation time of the water protons in the coordination sphere of the metal ion. The term $T_{1M}$ is proportional to the reciprocal of $\tau$, the correlation time for the dipolar interaction. $\tau$, has been shown to be the rotational correlation time of water, $\tau$, for the manganous aquocation, and it is reasonable to assume that $\tau$ equals $\tau_M$ in the complexes as well. Since all values of $\epsilon$ which have been observed in binary and ternary complexes of manganese-nucleotide and manganese-nucleotide-creatine kinase are greater than 1, $1/T_{1P}$ is greater for all complexes than in the manganous aquocation. Of the three parameters, $P$, $\tau_M$, and $T_{1M}$, which may change in the complex, $P$ can only decrease, which would decrease $1/T_{1P}$; $\tau_M$ in the aquocation is so small that it may be neglected and thus a further decrease in the complex would have no effect, although an increase in $\tau_M$ in the complex would cause a corresponding decrease in $\epsilon$; $\tau$, i.e. $1/T_{1M}$, must therefore increase in all the complexes in order to explain the observed enhancement, greater than 1, of all the complexes. The increase in $\tau$, which is found in all complexes investigated in this study indicates that the rotational motion of the water coordinated to the metal ion is hindered in all complexes.

In the ternary complexes, unlike the binary complexes, $\epsilon$ values change with changes in the nucleoside structure. Since $P$ remains constant, a decrease in $\epsilon$, can result only from an increase in $\tau_M$ or a decrease in $\tau$, so that $\tau$ or $\tau_M$ or both must be a function of nucleoside structure. Steric changes have been induced by the different nucleotides; either these changes hinder the rotational motion of the water to different extents, or the hindrance of rotational motion may remain unchanged as an increase proceeds from ADP to IDP, and the chemical exchange rate of the water, $1/T_{1M}$, decreases in an ordered manner for the series of ternary complexes. Another alternative is that both $\tau$ and $\tau_M$ have changed. This ambiguity should eventually be resolved by temperature dependence studies (cf. the preceding paper (7)). Whether the decrease in enhancement within nucleotide series is due to increased rotational motion or to an increased chemical exchange rate or both is not crucial to the argument. In either case, a steric effect of the nucleoside moiety on the ternary complexes and not in the binary complexes must be invoked to explain the observations, and it is reasonable to conclude that the steric changes are due to changes in the conformation of the additional component, namely the protein, in the region of binding of the metal-nucleotide.

An increase in the reactivity of an amino acid residue, as served with the cysteine residues of creatine kinase in this investigation upon binding of metal-nucleoside diphosphate substrates, cannot be explained on the basis of steric hindrance of the substrate, as previously pointed out by Yankelev and Koshland in an investigation of phosphoglucomutase (18). The data for each of the nucleoside di- and triphosphates, both binary and metal complexes, fall into a pattern when one considers the nucleoside di- and triphosphates, both binary and metal complexes, fall into a pattern when one considers the nucleoside structure. Steric changes have been induced by the different nucleotides; either these changes hinder the rotational motion of the water to different extents, or the hindrance of rotational motion may remain unchanged as an increase proceeds from ADP to IDP, and the chemical exchange rate of the water, $1/T_{1M}$, decreases in an ordered manner for the series of ternary complexes. Another alternative is that both $\tau$ and $\tau_M$ have changed. This ambiguity should eventually be resolved by temperature dependence studies (cf. the preceding paper (7)). Whether the decrease in enhancement within nucleotide series is due to increased rotational motion or to an increased chemical exchange rate or both is not crucial to the argument. In either case, a steric effect of the nucleoside moiety on the ternary complexes and not in the binary complexes must be invoked to explain the observations, and it is reasonable to conclude that the steric changes are due to changes in the conformation of the additional component, namely the protein, in the region of binding of the metal-nucleotide.
The paramagnetic ion in the region of the active site has proved the dynamic properties of water in the coordination sphere of a tial cysteine residues. Thus, a new technique which reflects is independent of the immediate steric environment of the essential for the activity of creatine kinase complexes, a parameter which enhancement of the proton relaxation rate of water in mannese-substrate-creatine kinase complexes, a parameter which exhibits the same independence on the structure of the nucleotide substrates as the essential cys- tine, is not solely a function of conformational change, the general usefulness of the PRR technique as a tool for investigating conformation at a particular binding site of the protein molecule remains to be explored. For creatine kinase, however, the finding that various nucleotide substrates affect both independent properties studied, in the same order as their relative velocities in the enzymatic reaction, gives strong support to the applicability of Koshland’s induced fit theory (6) to the active site conformation of creatine kinase as opposed to the template theory.

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REFERENCES

Nucleotide Specificity and Conformation of the Active Site of Creatine Kinase: MAGNETIC RESONANCE AND SULFHYDRL REACTIVITY STUDIES
W. J. O'Sullivan and Mildred Cohn


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