Magnetic Resonance Studies on Inactivated Forms of Creatine Kinase*

W. J. O'SULLIVAN‡ and MILDRED COHN§

From the Johnson Research Foundation, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

SUMMARY

Magnetic resonance and relative enzymatic velocity studies with the use of the paramagnetic manganese ion were carried out on creatine kinase inactivated by the specific —SH reagents iodoacetic acid and dinitrofluorobenzene and by the nonspecific reagents urea and decyl sulfate. Modification of creatine kinase at the two essential —SH groups by iodoacetic acid or dinitrofluorobenzene affected the enzymatic velocity, but had little, if any, effect on the binding constants or the environment at the binding sites of Mn-ADP-, ADP3-, Mn-2'-dADP-, or Mn-ATP2-, as determined from the enhancement of the relaxation rate of the water protons due to formation of ternary complexes of enzyme, manganese, and nucleotide.

The addition of creatine to the ternary Mn-ADP-enzyme complex with the native enzyme caused a change in the proton relaxation rate measured over the temperature range, 2°-45°; as the temperature decreased the binding constant of Mn-ADP changed slightly, but the creatine-binding constant increased by a factor of about 30. The effect of creatine on the proton relaxation rate of water could be ascribed to a decreased rate of exchange of the manganese water ligands with the solvent water in the quaternary complex relative to the ternary complex and to a longer correlation time for the dipole-dipole interaction between manganese and water in the coordination sphere because of increased immobilization at the binding site of Mn-ADP. No such effect of creatine was observed for enzyme with the two essential cysteine residues carboxymethylated, indicating either that creatine is no longer bound to this form of the enzyme or that carboxymethylation of the essential —SH groups prevents interaction between the creatine and manganese-nucleotide-binding sites.

The decrease in the enhancement of the proton relaxation rate of the ternary complex with increasing urea concentration was correlated with loss of activity. The inactivation of creatine kinase by high concentrations of urea was reversible. The inactivation of creatine kinase by decyl sulfate was irreversible. In the presence of 0.01 M decyl sulfate, relatively high values were obtained for the enhancement of the proton relaxation rate of the manganese-enzyme complex, and this enhancement decreased on the addition of ADP, the reverse of the behavior of the native enzyme.

The use of magnetic resonance techniques to study the interaction between creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) and its manganese-nucleotide substrates has been described in a number of recent communications from this laboratory (1-4). In particular, the measurement of the proton relaxation rate of water was used to show that there is little direct interaction between the enzyme and the activating metal, Mn²⁺. Rather, the enzyme interacts with the metal complex form of the adenine nucleotides, viz. Mn-ADP²⁻ and Mn-ATP²⁻, and the respective binding constants can be evaluated. The formation of the abortive quaternary complex, Mn-ADP-creatine kinase-creatine, can also be investigated by its effect on the PRR of water.

To explore the potential use of PRR measurements in specifying the binding sites of the substrates, the PRR measurements have been extended to the study of the binding of substrates to modified forms of creatine kinase. For the two reagents, iodoacetic acid and dinitrofluorobenzene, conditions have been described such that the reaction with creatine kinase can be restricted to the two essential —SH groups of the enzyme (5-7). In this paper results are presented on the binding of substrates,
namely ADP, 2′dADP, ATP, and creatine, to creatine kinase which had been partially or wholly modified at its essential —SH groups by these reagents. Some experiments with less specific reagents, urea and decyl sulfate, are also reported.

**EXPERIMENTAL PROCEDURE**

**Materials**

Dinitrofluorobenzene was obtained from Calbiochem. Urea (reagent grade) was recrystallized from hot water and aqueous solutions were prepared daily as required. Sodium decyl sulfate was a gift from Dr. F. Karush. Other reagents were as described previously (3, 4). The crystalline creatine kinase used had a specific activity of 60 to 65 Kuby units (8).

**Methods**

Reaction of Creatine Kinase with Iodoacetic Acid—The reaction between creatine kinase and iodoacetate was carried out essentially as described by Mahowald, Noltmann, and Kuby (5) and by Thomson, Eveleigh, and Miles (6). Enzyme at 40 mg per ml (0.5 mM, corresponding to 1.05 μmol iodoacetate per ml) at 0.05 M N-ethylmorpholine-HCl, pH 8.0, in a total volume of 0.2 ml for 30 min at 30°.

Reaction of Creatine Kinase with Dinitrofluorobenzene—Dinitrofluorobenzene was diluted in ice-cold isopropyl alcohol immediately before use. In separate experiments, enzyme at 40 mg per ml was allowed to react with 0.5 mM or 0.75 mM dinitrofluorobenzene in 0.05 M N-ethylmorpholine-HCl, pH 8.0, in a total volume of 0.1 ml for 40 min at 0° (5).

Assay of Creatine Kinase Activity—Creatine kinase activity was followed with a Radiometer pH-stat TTT1c as described previously (3).

**Magnetic Resonance Measurements**—The longitudinal proton relaxation rate, 1/\(T_1\), of water protons was measured by a pulsed nuclear magnetic resonance method at 25 megacycles per sec (9). The observed enhancement of the PRR, \(e^*\), in systems containing manganese and a complexing agent is defined (10) as the ratio of the paramagnetic contribution to the relaxation rate, 1/\(T_1\), for the solution containing the complexing agent to 1/\(T_1\) in the same solution in the absence of the complexing agent.

\[
e^* = \frac{1/T_{1p}}{1/T_{1}}
\]

The observed relaxation rate, 1/\(T_{1p}\), is the sum of contributions from the paramagnetic species, the complexing agent, and water; to obtain \(e^*\) from the observed relaxation rates it is assumed that each contribution is independent.

\[
e^* = \frac{1/T_{1p}}{1/T_{1}} = \frac{1/T_{1}}{} - \frac{1/T_{10}}{1/T_{1}} = 1/\frac{T_{1} - 1/\frac{T_{10}}{}}{1/\frac{T_{10}}{}}
\]

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 complexing agent. In a solution, 1/\(T_{1p}\) and therefore \(e^*\), will be a weighted average of each paramagnetic species present (cf. Equation 2 of Reference 9), i.e.

\[
e^* = \frac{M_{n}}{M_{n} + M_{n} + M_{n} + M_{n} + M_{n} + M_{n}}
\]

Free manganese concentration was measured by the intensity of its electron paramagnetic resonance spectrum with the use of a Varian EPR Spectrometer model V 4500 A at 9.5 × 10⁹ cyklos per sec.

Experiments were carried out in 0.05 M N-ethylmorpholine-HCl, pH 8.0, with a total concentration of MnCl₂ of 0.1 mm and various concentrations of nucleotide and creatine kinase.

**RESULTS**

Formation of Ternary Enzyme-Metal-Substrate Complexes with Modified Forms of Creatine Kinase—Experiments were carried out on three chemically modified forms of the enzyme: Enzyme I, product of reaction with 1.0 mole of dinitrofluorobenzene per mole of enzyme, relative activity 0.47; Enzyme II, product of reaction with 1.5 moles of dinitrofluorobenzene per mole of enzyme, relative activity 0.25; Enzyme III, product of reaction with 2.1 moles of iodoacetic acid per mole of enzyme, relative activity <0.01. In Table I, the enhancements of the ternary enzyme-Mn-ADP complexes obtained with these enzymes are compared with the enhancements of the native enzyme under the same conditions and with the same concentrations of manganese and ADP. Values of \(e^*\), the enhancement at infinite metal-substrate concentration with a finite concentration of enzyme, are included in this table.

**Table I**

<table>
<thead>
<tr>
<th>[ADP] (mM)</th>
<th>(e^*) (native enzyme)</th>
<th>(e^*) (modified enzyme I)</th>
<th>(e^*) (modified enzyme II)</th>
<th>(e^*) (modified enzyme III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.26</td>
<td>1.36</td>
<td>1.20</td>
<td>1.12</td>
</tr>
<tr>
<td>0.2</td>
<td>2.81</td>
<td>3.15</td>
<td>3.05</td>
<td>2.61</td>
</tr>
<tr>
<td>0.4</td>
<td>3.98</td>
<td>4.40</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>0.5</td>
<td>4.89</td>
<td>5.11</td>
<td>4.73</td>
<td>4.41</td>
</tr>
<tr>
<td>0.7</td>
<td>6.00</td>
<td>5.88</td>
<td>5.39</td>
<td>5.29</td>
</tr>
<tr>
<td>1.0</td>
<td>7.1</td>
<td>6.52</td>
<td>6.52</td>
<td>6.52</td>
</tr>
<tr>
<td>1.4</td>
<td>6.00</td>
<td>5.82</td>
<td>5.82</td>
<td>5.82</td>
</tr>
<tr>
<td>(e^*)</td>
<td>10.5</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

From the results shown in Table I, it is clear that the modified forms of creatine kinase were still capable of binding Mn-ADP⁻. Indeed, by the criterion of PRR measurements, there were only slight differences in the binding of Mn-ADP⁻ to the native enzyme and to any of the forms which had been modified at the essential —SH groups. Thus it would appear that the environment of the Mn-ADP⁻ in the ternary complex is similar in each case. A small decrease of approximately 15% in \(e^*\) was observed consistently with the E-Mn-ADP complex of creatine kinase III as compared to the native enzyme. This difference is beyond the
Further, there was no significant difference in the dissociation constant, $K_s$, of Mn-ADP from the EMS complex with the inactive enzyme as compared to the native enzyme. Calculations from two experiments gave values of $0.8 \times 10^{-4}$ M and $0.4 \times 10^{-4}$ M of $K_s$ for the fully inactivated enzyme (III). These values are in good agreement with the value of $0.5 \times 10^{-4}$ M reported by O'Sullivan and Cohn (3) for the native enzyme.

It was found that in a titration of the completely inactivated enzyme (III) with ADP at constant MnCl$_2$ concentration (Column 5 in Table I), the enhancement first increased with increasing ADP concentration, reached a maximum, and then decreased. This pattern is the same as that observed with native creatine kinase (2, 3) due to competition between metal-ADP and free ADP, thus indicating that ADP$^\ast$, as well as Mn-ADP$^\ast$, binds to the modified enzyme. The fully inactivated enzyme was also able to bind Mn-2'-dADP with no significant difference from the results obtained with unmodified enzyme.

The carboxymethylated derivative of creatine kinase was also able to bind Mn-ATP. With fully inactivated creatine kinase at approximately 4 mg per ml (0.05 mM) and MnCl$_2$ (0.1 mM) in 0.05 N-ethylmorpholine-HCl, pH 8.0, enhancements of 2.6 and 1.7 were obtained with ATP concentrations of 0.1 and 0.02 mM, respectively. Corresponding values with the unmodified enzyme under the same conditions were 4.1 and 1.9, respectively. Care had to be exercised to obtain initial values of $e^\ast$ in experiments with ATP, since the value of $t$ for the E-Mn-ATP complex increased with time as had been observed with the native enzyme.

### Formation of Abortive Quaternary Complex, Mn-ADP-Enzyme-Creatine: Experiments with Native and Carboxymethylated Creatine Kinase

The addition of creatine to a solution containing creatine kinase, manganese, and ADP caused a decrease in the activity of creatine kinase.

In the earlier papers from this laboratory on creatine kinase (2, 3), the calculation of $K_s$, the dissociation constant of the metal-nucleotide from the EMS complex by Procedure I (3) was incorrectly formulated. At the half-way point in the titration curve, i.e. where $e^\ast = (e_0 + e_i)/2$, half of the metal nucleotide is bound to the enzyme and half is free; therefore

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

and not [M][S] as stated. The value of $K_s$ for Mn-ADP (Reference 3, p. 3109) remains unchanged, but the value of $K_s$ for Mn-ATP (ibid., p. 3110) is changed slightly when calculated by Procedure I from $0.4 \times 10^{-4}$ M to $0.7 \times 10^{-4}$ M, and the average value in Table II (ibid., p. 3109) would be $1.0 \times 10^{-4}$ M rather than $1.1 \times 10^{-4}$ M. Similarly, in Table I of Reference 4, the value of $K_s$ for Mn-ADP and 3'-dADP remain unchanged, and the values for 2'-dADP and IDP are changed from $0.9 \times 10^{-4}$ M and $10 \times 10^{-4}$ M to $0.8 \times 10^{-4}$ M, and $7 \times 10^{-4}$ M, respectively.

A small increase in enhancement of the ternary complex of the carboxymethylated enzyme and Mn-ATP was observed as a function of time; a similar phenomenon had been observed with the native enzyme (3). In the latter case, this phenomenon was attributed to a weak ATPase activity of the enzyme with consequent formation of E-Mn-ADP, which has a higher value of $t$ than the ternary complex of ATP. It may be inferred that the carboxymethylated enzyme retains the ATPase activity, although such a result would appear to be inconsistent with the results of Sasa and Noda (11) on the effect of various —SH inhibitors on the ATPase activity of creatine kinase.

### Table II

<table>
<thead>
<tr>
<th>ADP</th>
<th>Native enzyme</th>
<th>Carboxymethylated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>$e^\ast$</td>
<td>$e^\ast$</td>
<td>$\Delta e^\ast$</td>
</tr>
<tr>
<td>$x \times 10^4$ M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>4.70</td>
<td>2.35</td>
</tr>
<tr>
<td>0.5</td>
<td>5.39</td>
<td>3.90</td>
</tr>
<tr>
<td>1.0</td>
<td>7.12</td>
<td>3.60</td>
</tr>
<tr>
<td>1.5</td>
<td>7.06</td>
<td>3.23</td>
</tr>
</tbody>
</table>

Enhancements for the native creatine kinase and for a modified enzyme (completely inactivated by carboxymethylation of two essential —SH groups with iodoacetic acid) was measured in the absence of creatine, $e^\ast$ and in the presence of 20 mM creatine, $e^\ast$, at the ADP concentrations indicated. The concentration of MnCl$_2$ was 0.1 mM; other experimental conditions were the same as in Table I.
TABLE III
Variation of PRR enhancement of E-Mn-ADP complex with creatine concentration

Various concentrations of creatine as indicated were added to solutions containing 0.11 mM ADP and 0.1 mM MnCl₂ with native creatine kinase or with a modified enzyme (the two essential —SH groups carboxymethylated). The temperature was 19°; other conditions were as in Table I. Free manganese concentrations were obtained by EPR measurements. Δκ is decrease in ε upon addition of creatine.

<table>
<thead>
<tr>
<th>Creatine (mM)</th>
<th>Native enzyme</th>
<th>Carboxymethylated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ε₀</td>
<td>Δε₀</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
<td>1.8</td>
</tr>
<tr>
<td>10</td>
<td>6.7</td>
<td>2.4</td>
</tr>
<tr>
<td>20</td>
<td>5.4</td>
<td>2.7</td>
</tr>
<tr>
<td>50</td>
<td>5.3</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Fig. 1. Double reciprocal plot of Δε₀, the change in observed PRR enhancement of E-Mn-ADP solutions at various creatine concentrations, against creatine (Cr) concentration. The plot is made from data given in Table III.

Its absence. A calculation, based on this hypothesis, by Procedure III of Reference 1 showed that the dissociation constant of Mn-ADP⁻ from the quaternary complex (with creatine 50 mM) was approximately half of that from the ternary complex. The kinetic studies of Morrison and James (13) had indicated that Mg-ADP⁻ is bound more strongly to creatine kinase in the presence of creatine than in its absence, although the difference was small, viz. dissociation constants of 1.2 × 10⁻⁴ M and 1.7 × 10⁻⁴ M, respectively.

Effect of Temperature on PRR of Various Creatine Kinase Complexes—It was pointed out in a previous paper (3) that the magnitude of the creatine effect on the E-Mn-ADP complex is temperature dependent. The difference between the observed enhancements in the presence and in the absence of creatine was found to change from positive to negative values as the temperature was lowered. Recent studies (14) have indicated that eₐ, the enhancement of the ternary Mn-ADP-enzyme-creatine complex, are equal in magnitude at −20°. Below 20°, eₐ becomes increasingly lower than e₀ as the temperature is lowered; above 25°, eₐ is greater than e₀.

Thus, the failure to observe significant differences, firstly between the ε values of Mn-ADP complexes of the native and modified enzymes, and secondly between the ε values of the ternary EMS complex and quaternary EMS-creatine complex of the carboxymethylated enzyme, might be a fortuitous result due to the particular temperature of the measurements. To eliminate this possibility, PRR measurements were extended to cover the range, 2–43°. The results of the experiments over this temperature range are illustrated in Fig. 2 for four sets of conditions: all solutions contained 0.1 mM MnCl₂ and 0.1 mM ADP in 0.05 M N-ethylmorpholine, pH 8.0; in addition they contained 1a, native enzyme, 4 mg per ml; 1b, same as 1a with 30 mM creatine; 2a, inactive, carboxymethylated enzyme, 4 mg per ml; 2b, same as 2a with 30 mM creatine. The logarithm of the paramagnetic contribution to the relaxation rate, 1/T₁p, is plotted against the reciprocal of the absolute temperature (cf. Reference 15). Unlike the native enzyme, at all temperatures tested, no significant change in 1/T₁p was observed upon addition of creatine for the carboxymethylated enzyme (cf. Curves 2a and 2b). Furthermore, over the whole temperature range the curves containing the ternary complexes of the modified and unmodified enzyme (Curves 1a and 2a) remain parallel, but a very dramatic difference is found for the corresponding quaternary complexes (Curves 1b and 2b).

It should be borne in mind that the experiments in Fig. 2 were carried out with creatine kinase, 4 mg per ml (0.05 mM enzyme, equivalent to 0.1 mM active sites); MnCl₂, 0.1 mM; and ADP, 0.1 mM; in 0.05 M N-ethylmorpholine- HCl, pH 8.0. 1a (•), unmodified enzyme; 1b (○), unmodified enzyme in the presence of creatine (30 mM); 2a (△), inactive, carboxymethylated enzyme; 2b (◇), inactive, carboxymethylated enzyme in the presence of creatine (30 mM).
carried out only at one set of concentrations of the reaction components. Under these conditions ~35% of the manganese was in the ternary EMS complex, and each point on the curve represents a composite of several species. If one changes the concentration of the components, the shape of the curve changes; not only are there changes in dissociation constants of the various complexes with temperature, but the temperature coefficient of the PRR is different for each species. The data in Fig. 2 are strictly valid for comparative purposes, but insufficient for calculating quantitatively the temperature dependence of the PRR of the individual species. In a more extensive investigation (14) the temperature dependence of the ternary and quaternary complexes for the unmodified enzyme were obtained both in the presence and in the absence of saturating concentrations of creatine, and the shape of the curves is qualitatively similar although the temperature of cross-over, with and without creatine, is different. As shown in Fig. 1 of Reference 14, the relaxation rates (1/TIP) of free Mn, Mn-ADP, and the ternary creatine kinase-Mn-ADP complex all have negative temperature coefficients; the quaternary complex of the native enzyme in the lower temperature region is exceptional in that it has a positive temperature coefficient.

As discussed in previous papers (3, 14, 15), PRR temperature dependence data can be analyzed from the relationship

\[ 1/T_{IP} = \frac{p}{T_{IM} + \tau_M} \]  

(2)

where \( T_{IM} \) is the relaxation time of a water proton in the first coordination sphere of manganese; \( \tau_M \) is the residence time of a water proton in the coordination sphere, i.e. the reciprocal of the rate of chemical exchange of the water protons between the coordination sphere and the solvent; and \( p \) is the mole fraction of water protons or protons exchangeable with water in the first coordination sphere. Since the temperature coefficient of \( 1/T_{IM} \) is negative and the temperature coefficient of \( 1/\tau_M \) is positive, when the observed temperature coefficient of \( 1/T_{IP} \) is negative, it follows that its value is dominated by \( T_{IM} \) and when the temperature coefficient is positive, the value of \( 1/T_{IP} \) is dominated by \( \tau_M \).

From these considerations as shown below, certain relationships may be derived for the relative values of \( 1/T_{IM} \) for the ternary E-Mn-ADP complex and the quaternary E-Mn ADP-creatine complex; similarly, the relative values of \( 1/\tau_M \) for the two complexes may be derived. If the curves for the native enzyme-Mn-ADP complex with creatine (Ib) and without creatine (Ia) in Fig. 2 are compared, since \( p \) can be assumed to be the same for Ia and Ib, it can be seen that in the low temperature region

\[ 1/pT_{IP}(Ia) > 1/pT_{IP}(Ib) \]  

(3)

from Equation 2 and the positive temperature coefficient of Ib

\[ 1/pT_{IP}(Ib) \approx \frac{1}{\tau_M(Ib)} \]  

(4)

from Equation 2 and the negative temperature coefficient of Ia

\[ 1/pT_{IP}(Ia) \approx \frac{1}{T_{IM}(Ia)} > \frac{1}{\tau_M(Ia)} \]  

(5)

therefore

\[ 1/\tau_M(Ia) > 1/\tau_M(Ib) \]  

(6)

Thus, the addition of creatine to the EMS complex decreases the rate of chemical exchange of the water protons, \( 1/\tau_M \), as deduced from the behavior in the low temperature region. In the high temperature region, the temperature coefficients for Ia and Ib are both negative and \( 1/T_{IP}(Ib) > 1/T_{IP}(Ia) \); therefore, the relaxation rate in the first coordination sphere, \( 1/T_{IM} \), is greater in the presence of creatine (Ib) than in its absence (Ia). Since \( 1/T_{IM} \) is proportional to \( \tau_M \), which is probably a rotational correlation time, the addition of creatine to the EMS complex also decreases the rate of rotational motion.

The experiments with the unmodified enzyme were extended by titration of the creatine effect. It was found that, with addition of various concentrations of creatine to Mn-ADP-enzyme, the change in enhancement tended to a limiting value with increasing creatine concentration. Double reciprocal plots of \( \Delta^* \), the observed change in enhancement, against creatine concentration were used to determine the enhancement at infinite creatine concentration and also an apparent dissociation constant for creatine. The results of these experiments are shown in Table IV. The numbers are approximate, since the calculations were based on the approximation (discussed below) that the concentration of EMS remains constant over the temperature range, 2-43°. In any case, it is clear that the binding of creatine to the ternary complex does vary appreciably with temperature. The values listed in Table IV are probably not grossly in error, since preliminary calculations have indicated that the variation of the dissociation constant of MS from EMS varies less than 2-fold from 2° to 36°. The validity of the approximation is further reinforced by the fact that the magnitude of the observed enhancements shown in Table IV is a fairly constant fraction (approximately one-third) of the extrapolated values, \( \epsilon_0 \), for the ternary complexes. Further it is interesting to note the good agreement obtained by Kuby, Mahowald, and Noltmann (16) from equilibrium dialysis experiments on the binding of Mg-ADP to creatine kinase at 4°, to the results from kinetic experiments carried out at 30° (12, 17).

Some tentative thermodynamic parameters may be calculated from the data in Table IV. A plot of \( \log K \) for creatine against \( 1/T \) gives a straight line, from the slope of which a value of \( \Delta H \).
the enthalpy change for the binding of creatine, was calculated as approximately \(-15\) kcal per mole. \(\Delta F\) for the binding of creatine to the EMS complex at 27\(^\circ\) is approximately \(-3\) kcal (from \(\Delta F = RT \ln K_d\)). Thus \(\Delta S\) for the binding of creatine, \((\Delta H - \Delta F)/T\), equals approximately \(-40\) e.u., a large decrease in entropy which would be consistent with a contribution from a conformational change of the protein.

**Effect of Urea on PRR Enhancement and on Enzyme Activity—** The presence of urea was found to affect both the observed enhancement and the enzymatic activity to approximately the same extent in each case. Qualitatively, the enhancements obtained in the presence of urea followed the same pattern as with the native enzyme. Thus, little effect was observed with creatine kinase and manganese alone, and a much greater effect was observed with creatine kinase after it had been completely inactivated by dinitrofluorobenzene with the essential sulfhydryl groups of creatine kinase (3, 4), it has been shown that measurements of the PRR of water and of EPR spectra can provide information on the interaction of the enzyme with manganese and the substrates at the active site. In all cases, dissociation constants of the various complexes may be obtained which compare favorably with those derived from kinetic experiments and classical equilibrium investigations. The value of the enhancement of the PRR of water is highly sensitive to the environment of manganese in the samples; in some cases the enhancement of the PRR may be directly related to the conformation of the protein at its active site, but in other cases it is limited by the rate of chemical exchange of the water ligands with the bulk solvents (4, 14).

An obvious extension of this technique for investigation of active sites is to study the effect of enzyme modifications on the parameters of the enzyme-metal-substrate complexes. Some attempts to study the binding of Mn-ATP\(^{3-}\) to various modified forms of adenylate kinase\(^{4}\) have been described (18). For creatine kinase, the effect of the reaction of iodoacetic acid and dinitrofluorobenzene with the essential sulphydryl groups of creatine kinase on the binding of various substrates was chosen since it had been demonstrated by Mahowald, Noltmann, and Kuby (5) that conditions could be found such that these two reagents reacted stoichiometrically with the two essential \(-\text{SH}\) groups of creatine kinase with concommitant loss of enzymatic activity. These results have been confirmed by work in a number of other laboratories, including our own (6, 12).

The PRR experiments provide unequivocal proof that Mn-ADP\(^{3-}\), ADP\(^{3-}\), Mn-ATP\(^{3-}\), and Mn-2'-dADP\(^{3-}\) can still bind to creatine kinase after it has been completely inactivated by iodoacetic acid. The same appears to be true for enzyme inactivated by dinitrofluorobenzene. It would appear that blocking of the essential \(-\text{SH}\) groups has little if any effect on the binding of the metal-nucleotide substrate, i.e., the nucleotide does not bind to the \(-\text{SH}\) moiety, a conclusion inferred previously from studies on the kinetics of the inactivation of creatine kinase by iodoacetic acid in the presence or absence of various substrates

---

**DISCUSSION**

In a series of previous investigations on manganese-activated creatine kinase (3, 4), it has been shown that measurements of the PRR of water and of EPR spectra can provide information on the interaction of the enzyme with manganese and the substrates at the active site. In all cases, dissociation constants of the various complexes may be obtained which compare favorably with those derived from kinetic experiments and classical equilibrium investigations. The value of the enhancement of the PRR of water is highly sensitive to the environment of manganese in the samples; in some cases the enhancement of the PRR may be directly related to the conformation of the protein at its active site, but in other cases it is limited by the rate of chemical exchange of the water ligands with the bulk solvents (4, 14).

An obvious extension of this technique for investigation of active sites is to study the effect of enzyme modifications on the parameters of the enzyme-metal-substrate complexes. Some attempts to study the binding of Mn-ATP\(^{3-}\) to various modified forms of adenylate kinase\(^{4}\) have been described (18). For creatine kinase, the effect of the reaction of iodoacetic acid and dinitrofluorobenzene with the essential sulphydryl groups of creatine kinase on the binding of various substrates was chosen since it had been demonstrated by Mahowald, Noltmann, and Kuby (5) that conditions could be found such that these two reagents reacted stoichiometrically with the two essential \(-\text{SH}\) groups of creatine kinase with concommitant loss of enzymatic activity. These results have been confirmed by work in a number of other laboratories, including our own (6, 12).

The PRR experiments provide unequivocal proof that Mn-ADP\(^{3-}\), ADP\(^{3-}\), Mn-ATP\(^{3-}\), and Mn-2'-dADP\(^{3-}\) can still bind to creatine kinase after it has been completely inactivated by iodoacetic acid. The same appears to be true for enzyme inactivated by dinitrofluorobenzene. It would appear that blocking of the essential \(-\text{SH}\) groups has little if any effect on the binding of the metal-nucleotide substrate, i.e., the nucleotide does not bind to the \(-\text{SH}\) moiety, a conclusion inferred previously from studies on the kinetics of the inactivation of creatine kinase by iodoacetic acid in the presence or absence of various substrates

(4, 12). Furthermore, since the enhancement of the PRR of the ternary complex does not change appreciably upon carboxymethylation of the essential cysteine residues, this modification does not appreciably affect those structural parameters of the protein at the binding site of Mn-ADP which determine the rotational motion, i.e. $\tau_\text{m}$, involved in the proton relaxation mechanism.

As shown in Fig. 2, the PRR enhancement of the ternary E-Mn-ADP complex of the modified enzyme differs only slightly from that of the native enzyme over the temperature range investigated. The 15% difference observed is beyond the experimental error and may be attributed to several possible sources. (a) The carboxymethyl group on the cysteine residue may be sufficiently near the binding site for Mn-ADP$^-$ to cause a perturbation in the dynamic properties of the ternary complex; this possibility was considered unlikely, as the relatively large dinitrobenezene groups did not produce lower values of $E^*$.

(b) The iodoacetic acid may have reacted to a slight extent with non-essential—SH groups or with other amino acid moieties at or near the active site (cf. Reference 5). (c) The comparatively vigorous essential—SH groups with other amino acid moieties at or near the active site (cf. Reference 5).

The loss of the creatine effect with the carboxymethylated enzyme into subunits reversibly (24), a progressive lowering of the PRR of the ternary E-Mn-ADP complex but that the absence of the —SH group prevents the interaction between the two sites. That the —SH group mediates such an interaction and concomitantly causes a change in the conformation of creatine kinase has been suggested by Watts and Rabin (20). A similar concept, that the —SH group is essential for the maintenance of the correct configuration of the enzyme in the presence of these two substrates, has been suggested by Noda, Nishi, and Moore (21) and by Krass, Rato, and Noda (22). Some information on this question could, in principle, be obtained by a direct comparison of the binding of creatine to native and modified creatine kinase. This experiment had not been attempted because of the weak binding of creatine to creatine kinase (16). The finding (Table IV) that creatine binds relatively strongly to Mn-ADP-creatine kinase at low temperature suggests that a direct binding experiment would be feasible at low temperature.

Although the experiments on the effect of urea and of decyl sulfate on the PRR enhancement of the ternary E-Mn-ADP system are only exploratory and cannot yet be analyzed in terms of detailed structural changes of the enzyme, it is apparent that this parameter may serve to differentiate among various types of changes which occur when enzyme activity is lost by different treatments. Structural differences in the monomeric forms produced in dissociation by urea and lauryl sulfate, as reflected in sedimentation behavior, have been investigated in detail by Yue et al. (23). The drastic change in pattern of PRR behavior after treatment with decyl sulfate, with the appearance of altered binding sites for the manganese ion and loss of the nucleotide-binding site, is consistent with the profound changes in structure reflected in the irreversibility of the inactivation. On the other hand, after treatment with urea, which has been shown to dissociate the enzyme into subunits reversibly (24), a progressive lowering of the PRR of the ternary E-Mn-ADP complex which parallels the loss of enzyme activity is observed, but no change in the pattern of PRR enhancement of the binary and ternary complex. The apparent lack of binding of Mn-ADP to the monomeric form deduced from the lowered enhancement in urea solutions is being investigated further.

REFERENCES

2. COHN, M., Biochemistry, 2, 623 (1963).
Magnetic Resonance Studies on Inactivated Forms of Creatine Kinase
W. J. O'Sullivan and Mildred Cohn


Access the most updated version of this article at [http://www.jbc.org/content/243/10/2737](http://www.jbc.org/content/243/10/2737)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/243/10/2737.full.html#ref-list-1](http://www.jbc.org/content/243/10/2737.full.html#ref-list-1)