Magnetic Resonance and Kinetic Studies Related to the Manganese Activation of the Adenylate Kinase Reaction*

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SUMMARY

Proton relaxation rate (PRR) studies indicated that there was little direct interaction between manganous ion and adenylate kinase. However, a large enhancement of the PRR of water was observed in the presence of ATP; for the enzyme combined with manganous-ATP complex rather than directly with the metal ion. Thus adenylate kinase is similar to creatine kinase but not to pyruvate kinase, with respect to the formation of the ternary enzyme-metal-substrate complex.

The enhancement of the PRR of the ternary complex, MnATP-adenylate kinase, was determined to be 35 at 25 MC per sec, pH 8.0, and 24°. The dissociation constant of MnATP2− from the ternary complex was determined to be 0.5 × 10−4 M from the PRR data.

A combination of PRR measurements and of free manganese determinations by electron paramagnetic resonance was used to determine the dissociation constant for MnATP2− and also for the free nucleotide from the enzyme. Values of 0.9 × 10−4 M and 0.3 × 10−4 M, respectively, were obtained. These agree reasonably with previous results from kinetic and thermodynamic data.

The enhancement factors, ε, of the PRR of the ternary complexes of adenylate kinase with the manganese complexes of a number of nucleoside triphosphates were determined. The order of ε was found to be: MnATP (35) > Mn-2′-dATP (20) > MnGTP (8) > MnITP (5) > MnCTP (4) and MnUTP (4) at 24°. Also, a value of 8 for the ternary complex formed between adenylate kinase, manganous ion, and tripolyphosphate was obtained. A comparative kinetic study was carried out with these compounds with manganous ion as activator. For the purine nucleotides the order of maximum velocities was ATP > 2′-dATP > GTP > ITP. The correlation between ε and the maximum velocities could be interpreted in part as indicative of some graded degree of conformational change induced in the protein by these substrates. This correlation could not be extended to the pyrimidine nucleotides, and tripolyphosphate did not give a measurable velocity.

A temperature study of the variation of the PRR for the ternary complexes formed with adenylate kinase and MnATP2− and Mn-2′-dATP2− was carried out over the range 2–42°. The PRR increased with increasing temperature for both complexes, which is characteristic of a chemical exchange-dominated process.

The enzyme, ATP:AMP phosphotransferase (adenylate kinase or myokinase, EC 2.7.4.3) as isolated from rabbit muscle requires a divalent metal ion for activity. This requirement can be met by either magnesium or manganous ions. A kinetic study of the magnesium activation was interpreted to indicate that the active substrates for the reaction were MgADP− and ADP2− for the forward reaction and MgATP2− and AMP3− for the reverse reaction (1, 2).

The application of magnetic resonance techniques to enzyme reactions which can utilize a paramagnetic ion as the activating ion has been demonstrated in a number of studies. Measurement of the proton relaxation rate of the nuclear magnetic resonance of water, with manganese as the activating ion, has been demonstrated in a number of studies. Measurement of the proton relaxation rate of the nuclear magnetic resonance of water, with manganese as the activating ion, has been shown to be a very useful tool in the study of those enzymatic reactions which are concerned with the reversible transfer of a phosphoryl group from ATP (3, 4). It has been shown that PRR measurements can conveniently distinguish between

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1 The abbreviations used are: PRR, proton relaxation rate; EPR, electron paramagnetic resonance; HMB, p-hydroxymercuribenzoate; PPP, tripolyphosphate. In addition, throughout the text, ES, EM, MS, and EMS are used to designate the enzyme-
enzymes such as pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), where the essential metal ion is bound directly to the enzyme, and those such as creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2), where the enzyme reacts with a metal-nucleotide substrate rather than with the free metal ion. More detailed studies utilizing both PRR and electron paramagnetic resonance with pyruvate kinase (5, 6) and creatine kinase (7) have shown that magnetic resonance techniques can provide convenient and reliable values of the binding constants between these enzymes and both the manganous ion and the relevant manganese complexes formed during the respective reactions.

This paper is concerned with a magnetic resonance study of the complexes formed in the manganese-activated adenylate kinase reaction and, in particular, with the interaction between MnATP$^2+$ and the enzyme. The interactions between adenylate kinase and the manganese complexes of a number of other possible substrates of adenylate kinase were studied. Some observations on the kinetic behavior of these compounds are also reported.

**EXPERIMENTAL PROCEDURE**

**Materials**

Manganous chloride was a spectrographically standardized reagent from Johnson, Matthey and Company, Ltd., London. Other metal salts used were reagent grade.

N-Ethylmorpholine was purchased from Eastman, and Tris from Sigma. Buffers (N-ethylmorpholine and Tris) were made up at 0.5 M concentration after adjustment to the required pH with concentrated HCl, and the pH was checked on dilution.

ATP, AMP, ITP, and p-hydroxymercuribenzoate were obtained from Sigma; 2'-$d$ATP, GTP, CTP, UTP, and 2'-$d$AMP from Schwarz BioResearch. Tripolyphosphate as obtained from Monsanto Chemical Company was recrystallized from alcohol-water.

Adenylate kinase was prepared as described by Kress, Bono, and Noda (8). The stock preparation used for the magnetic resonance studies was at a concentration of 47 mg per ml in 0.001 m succinate buffer, pH 6.4, and had a specific activity of 1840 units per mg. For the kinetic studies a similar preparation at 25 Mc per sec, as described previously (9).

**Methods**

**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 Ge per sec. The bound manganese was studied by its effect on the PRR of water by a pulsed nuclear magnetic resonance method usually in a total volume of 0.1 ml.

**Calculation of Binding Constants**

Two methods were used to calculate dissociation constants.

- **Procedure 1**—The dissociation constant $K_2$ of EMS to yield $E$ and $MS$ may be obtained from the half-maximal value of $1/\tau_1$ against $1/(E)^2$ (or $1/\tau_1 = 1$ against $1/(E)^2$) of the data in the ascending limb of the titration curve.

  Then, extrapolation of a plot of $1/\tau_1$ against $1/(E)^2$ yields

  $$1/\tau_1^* - 1/\tau_1 = K_2 (E)$$

  where $T_1$ and $T_1(0)$ 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.

  Unless otherwise indicated, all PRR measurements were carried out on solutions with 0.05 m N-ethylmorpholine-HCl buffer, pH 8.0, with a total concentration of MnCl$_2$ of 0.1 m and various concentrations of nucleotide and adenylate kinase, usually in a total volume of 0.1 ml.

  **Evaluation of Enhancements**

  The procedure for determining the values of the enhancement of PRR of binary manganese-substrate complexes, $e_1$, and dissociation constants ($K_2$) have been presented by Mildvan and Cohn (5, 9).

  The general expression for the observed enhancement, $e^*$, is

  $$e^* = \frac{[M][S]}{[M][S] + [EM][S] + [EMS][S]}$$

  where $e_1$, enhancement of free Mn$^{2+}$, equals 1 by definition and $e_0$, $e_1$, and $e_2$ are enhancements of $MS$, $EM$, and $EMS$ complexes, respectively. In the case of adenylate kinase, the term containing $e_2$ was found to be negligible compared to the other terms in Equation 2. (See "Interaction between Manganese and Adenylate Kinase" under "Results.") Thus Equation 2 simplifies to

  $$e^* = \frac{[M][S]}{[M][S] + [EM][S] + [EMS][S]}$$

  The method for the evaluation of $e_2$ and the relevant theory have been described in detail for the case of creatine kinase by O’Sullivan and Cohn (7, 9), and the same procedure was used for the results reported in this paper. For a particular enzyme concentration, with constant manganese concentration, $e^*$ first increases with increasing substrate concentration and then decreases, presumably because of competition between $S$ and $MS$ for the same site on the enzyme. The enhancement, $e_2^*$, of the $EMS$ complex at $[S]_o$ and finite $[S]$ is found from extrapolation of linear plot of $1/e_2^*$ against $1/(E)$. If $[ES]$ is assumed to be negligible, half of the metal may be considered in the complexes formed in the manganese-activated adenylate kinase reaction and, in particular, with the interaction between MnATP$^2+$ and the enzyme. The interactions between adenylate kinase and the manganese complexes of a number of other possible substrates of adenylate kinase were studied. Some observations on the kinetic behavior of these compounds are also reported.

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  Then, extrapolation of a plot of $1/\tau_1$ against $1/(E)^2$ (obtained for different values of $[E]$) against $1/(E)^2$ yields

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the form of EMS, and half as MS. Then, as there is no free [M] present, $K_2 = [M][E]/[EMS] = [E]$, where $[E] = [E]_T - [EMS] = [E]_T - [M]_T$.

Procedure II (Procedure III of Reference 7)—If the value of free [M] is determined experimentally by EPR, the first term in Equation 3 may be subtracted from $e^*$ to give the enhancement of the total bound $M e^*(M)_T$.

$$e^*(M)_T = \frac{[M][e^* + [EMS]/[M]_T}$$

By successive use of the respective conservation equations for $[M]_T$, $[S]_T$, and $[E]_T$ it is possible to calculate the concentration of all species present in solution (see O'Sullivan and Cohn (7)). Thus the value of $K_2$ and also of $K_e$, where $K_e = [E][S]/[ES]$ can be calculated directly.

Temperature Dependence of Proton Relaxation Rate

The interpretation of the variation of the PRR with temperature is based on the analysis presented by Luz and Meiboom (11) of their experiments with the cobalt chloride-methanol system. The application to enzyme complexes containing divalent manganese has recently been discussed by Cohn (12).

For any molecular species, e.g. water, undergoing chemical exchange between two environments, the solvent and the coordination sphere of a paramagnetic metal ion, it was shown (11) that the paramagnetic contribution, $1/T_1P$, to the observed longitudinal relaxation rate of the protons of water, $1/T_1$, may be expressed as

$$\frac{1}{T_1} = \frac{1}{T_1^i} - \frac{1}{T_1^o} = \frac{P}{\tau_M + T_1M}$$

where $1/T_1(0)$ is defined above in connection with Equation 1. $P$ is the ratio of the number of ligands in the first coordination sphere of the paramagnetic metal ion to the total number in the solution, $\tau_M$ is the residence time of a ligand in the first coordination sphere of the metal ion (i.e., the reciprocal of the chemical exchange rate), and $T_1M$ is the longitudinal relaxation time of the ligand protons in the coordination sphere of the metal ion. $T_1M$ is inversely proportional to $\tau_o$, the correlation time for the dipolar interaction (12, 13).

Luz and Meiboom (11) discussed their results in terms of three temperature regions. In a region at very low temperatures (Region I), there was negligible chemical exchange; two separate resonance lines for the bulk solvent and the solvent in the first coordination sphere were observed. The paramagnetic ion exerted its effect on the relaxation rate of the solvent molecules through a dipolar interaction with solvent molecules outside the first coordination sphere. In this region $1/T_{1p}$ decreases with increasing temperature. As the temperature is increased, exchange starts to set in and the relaxation rate increases with increasing temperature (Region II) as $\tau_M$ decreases with increasing temperature. At relatively high temperatures (Region III), chemical exchange becomes very rapid, viz. $\tau_M \ll T_1M$, so that $1/T_{1p}$ is determined by $P/T_1M$ and again decreases with increasing temperature.

Thus, if $\log (1/T_{1p})$ is plotted as a function of reciprocal temperature, in Regions I and III this plot will have a negative slope, and in Region II a positive slope. Regions II and III are particularly relevant to the results obtained with adenylate kinase.

Measurement of Velocity of Enzymatic Reaction

Kinetic experiments were carried out with the microcolumn method of Noda (2). The reaction mixtures contained 0.004 M AMP, 0.05 M Tris-HCl, pH 7.9, and various concentrations of nucleoside triphosphates, in a total volume of 2.0 ml. MnCl$_2$ was added at a concentration equal to the total nucleoside triphosphate concentration. Experiments were run at 25° with adenylate kinase at a final concentration of 0.0155 mg/ml for the adenine nucleoside triphosphates and 2 to 20 times this concentration for the other triphosphates. The stock enzyme was first diluted (5 ml to 3.0 ml) into a solution containing 0.02 M system, 1% bovine serum albumin, 0.005 M EDTA, and 0.01 M Tris at pH 7.2. Further dilutions were made into the same mixture without EDTA. Aliquots (0.4 ml) were removed from the incubation mixtures at various time intervals and inactivated by 0.6 ml of 1 M NH$_2$OH, pH 8.0, and the nucleotides were separated on small columns of Dowex 1-X4 (Cl$^-$ form).

After washing with 1.0-ml portions of water, AMP was removed with 0.004 M HCl, and ADP was eluted with 0.006 M HCl-0.05 M NaCl and measured by its absorption at 267 mg. Some small modifications were necessary for the experiments with the monadenine nucleotides. It was found that IDP and UDP were eluted with ADP, and allowance was made for the different molar extinctions in measuring the nucleotide present. Some trace amounts of CTP were eluted with ADP, but the ADP formed could be calculated by taking measurements at 280 mg in addition to 230 mg. Neither GDP nor GTP interfered with the assay for ADP under the experimental conditions.

RESULTS

Binary Complexes of Mn$^{2+}$ with Components of Adenylate Kinase Reaction

Preliminary investigations showed that succinate buffer at the concentrations (0.05 to 0.2 mm) added with the adenylate kinase would not have any significant effect on the free manganese concentration under the conditions of the experiments reported below. It was shown previously (7) that 0.05 M N-ethylmorpholine-HCl, pH 8.0, also had a negligible effect on the free manganese.

The enhancements, $e_1$, of the binary complexes of manganese and ATP, 2'-dATP, ITP, and GTP were previously determined as approximately 1.7 (7, 14) and it was assumed that $e_1$ for MnCTP$^-\cdot$ and MnUTP$^-\cdot$ did not differ significantly from this number. The values of $e_1$ for the complexes MnADP$^-\cdot$ (1.6), MnAMP (1.3), MnPPP (1.3), and MnPP (1.2), relevant to the experiments with adenylate kinase, have also been reported (7). The stability constant for the formation of MnATP$^-\cdot$ was taken as $10^9$ M$^{-1}$, and the same value was also assumed for the stability constants of the other manganese-nucleoside triphosphate complexes (7, 14, 15). The stability constant of MnAMP, determined from the observed decrease in EPR signal on the addition of AMP to solutions of MnCl$_2$ in 0.05 M N-ethylmorpholine-HCl, pH 8.0, at 25°, was calculated to be 330 M$^{-1}$.

Interaction Between Manganese and Adenylate Kinase

As measured by PRR, there was only a slight interaction between Mn$^{2+}$ and adenylate kinase. Experiments were carried out holding the Mn$^{2+}$ concentration constant (0.1 mm) and
TABLE I
Concentration of various species in solution and calculation of \( K_s \) and \( K_e \)

<table>
<thead>
<tr>
<th>([E]_0^a)</th>
<th>([S]_0^b)</th>
<th>([e]^*)</th>
<th>([M])</th>
<th>([e]^*([M]))</th>
<th>([MS])</th>
<th>([[EM]])</th>
<th>([ES])</th>
<th>([KS])</th>
<th>([KE])</th>
<th>([K_e])</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
<td>0.7</td>
<td>5.42</td>
<td>0.49</td>
<td>4.93</td>
<td>0.39</td>
<td>0.12</td>
<td>0.08</td>
<td>0.19</td>
<td>0.34</td>
<td>1.0</td>
</tr>
<tr>
<td>0.80</td>
<td>0.4</td>
<td>4.81</td>
<td>0.80</td>
<td>4.01</td>
<td>0.09</td>
<td>0.11</td>
<td>0.01</td>
<td>0.21</td>
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<td>0.86</td>
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<td>5.57</td>
<td>0.18</td>
<td>0.26</td>
<td>0.05</td>
<td>0.16</td>
<td>0.44</td>
<td>0.4</td>
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<td>4.18</td>
<td>0.78</td>
<td>3.40</td>
<td>0.13</td>
<td>0.09</td>
<td>0.02</td>
<td>0.06</td>
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<td>1.5</td>
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<td>0.76</td>
<td>4.45</td>
<td>0.12</td>
<td>0.12</td>
<td>0.02</td>
<td>0.14</td>
<td>1.04</td>
<td>1.0</td>
</tr>
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<td>1.0</td>
<td>8.62</td>
<td>0.43</td>
<td>8.19</td>
<td>0.35</td>
<td>0.22</td>
<td>0.08</td>
<td>0.35</td>
<td>0.73</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\( a \) Concentration expressed in active sites, i.e., twice the molar concentration.

\( b \) Calculated using 10^5 for the stability constant of MnATP^- (7).

The dissociation constant \( K_s \) was obtained directly from Fig. 2 (Procedure I). For the condition that \([MS] = [EM]]\), \( e^* = (e_i + e_s)/2 = 19.8 \), and \( 1/e^* = 0.0505 \). At this value of \( 1/e^* \), \([E]_T = 0.51 \times \)

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Varying the adenylate kinase concentration over the range 0.037 mm to 0.224 mm. The highest enhancement observed (at an adenylate kinase concentration of 0.224 mm) was 1.22. Extrapolation of a reciprocal plot of enhancement against enzyme concentration (5) to infinite enzyme concentration gave a value of 1.3 for \( e_i \), the enhancement of the binary manganese-protein complex. This is less than the value of 1.6 obtained for creatine kinase (7) and at a different order of magnitude from that of 30 for manganese-pyruvate kinase (5).

Enhancement of Adenylate Kinase-MnATP Complex

A large increase in enhancement was observed when both ATP and adenylate kinase were added to MnCl₂. This is consistent with manganese being bound to the enzyme through the nucleotide as is the case with creatine kinase.

The PRR at first increased with increasing ATP concentration, passed through a maximum, and then decreased as the concentration of ATP increased. This behavior is the same as observed with creatine kinase (7, 4), and the decrease in enhancement was attributed to competition by free ATP^- with MnATP^- for the same site on the enzyme. Data are presented in Table I.

Double reciprocal plots, of the variation of \( (E^* - 1) $3 with ATP concentration, for various concentrations of adenylate kinase, are shown in Fig. 1. The extrapolation to infinite concentration of ATP was made from the region of increasing \( e^* \). The extrapolated lines gave values of \( e^* \), the observed enhancement found at infinite substrate and finite enzyme concentrations of 8, 21, 28, and 31 for enzyme concentrations of 37, 56, 112, and 224 μM, respectively. A plot of \( 1/e^* \) against \( 1/[\text{enzyme}] \) (Fig. 2) yielded a value of 38 for the enhancement, \( e_i \), of the ternary enzyme-MnATP complex.

Treatment of the same data by plotting \( 1/e^* \) against 1-ATP gave \( e_i \) as 33, with an average of 35. A second experiment confirmed this result, an average value of \( e_i \) of 33 being obtained.

Dissociation Constant of MnATP^- from Enzyme

Determinations of the dissociation constant \( (K_e) \) for MnATP^- were obtained by the procedures described under "Methods." For these calculations, it was assumed that there were two catalytic sites, each capable of binding a molecule of MnATP^- (16, 17).

\( K_e \) may be obtained directly from Fig. 2 (Procedure I). For the condition that \([MS] = [EM]]\), \( e^* = (e_i + e_s)/2 = 19.8 \), and \( 1/e^* = 0.0505 \). At this value of \( 1/e^* \), \([E]_T = 0.51 \times \)

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**Fig. 1.** Titration of 0.1 mm MnCl₂ with ATP at four different concentrations of adenylate kinase. \( 1/(e^* - 1) \) is plotted against \( 1/[\text{ATP}]_{1} \). The concentrations of adenylate kinase were: Curve 1, 224 μM; Curve 2, 112 μM; Curve 3, 56 μM; Curve 4, 37 μM. The titrations were carried out in 0.05 M N-ethylmorpholine-HCl, pH 8.0, in a total volume of 0.1 ml at 25°C.

**Fig. 2.** Double reciprocal plot of \( e^* \) values obtained from Fig. 1 against adenylate kinase concentration. The curve extrapolates to give a value of \( e_i \) equal to 38 at infinite enzyme concentration.
In a separate experiment, determinations of $e^a$ from PRR measurements and of free Mn$^{2+}$ from EPR measurements were made on the same samples. By the use of these measurements, it was possible to calculate the concentration of all species present (Procedure III of Reference 7) and thus to obtain both $K_2$ and also $K_3$, the dissociation constant of the enzyme-ATP-complex. Values of $0.9 \times 10^{-4}$ m and $3.0 \times 10^{-4}$ m (average of six estimations) were obtained (Table I). It is interesting to note that ATP$^-$ appeared to bind slightly more strongly than MnATP$^-$ to adenylate kinase. These results may be compared to those from the ultracentrifuge studies of Kuby, Mahovolad, and Noltmann (17), who obtained $1 \times 10^{-4}$ for the dissociation constants of both MgATP$^-$ and ATP$^-$ from their respective complexes with adenylate kinase.

**EPR Spectrum of MnATP-Adenylate Kinase**

It was demonstrated with creatine kinase that although the binding of MnADP$^-$ to the enzyme produced a dramatic change in the PRR, there was no change in the EPR signal of MnADP (3, 4). This would indicate that the manganese bears the same relationship to ADP in the ternary complex as in the binary complex, i.e., the bonding atoms and symmetry in the ligand environment are the same.

A similar observation was made with MnATP$^-$ and adenylate kinase. An experiment was carried out with MnCl$_2$ at 0.5 m and ATP at 1 m. Under these conditions, the dominant form of manganese is MnATP$^-$; the EPR spectrum of which has broader lines than free manganese. The further addition of adenylate kinase at a final concentration of 12 mg per ml (1.1 m) which would result in approximately 40% of MnATP$^-$ bound to the enzyme, did not cause any alteration in the spectrum, either of the usual first derivative curve or of the second derivative curve. It was concluded that there was no evidence for any change in the parameters of the MnATP$^-$ complex, which determine the EPR signal, when it was bound to adenylate kinase.

**Table II**

Comparison of enhancement (PRR), velocity, and dissociation constants of adenylate kinase-manganese-nucleoside triphosphate complexes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$e_m$</th>
<th>{{\text{10}}^{10}} M$^{-1}$</th>
<th>$v_{max}$</th>
<th>{{\text{10}}^{10}} M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnATP$^-$</td>
<td>35</td>
<td>0.5</td>
<td>6200</td>
<td>0.8</td>
</tr>
<tr>
<td>Mn-2'-dATP*</td>
<td>20</td>
<td>0.35</td>
<td>7400</td>
<td>0.4</td>
</tr>
<tr>
<td>MnGTP$^-$</td>
<td>8</td>
<td>2.5</td>
<td>2000</td>
<td>2.2</td>
</tr>
<tr>
<td>MnUTP$^-$</td>
<td>5</td>
<td>3</td>
<td>1000</td>
<td>5</td>
</tr>
<tr>
<td>MnCTP$^-$</td>
<td>4</td>
<td>1.5</td>
<td>4800</td>
<td>4</td>
</tr>
<tr>
<td>MnUTP$^-$</td>
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</tr>
<tr>
<td>MnTPP</td>
<td>8</td>
<td>1</td>
<td>N.D.$^c$</td>
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</tbody>
</table>

* $K_2$ is the dissociation constant for EMS $\rightarrow E + MS$ as determined from PRR measurements.

1 The maximum velocities are expressed as moles of AMP used per min per mole of enzyme. The $v_{max}$ and $K_4$ values were obtained from Lineweaver-Burk plots of velocity against manganese-nucleoside triphosphate. The concentrations of the metal nucleoside triphosphates present were calculated with $10^{10}$ m$^{-1}$ as the stability constant for all of these complexes and the value of $330$ m$^{-1}$ for the stability constant of MnAMP. The concentrations of free nucleotides present in solution were also taken into account by making the assumption that they competed for the same site on the enzyme and with the same binding constant as the metal complex form.

2 N.D., not detected.
of different nucleoside triphosphates, which could be correlated with the enhancements reported in Table II, was available from the literature, such a study was initiated.

The results of these kinetic experiments are included in Table II. Apart from ATP, the purine nucleotides (2'-dATP, GTP, and ITP) and the pyrimidine nucleotides (CTP and UTP) were found to act as substrates of adenylate kinase. The $K_m$ values are in agreement with the results from the magnetic resonance experiments and provide strong support for the concept that the latter measurements are related to the kinetically active species. PPP was not a substrate under the conditions tested.

It is seen from Table II that a correlation does exist between the enhancement, $e_t$, and the maximum velocity at 24° for the purine nucleotides. In each case, the order ATP $>$ 2'-dATP $>$ GTP $>$ ITP occurs. With the exception of 2'-dATP this correlation is very close and may be considered quantitative.

A quantitative correlation in the two parameters, $e_t$ and $V_{\text{max}}$, in the case of creatine kinase was interpreted as reflecting different degrees of conformational change induced in the enzyme by the substrates. The same proposition could be advanced for adenylate kinase. For the nucleotides, ATP, GTP, and ITP, relatively small changes in the purine moiety are manifested in the latter measurements are related to kinetically active species.

Experiments with 2'-dAMP indicated that it could substitute for AMP. Rates of approximately 70% of that obtained with AMP were found for the 2'-dAMP under the same conditions.

Temperature Dependence of PRR of Ternary Complexes of Adenylate Kinase

The variation of the proton relaxation rate with temperature for the ternary complexes formed between adenylate kinase and the manganese complexes of ATP and 2'-dATP, respectively, is illustrated in Fig. 3. According to the procedure of Luz and Meiboom (11), the logarithm of $1/T_1$ is plotted against the reciprocal of absolute temperature. Results for MnCl$_2$ and MnATP$^-$ are included. Measurements were made over the temperature range 2-42° and, in the case of the ternary complexes, with different concentrations of nucleotide and enzyme. Values of $e_t$, and thus of $1/T_1$, (see Equation 1), were obtained in each case by the type of reciprocal plots illustrated in Figs. 1 and 2. For MnATP-adenylate kinase, $e_t$ was determined as $10, 15, 35, 53$, and 170 at temperatures of 2, 13, 24, 35, and 42°, respectively. For Mn-2'-dATP-adenylate kinase, $e_t$ was determined as $5, 10, 18, 20$, and 28 at temperatures of 6, 13, 24, 35, and 41°, respectively. Values of $K_2$ were also obtained from these plots by means of Procedure I. For MnATP-adenylate kinase, $K_2$ was estimated as $\sim 0.2, 0.25, 0.52, 1.2$, and $3.5 \times 10^{-4}$ m at temperatures of 2, 13, 24, 35, and 42°, respectively. For Mn-2'-dATP-adenylate kinase, $K_2$ was determined as $\sim 0.1, 0.2, 0.35, 0.6$, and $2.0 \times 10^{-4}$ m at temperatures of 6, 13, 24, 35, and 41°, respectively.

Recently, Su and Russell (20) have reported that adenylate kinase isolated from baker's yeast can utilize the nucleotides, ATP $>$ 2'-dATP $>$ GTP $>$ ITP, as substrates.

Fig. 3 contains a few minor corrections to the version published April 10, 1968.
the temperature curve, and this should be borne in mind when
comparing results obtained for these complexes at a single
temperature (e.g. Table II).

The first two points are discussed immediately below. The
significance of Point 3 is further considered under “Discussion.”

Point 1—The temperature behavior of the manganous ion
aqocation in agreement with the detailed studies of Bernh-eim
et al. (21) and with the nO studies of Swift and Connick
(22). For manganous ions in water at 25°, Bernheim et al. (21)
determined $\tau_M$ as $2.5 \times 10^{-4}$ sec and $T_{1 M}$ as $2 \times 10^{-4}$ sec, i.e.
$\tau_M < T_{1 M}$ and has a negligible effect in determining $1/T_{1 P}$.

Under such circumstances, Equation 6 reduces to

$$\frac{1}{T_{1 P}} = \frac{P}{T_{1 M}} = P \tau_e$$  \hspace{1cm} (7)

We may further note that, in the manganous aqocation, the
correlation time, $\tau_e$, is determined by $\tau_M$, the rotational
correlation time, as

$$\frac{1}{\tau_e} = \frac{1}{\tau_M} + \frac{1}{\tau_i}$$  \hspace{1cm} (8)

where $\tau_i$, the electron spin relaxation time, is of the order of $10^{-8}$
sec and $\tau_M$ is of the order of $10^{-11}$ sec (21).

The enhancement of MnATP$^-$ at 25° is 1.68. As with Mn$^{2+}$,
it appears that $\tau_M \ll T_{1 M}$. Then, from Equations 6 and 7, the
expression for the enhancement (Equation 1) becomes

$$\epsilon = \frac{1/T_{1 P}}{1/T_{1 M}} = \frac{P \tau_e}{T_{1 M}} = \frac{P \tau_e}{P \tau_M} = \frac{T_{1 M}}{T_{1 M}}$$  \hspace{1cm} (9)

(\text{Note that the terms with an asterisk refer to the complexed
species; the terms without an asterisk refer to the Mn(H$_2$O)$_6^{2+}$.)

If $P^*$ is taken as 3, i.e. 3 water molecules of the Mn(H$_2$O)$_6^{2+}$
have been replaced by ligands from ATP$^-$ (23), then the forma-
tion of MnATP$^-$ has decreased the value of $T_{1 M}$ for the water
molecules remaining in the first coordination sphere of the
complexed manganese by a factor slightly greater than 3, or it
has increased the value of $\tau_e$ by the same factor. Taking the
value of $\tau_e$ at 25° as approximately $3 \times 10^{-11}$ see (24), then the
correlation time for the water protons in the MnATP$^-$ complex
is found to be slightly greater than $10^{-10}$ sec, which may be
compared with the value of 2 to $3 \times 10^{-10}$ sec obtained by
Sternlicht, Shulman, and Anderson (25) under slightly different
conditions (e.g. much higher concentrations of ATP).

It is to be expected that the major contribution to the change
in $\tau_e$ would be from a change in the rotational correlation time,
$\tau_i$. Some change in $\tau_i$ does take place on the binding of Mn$^{2+}$ to
ATP, as evidenced by broadening of the individual lines of the
EPR spectrum. However, this effect is not large, namely, of the
order of a 3-fold increase in the line width and thus a similar
decrease in $\tau_i$, so that $\tau_e$ would still be dominated by $\tau_i$ (Equation
8).

Point 2—The fact that $1/T_{1 P}$ is dominated by $\tau_M$ for the
ternary complexes over a significant portion of the temperature
range studied would indicate that $T_{1 M}$ is low for these complexes;
i.e. $\tau_e$ is high, only a lower limit being observed. In both
cases, the manganese may be considered as being in a compara-
tively highly immobilized structure at the active site of the
enzyme with respect to free manganese. At 42°, the highest
temperature studied before protein denaturation occurred, the

enhancement of the MnATP-enzyme complex was 170, the
highest enhancement so far recorded. Again taking $P^*$ as 3,
$T_{1 M}$ must have been decreased by a factor of more than 340
since $\tau_M$ still dominates $1/T_{1 P}$. Thus $\tau_e$ of the water protons in
the ternary complex has increased by more than 340 with re-
spect to Mn(H$_2$O)$_6^{2+}$. As the EPR spectrum of MnATP$^-$ is
apparently unchanged on binding to adenylate kinase (see “EPR
Spectrum of MnATP-Adenylate Kinase”) so that there is no change in $\tau_e$ then the principal change must have been in the
rotational correlation time, $\tau_i$. The same conclusion was
reached for the ternary complex, MnADP-creatine kinase (3, 4).

Approximate values of $\tau_M$ can be obtained directly from the
portion of Fig. 3, where $1/T_{1 P}$ is increasing with increasing
temperature for the two ternary complexes. The values of $\tau_M$
would be subject to a small correction because of outer sphere
relaxation. Such a correction can be obtained from extrap-
olation of Region I but has not been attempted because of
insufficient data (see Reference 11). It is also possible to obtain
$E_a$, the energy of activation of the exchange process, from the
straight line portions of the plot. Hence it is possible to calcu-
late the values for the enthalpy (ΔH) and the entropy (ΔS) of the
exchange process.

The values so obtained are summarized in Table III. Esti-
mates for the ternary complexes of adenylate kinase are compared
with values for the manganese aqocation from the nO studies of
Swift and Connick (22) and with two other enzyme systems, the
quaternary complex, MnADP-creatine kinase-creatine, and the
binary complex, manganese-pyruvate kinase (12, 27). The
values are calculated for 17° (290° K; 1/T = 3.45 $\times 10^{-4}$), so
that a valid comparison between the two ternary adenylate
kinase complexes can be made. (See Point 3, “Discussion,”
and Reference 12.)

It should be noted that for the enzyme complexes, the nu-
merical value recorded in Table III should be regarded as
approximate only, as each point of the original graph is obtained
from a double extrapolation and thus is subject to considerable
systematic error.

Other Ternary Complexes

No direct estimation of a MnADP-adenylate kinase was
possible. The addition of 0.1 mM ATP to 0.1 mM MnCl$_2$ in the
presence of 112 μM adenylate kinase gave an enhancement of
3.3. Based on the assumption that the dissociation constant of the
ternary complex with manganese substrates did not differ signifi-
cantly from those with the magnesium substrates, an approxi-
mate calculation of the species present (1) indicated that this
could be largely attributed to the MnATP·E present. It
was concluded that $\epsilon$, for MnATP·E, if it formed, was con-
siderably less than 20; i.e. the reverse of the order obtained with
ATP and ADP, respectively, in the creatine kinase system was
observed. However, such a comparison may not have any
validity as it makes a number of presuppositions, e.g. that the
enhancement of a substrate in the inactive ternary complex is
the same as it would be for the working enzyme, that MnADP$^-$ is
only bound at two sites and not at the second nucleotide site, and
that a ternary MnADP-enzyme complex is in the same temper-
ature region as MnATP-enzyme.

Under the experimental conditions used, no ternary complexes
were detected with adenylate kinase and the manganese
complexes of AMP or pyrophosphate.
TABLE III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Complex</th>
<th>( \tau_m )</th>
<th>( E_a )</th>
<th>( \Delta H^a )</th>
<th>( \Delta S^a )</th>
<th>( -T \Delta S^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Mn(^2+) c</td>
<td>6 \times 10^{-4}</td>
<td>8.7</td>
<td>8.1</td>
<td>4</td>
<td>-1.2</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>M(n)ATP-E</td>
<td>2.7 \times 10^{-2}</td>
<td>10.5</td>
<td>0.0</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mn-(^2+)-dATP-E</td>
<td>5 \times 10^{-3}</td>
<td>8.4</td>
<td>7.8</td>
<td>-1</td>
<td>0.3</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>MnADP-E-creatine</td>
<td>5.3 \times 10^{-3}</td>
<td>11.9</td>
<td>11.3</td>
<td>13</td>
<td>-3.8</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Mn-E</td>
<td>1.5 \times 10^{-4}</td>
<td>19.9</td>
<td>19.3</td>
<td>48</td>
<td>-14</td>
</tr>
</tbody>
</table>

\( a \) Calculated from \( \Delta H^a = E_a - RT \), where \( R = 1.98 \text{ cal degree}^{-1} \text{ mole}^{-1} \) and \( T \) is absolute temperature.

\( b \) Calculated from the expression \( \frac{1}{\tau_m} = \frac{kT}{h} e^{-\frac{\Delta H^a}{RT}} \) (see Reference 11). The following values were used for the respective constants: \( h = 6.626 \times 10^{-34} \text{ erg per sec}; k = 1.38 \times 10^{-16} \text{ erg degree}^{-1}; R = 1.98 \text{ cal degree}^{-1} \text{ mole}^{-1}; \) and \( T \) is absolute temperature (20).

HMB Derivatives of Adenylate Kinase

Kress et al. (8) have shown that it is possible to modify adenylate kinase with p-hydroxymercurobenzoate at the two reactive sulphydryl groups. The resultant adenylate kinase-mercurial complex had about 45% of the activity of the native enzyme. It would be expected that these derivatives would still bind M\(n\)ATP \(^-\) at least to some extent, and that PRR studies could be used to determine the extent of binding.

For PRR measurements it was necessary to use somewhat higher concentrations of adenylate kinase than in the experiments of Kress et al. (8). Thus, adenylate kinase at 4.7 mg per ml (2.2 \times 10^{-4} \text{ ml} \text{ corresponding to} 4.4 \times 10^{-4} \text{ ml active sites}) was incubated at 0\(^\circ\) in 0.05 \text{ m N-ethylmorpholine-ICl (pH 8.0)}-0.5 \text{ m NaCl for 3 min with 0.1 \text{ m MnCl}_2 and 0.2 \text{ m ATP}} \)- was reduced by approximately 25%.

Activation of Adenylate Kinase by Different Metal Ions

Manganese chloride was found in the present experiments to be about one-third (cf. less than one-half previously reported (2)) as effective as magnesium chloride as an activator of adenylate kinase catalyzing the reaction in the direction of utilization of ATP. Calcium chloride as activator for the reaction in the direction of formation of ATP was about one-tenth as effective as magnesium chloride (metal ion equal to one-half the ADP concentration), whereas for the reverse reaction (utilization of ATP with metal ion equal to ATP concentration) calcium was found to be as effective as magnesium. The failure of Noda in a previous report (2) to confirm the findings of Bowen and Kerwin (28) may in part be explained by the lower order of calcium activation for the reaction in the direction of formation of ATP. Cobalt chloride was found to be about one-tenth as good as magnesium as an activator for the reaction in the direction of utilization of ATP. The effectiveness of the metal ions for the reaction utilizing ATP (1 mm ATP, 1 mm metal ion, and 4 mm AMP at pH 8) was in the order Mg\(^{2+}\) > Ca\(^{2+}\) > Mn\(^{2+}\) > Co\(^{2+}\).

DISCUSSION

As inferred from previous kinetic (2) and thermodynamic studies (17), the results of the magnetic resonance experiments reported in this paper indicate that adenylate kinase forming ternary enzyme-metal-substrate complexes does not bind the metal ion directly but rather via the nucleotide substrate. The PRR enhancement factor used to calculate \( K_s \), the dissoci-
tion constant of MnATP$^*$ from the EMS complex. A combination of PRR and EPR measurements was used to determine $K_2$ and also $K_*$, the dissociation constant of free ATP$^*$ from the enzyme. For the calculation it was assumed that there were two active sites per molecule of adenylate kinase (16) and that only 1 molecule of MnATP$^*$ or ATP$^*$ was bound at each site, i.e. neither MnATP$^*$ nor ATP$^*$ competed for the AMP$^*$ site (cf. Reference 2). The results so obtained are in quantitative agreement both with values obtained by using the classical techniques of equilibrium dialysis and sedimentation gradient with magnesium ion (17) and with the kinetically determined constant (Table II).

The study was extended to a number of other nucleoside triphosphates, and characteristic enhancements were obtained for the ternary complexes of adenylate kinase with manganese and 2'-dATP, GTP, ITP, CTP, UTP, and PPP. The ability of adenylate kinase to utilize these nucleotides as substrates for AMP$^*$ sites as triphosphates, and characteristic enhancements were obtained (2). The results so obtained are in quantitative agreement both with values obtained by using the classical techniques of equilibrium dialysis and sedimentation gradient with magnesium ion (17) and with the kinetically determined constant (Table II).

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The PRR for the MnATP-enzyme and Mn-2'-dATP-enzyme was dominated by the chemical exchange time of the water molecules, from the first coordination sphere to the bulk solution, over much of the temperature range studied (see Equation 6). Thus, it was possible to calculate estimates for the chemical exchange time and for the kinetic quantities associated with this exchange (Table III).

Because of the possible large errors associated with the values in Table III, they should be regarded as approximate. However, it is interesting to note that for none of the complexes does the chemical exchange time, at 17°, differ from that of the manganous aquocation by more than one order of magnitude. Furthermore, the energy of activation of the exchange process varies little in going from Mn(H2O)62+ to Mn-adenylate kinase. There is a small and probably significant difference between the calculated thermodynamic values for MnATP-adenylate kinase and Mn-2'-dATP-adenylate kinase, and this could well be attributed to differing conformations of the respective ternary complexes. As with Mn(H2O)62+, the magnitude of $\Delta F^\ddagger$ is dominated by the enthalpic term. A somewhat greater difference is seen with the quaternary MnADP-creatine kinase-creatine complex, and a striking difference with the binary manganese- pyruvate kinase complex. In the latter case, the value of $E_a$ is considerably larger and $\Delta F^\ddagger$ is dominated by the entropic term.

Again, a more detailed analysis of the variation of PRR with temperature for adenylyl kinase complexes must await further studies. Of particular interest might be a study of the temperature behavior of the MnPPI-creatine kinase complex. This complex not only has a fairly large enhancement; but the MnPPI binds to the enzyme nearly as strongly as MnATP, so that detailed information about the phosphate binding site could well be forthcoming.

The finding that Ca2+ also activates the adenylyl kinase reaction correlates with the empirical classification of Cohn (4, 12) of enzyme-metal-substrate interactions into two groups, based on PRR measurements. The first group, where the enhancement of the PRR of the ternary EMS complex is much greater than that of the binary EM complex, is activated by Ca2+ in addition to Mg2+ and Mn2+. Examples of this type are creatine kinase and 3-phosphoglycerate kinase (EC 2.7.2.3) (4), and adenylyl kinase may be added to this list. Recently, it has also been found that arginine kinase (ATP: L-arginine phosphotransferase, EC 2.7.3.3) as isolated from both Homarus vulgaris and Homarus americanus is also of this type. Examples of the second kind, where the enhancement of the PRR for EMS is much greater than that for EMS, are pyruvate kinase and phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase or enolase, EC 4.2.1.11) (4, 6, 12), and Ca2+ is an inhibitor of these enzymes.

Finally, we should like to draw attention to the PRR experiments with adenylyl kinase which had been modified with HMB. The PRR technique offers a very convenient and fast technique for studying the binding properties of enzymes with altered or negligible activity and could provide a very powerful tool in the study of modified enzymes. Studies in this direction have recently been reported for adenylyl kinase (30), creatine kinase (31), and pyruvate carboxylase (32).

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