NMR Studies of the Nucleotide Conformation and the Arrangement of Substrates and Activators on Phosphoribosylpyrophosphate Synthetase*

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The paramagnetic effects of Mn²⁺, an essential activator bound at the active site of phosphoribosylpyrophosphate synthetase, on the longitudinal relaxation rates of the phosphorus atoms of the bound substrates tetraaminecobalt(III)-βγ-phosphate-ATP and ribose 5'-phosphate, the substrate analog tetraaminecobalt(III)-βγ-phosphate-αβ-methylene-ATP, the products AMP and adenosine 5'-O-(thiophosphate) and the activator bound to calculate Mn²⁺-phosphorus distances. These data, together with the measured distances from Mn²⁺ to the protons of the substrate tetraaminecobalt(III)-βγ-phosphate-ATP were used to determine the preferred conformation of the enzyme-bound nucleotide. The metal-phosphorus distances (4.6 to 6.5 Å) indicate second sphere complexes of the nucleotide substrate and the AMP and adenosine 5'-O-(1-thiotriphosphate) products. The conformation of the enzyme-bound nucleotide is somewhat folded with a torsional angle at the glycosidic bond (x = 62 ± 5°) which differs by at least 20° from that found free in solution. The enzyme-nucleotide interaction that causes such a conformational change, as found with other ATP-utilizing enzymes, correlates with the high base specificity of these enzymes. Upon binding of ribose 5'-phosphate to phosphoribosylpyrophosphate synthetase, no movement of the adenine base portion of the bound nucleotide occurs, as indicated by the absence of changes in the Mn²⁺-proton distances, but a significant conformational change occurs in the polyphosphate chain as manifested in 1.2 Å and 0.8 Å increases in the distances from Mn²⁺ to the P₁ and P₃ atoms, respectively. The exchange rates of the nucleotide triphosphate substrates (10⁶ s⁻¹) and the nucleotide products (10⁸ s⁻¹) from their respective enzyme-Mn²⁺ complexes indicate that they are kinetically competent to participate in catalysis since k₄,n ~ 22 s⁻¹. The essential activator P₁ is found to bind not only at the active site 6.3 Å from the bound Mn²⁺, but also at the 5'-P site of ribose 5'-phosphate and at the P₇ site of tetraaminecobalt(III)-βγ-phosphate-ATP when these substrates are not present. The structural data are used to determine the arrangement of the substrates at the active site and, together with kinetic data, to examine further the role of the enzyme-bound metal.

The synthetase, which catalyzes a pyrophosphoryl transfer from ATP to the α1-OL group of ribose-5-P, is a member of that class of ATP-utilizing enzymes which requires two divalent cations per active site (1-3). Previous studies with PP-Rib-P synthetase have shown that the β isomer of the substitution-inert βγ-bidentate Co(NH₃)₄ATP is a slow substrate for the enzyme, with a maximal velocity of that found with MgATP. Activity was detected only in the presence of an added divalent cation, which was found to bind directly to the enzyme (3). Hence, one of the two metal ions required for activity is on the nucleotide substrate, while the other is located elsewhere on the enzyme. Kinetic studies with ATPαS using Mg²⁺ or Cd²⁺ as activators (4) have suggested that one of the two metal activators, possibly the enzyme-bound metal, interacts directly with the α-phosphoryl group of the nucleotide sometime during catalysis.

The availability of substitution-inert complexes of ATP with trivalent cations allows one to substitute a paramagnetic cation probe selectively at either the nucleotide-metal site or the enzyme-metal site and to determine distances from the probe to various atoms of the substrate molecules by NMR relaxation measurements. In a previous paper, studies of this kind were described that used PP-Rib-P synthetase and the αβγ-tridentate Cr(III)(H₂O)₄ATP complex (5). In the present work, Mn²⁺ was used as the paramagnetic probe and binding of Mn²⁺ to ATP was prevented by use of βγ-Co(III)(NH₃)₄ATP or its analogs. The results have been used to determine the spatial arrangement and conformation of enzyme-bound substrates and of enzyme-bound activators.

EXPERIMENTAL PROCEDURES

Materials—PP-Rib-P synthetase was purified to homogeneity from Salmonella typhimurium and assayed as previously described (6). AMP and ATP were purchased from Sigma, AMPCPP from P-L Biochemicals, AMPαS from Boehringer Mannheim, and Rib-5-P from Calbiochem. ATPαS was a generous gift from Prof. F. Eckstein, Max Planck Institute für Experimentelle Medizin, Göttingen, West Ger-

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many. The \( \beta,\gamma \)-bidentate \( \text{Co(NH}_3\text{)}_4\text{ATP} \) and \( \text{Co(NH}_3\text{)}_4\text{AMP} \text{CPP} \) complexes were prepared as previously described (7), and their structure and purity were established by thin layer chromatography and \( ^{31} \text{P} \) NMR. The \( \Delta \) isomer of \( \text{Co(NH}_3\text{)}_4\text{ATP} \) was prepared from the racemic mixture with hexokinase (3, 8).

Methods—Dissociation constants of various \( \text{Mn}^{2+} \) complexes were determined by EPR, which was used to monitor the free \( \text{Mn}^{2+} \) in solution, using a Varian E-4 spectrometer operating at 9.1 GHz. \( ^{31} \text{P} \) NMR spectra were recorded at 145.8 MHz on a Bruker WM 250 spectrometer and at 100 MHz on a Varian XL-100-FT spectrometer. \( ^{1} \text{H} \) NMR spectra were recorded at 250 MHz on a Bruker WM-250 spectrometer and at 100 MHz on a Varian XL-100-FT spectrometer. Longitudinal relaxation times \( (T_1) \) were measured by either 180°-\( \tau \)-90° or 90°-(homogeneity spoil)-\( \tau \)-90° pulse sequences. Transverse relaxation times \( (T_2) \) were measured from linewidths (9, 10). Solutions for the \( ^{31} \text{P} \) NMR measurements contained 20% \( \text{D}_2\text{O} \) to permit internal field-frequency locking. Solutions used in the \( ^{1} \text{H} \) NMR experiments were made up using \( \text{D}_2\text{O} \) (99.7%). Enzyme solutions were deuterated by repeated concentration and dilution by vacuum filtration using Millipore immersible ultrafiltration units. Unless otherwise noted, the experiments were carried out at 25°C to ensure the stability of the enzyme. After prolonged NMR experiments, PP-Rib-P synthetase was used as previously described (4).

RESULTS

\( ^{31} \text{P} \) NMR Characterization of the \( \text{Co(NH}_3\text{)}_4\text{ATP} \) and \( \text{Co(NH}_3\text{)}_4\text{AMP} \text{CPP} \) Complexes—The proton-decoupled \( ^{31} \text{P} \) NMR spectra at 145.8 MHz (pH 8.2) of \( \text{ATP} \), \( \text{Co(NH}_3\text{)}_4\text{ATP} \), \( \text{AMP} \text{CPP} \), and \( \text{Co(NH}_3\text{)}_4\text{AMP} \text{CPP} \) are shown in Fig. 1. Chemical-shift and spin-coupling data are given in Table I. Introduction of the methylene group between the \( \alpha \) - and \( \beta \)-phosphates of \( \text{ATP} \) causes a marked change in the chemical shifts of \( \text{P}_\alpha \) and \( \text{P}_\beta \) (i.e. 30.0 and 28.2 ppm downfield shifts, respectively) and in their spin-coupling constants (i.e. \( J_{\alpha \beta} \) decreases by 11.2 Hz, while \( J_{\beta \alpha} \) increases by 4.3 Hz). Upon formation of the \( \beta,\gamma \)-bidentate \( \text{Co(NH}_3\text{)}_4\text{amine complexes} \), \( \text{P}_\beta \) and \( \text{P}_\gamma \) shift downfield by 8.5 to 18.8 ppm and show small changes in the coupling constants. The different absolute configuration about the \( \beta \)-phosphorus in the \( \text{Co(NH}_3\text{)}_4\text{amine complexes} \) gives rise to chemical shift differences between the \( \text{P}_\alpha \) and \( \text{P}_\beta \) resonances of the two stereoisomers (\( \Delta \) and \( \Lambda \)) which are, respectively, 20, 10, and <2 Hz for \( \text{Co(NH}_3\text{)}_4\text{ATP} \), and 16, 14, and 6 Hz for \( \text{Co(NH}_3\text{)}_4\text{AMP} \text{CPP} \).

Studies of Binary \( \text{Mn}^{2+} \)-Ligand Complexes—As a control for the enzyme studies, the binary complexes of \( \text{Mn}^{2+} \) with \( \text{AMP} \text{S} \), \( \text{AMP} \text{S} \), Rib-5-P, and \( \text{P} \), were investigated. The dissociation constants of these complexes were determined by EPR and are given in Table II. The effects of \( \text{Mn}^{2+} \) on the \( ^{31} \text{P} \) longitudinal and transverse relaxation rates of the phosphorus nuclei in the above complexes were measured at varying \( \text{Mn}^{2+} \) concentrations. Calculated bound state relaxation rates are given in Table II. Since the affinity of PP-Rib-P synthetase for \( \text{Mn}^{2+} \) is high at saturating levels of \( \text{P} \) (\( K_\text{D} = 0.1 \mu \text{M} \), Ref. 3), under the present experimental conditions the enzyme, when present, bound a major fraction of the metal ion. The above dissociation constant and the parameters in Table II were used to correct the experimental \( ^{31} \text{P} \) relaxation rates in

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Complex & \( K_\text{D} \) & \text{Nucleus} & \( 1/T_{\text{lo}} \) & \( 1/T_{\text{tr}} \) & \hline
\text{Mn} \cdot \text{Co(NH}_3\text{)}_4\text{ATP} & 15 & \text{P}_\alpha & 0.9 & 0.3 \hline
\text{Mn} \cdot \text{AMP} & 8 & \text{P}_\alpha & 1.0 & 0.4 \hline
\text{Mn} \cdot \text{AMP} & 8 & \text{P}_\alpha & 1.5 & 0.2 \hline
\text{Mn} \cdot \text{Rib-5-P} & 5 & 5-P & 14.7 & 8.0 \hline
\text{Mn} \cdot \text{P} & 4 & \text{P} & 8.2 & 9.7 \hline
\end{tabular}
\caption{Dissociation constants of \( \text{Mn}^{2+} \) complexes and bound state \( ^{31} \text{P} \) relaxation rates at 145.8 MHz}
\end{table}

The uncertainty in the dissociation constants is \( \pm 30\% \) and in the bound state relaxation rates \( \pm 15\% \). pH 7.0, \( T = 25°C \).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{\( ^{31} \text{P} \) NMR spectra of nucleotides and their \( \text{Co(NH}_3\text{)}_4\text{amine complexes} \). The spectra were recorded at 145.8 MHz, at pH 8.2, \( T = 25°C \) in the presence of 1 mM EDTA. The inserts are the expanded multiplets and the bars are 20 Hz.}
\end{figure}
mixtures of Mn\(^{2+}\), enzyme, and ligands for the small contribution due to the formation of the weaker binary Mn\(^{2+}\)-ligand complexes. In the case of Co(NH\(_3\))\(_4\)ATP and Co(NH\(_3\))\(_2\)AMP•C\(_{PP}\), these contributions were found to be negligible. In the case of AMP, the correction was ~10%, while with AMP•S and Rib-5-P the corrections were ~30%. The maximal errors in the distances calculated from these relaxation rates (see below) due to uncertainties in the binding constants for Mn\(^{2+}\) are estimated to be less than 5%.

The 1/T\(_{1}\) values in Table II reflect structural differences in the binary Mn\(^{2+}\)-nucleotide complexes. For example, we have previously shown (12) that formation of the weak Mn\(^{2+}\)-Co(NH\(_3\))\(_4\)ATP complex involves interaction between the Mn\(^{2+}\) and the adenine ring only. The 40-fold greater effect of Mn\(^{2+}\) on the 31P of AMP reflects direct phosphoryl coordination. The 2.7-fold smaller effect of Mn\(^{2+}\) on 1/T\(_{1}\) of AMP•S as compared to AMP may be due to an 18% greater Mn\(^{2+}\) to phosphorous distance in this complex due to the presence of the sulfur atom. These complexes were not studied further.

NMR Studies of Enzyme Complexes—The effects on the 31P longitudinal and transverse relaxation rates of Mn\(^{2+}\) binding to such enzyme complexes as E-Co(NH\(_3\))\(_2\)AMP•C\(_{PP}\), Rib-5-P, E-Co(NH\(_3\))\(_2\)AMP•C\(_{PP}\), E-Co(NH\(_3\))\(_4\)ATP, E-AMP•S-Rib-5-P, E-AMP•S•P•P-Rib-5-P, and E-AMP•S were determined at 40.5 and 145.8 MHz. Data are exemplified in Fig. 2. Calculated bound-state relaxation rates are given in Tables III and IV. Typical concentrations used in the experiments were: enzyme, 70 to 86 µM; nucleotide, 10 to 35 mM; Rib-5-P, 10 to 30 mM; and inorganic phosphate, 40 to 45 mM. Note that Rib-5-P was added to enzyme solutions in the presence of the inactive Co(NH\(_3\))\(_2\)AMP•C\(_{PP}\) complex rather than the substrate Co(NH\(_3\))\(_2\)ATP to avoid occurrence of the reaction catalyzed by PP•Rib-5-P synthetase. Rib-5-P binding to the Mn-E-AMP complex could not be studied due to overlap (upon addition of Mn\(^{2+}\)) of the 31P resonances of Rib-5-P and AMP.

31P relaxation rates of Co(NH\(_3\))\(_2\)ATP (15.4, 17.1 mHz) were measured at 100 and 250 MHz in the presence of enzyme (73, 66 µM) and varying concentrations of Mn\(^{2+}\) (0 to 49 µM). Data are exemplified in Fig. 3. Calculated bound-state longitudinal relaxation rates and Mn\(^{2+}\)-proton distances are summarized in Table V. Addition of 10.6 µM Rib-5-P to the experimental solution resulted in insignificant changes in the proton relaxation rates of Co(NH\(_3\))\(_2\)ATP. Although a Ma-E-Co(NH\(_3\))\(_2\)ATP-Rib-5-P complex constitutes a reactive system (3), under the experimental conditions less than 20% of Co(NH\(_3\))\(_2\)ATP was estimated to be consumed by the enzyme in the short time required for this measurement.

Determination of Correlation Times and Intramolecular Distances—The longitudinal relaxation rates can be used to calculate distances from the enzyme-bound Mn\(^{2+}\) to the proton and phosphorus nuclei of the other enzyme-bound ligands using the relations (9):

\[ 1/T_{1} \text{obs} = 1/(T_{1\text{m}} + \tau) \]
\[ r = C(T_{1\text{m}} f(\tau))^{1/4} \]
\[ f(\tau) = 3 \tau c (1 + \omega_{c}^{2} \tau^{2}) + 7 \tau c \]

where \(\tau c\) is the mean lifetime of the ligand in the paramagnetic environment, \(\omega_{c}\) and \(\omega_{S}\) are the nuclear and electron Larmor frequencies, and \(C\) is a constant equal to 812 or 901 for Mn\(^{2+}\)-H or Mn\(^{2+}\)-31P interactions, respectively. The largest value of the paramagnetic contribution to the transverse relaxation rate within a given complex sets a lower limit on the dissociation rate (1/T\(_{1\text{m}}\)) of the ligand from that complex. Comparing the 1/T\(_{1}\) and 1/T\(_{2\text{P}}\) values (Tables III and IV), it is apparent that the longitudinal relaxation rates in the complexes studied are not limited by chemical exchange and may therefore be used for distance calculations. However, the large T\(_{2\text{p}}/T_{1}\) ratios for 31P (Tables III and IV) indicate significant contact contributions to 1/T\(_{1}\) which can occur with phosphorus even in second sphere complexes (9), rendering such ratios inappropriate for calculating dipolar correlation times. The correlation times that govern the paramagnetic relaxation rates were determined (9) by measuring the field (frequency) dependence of the longitudinal 31P relaxation rates of the enzyme-Mn\(^{2+}\) complexes of Co(NH\(_3\))\(_2\)ATP, AMP, and P, at 40.5 and 145.8 MHz.

These measurements yielded an average ratio T\(_{1}\) (145.8 MHz)/T\(_{1}\) (40.5 MHz) = 1.4 ± 0.1, with no systematic variation between the complexes studied (data not shown) suggesting very similar \(\tau\) values for both of the complexes. The correlation times of the enzyme-Mn\(^{2+}\)-Co(NH\(_3\))\(_2\)ATP complex in the absence and presence of Rib-5-P were also measured by the frequency dependence of the 1H longitudinal relaxation rate of Co(NH\(_3\))\(_2\)ATP, yielding a ratio of T\(_{1}\) (250 MHz)/T\(_{1}\) (100 MHz) = 2.2 ± 0.2 for both complexes (data not shown). This finding indicates that the binding of Rib-5-P to the enzyme-Mn\(^{2+}\)-Co(NH\(_3\))\(_2\)ATP complex does not alter \(\tau\). From these ratios, taking into account the two extreme limiting cases that \(\tau\) is either independent of or maximally dependent on frequency, an average correlation time was calculated to be 0.6 ± 0.3 ns at H\(_{0}\) = 23.5 kG, 1.5 ± 0.7 ns at 58.7 kG, and 2.7 ± 1.8 ns at 84.6 kG. The above three complexes which were used for \(\tau\) measurements represent the various types of complexes examined in these studies.

The errors in the distances (≤10%) take into account the errors in both \(\tau\), and in 1/T\(_{1}\). Distances calculated with these correlation times (Tables III and V) are significantly greater than the value (3 Å) expected for direct phosphoryl coordination by the enzyme-bound Mn\(^{2+}\), but are appropriate for second sphere complexes (4.5 to 6.0 Å) (11).

Conformation of the Enzyme-bound Nucleotide—The distances between the enzyme-bound Mn\(^{2+}\) and the phosphorus and proton nuclei of Co(NH\(_3\))\(_2\)ATP (Tables III and V) were used together with a computer search program to determine the preferred conformation of the enzyme-bound nucleotide. The search procedure has been described elsewhere (9, 10). Two sets of solutions, each unique to 5 to 15° about the six rotatable bonds of Co(NH\(_3\))\(_2\)ATP, were obtained, which yielded Mn\(^{2+}\)-nuclei distances within the experimental distances and their errors, and less than 0.35% van de Waals atomic overlap. Within their accuracies, the two solutions were indistinguishable except for the torsional angle at the glycosidic bond, which was anti \((x = 62 ± 5°)\) in one solution and syn \((x = 272 ± 5°)\) in the second. Since an anti conformation \((x = 15 to 44°)\) is usually found in adenine nucleosides and nucleotides in solution (12) and also in the crystalline state (13), this conformation (Fig. 4) is considered more likely. The conformation of the bound nucleotide thus differs significantly from that found free in solution.

The binding of Rib-5-P to the Mn-E-Co(NH\(_3\))\(_2\)ATP complex was found to have no significant effect on the distances from Mn\(^{2+}\) to the protons of Co(NH\(_3\))\(_2\)ATP (see above) suggesting little or no change in the conformation of the adenosine moiety of the nucleotide. However, significant increases were found in the distances between Mn\(^{2+}\) and P\(_{5}\) and P\(_{5}\) of the enzyme-bound Co(NH\(_3\))\(_2\)AMP•C\(_{PP}\) upon the binding of Rib-5-P (Table III) indicating a conformational change in the
Substrate Conformation and Arrangement on PP-Rib-P Synthetase

FIG. 2. Effects of Mn²⁺ on the 31P longitudinal relaxation rates of substrates, analogs, and inorganic phosphate in the presence of PP-Rib-P synthetase. A, Co(NH₃)₄AMPCPP (9.8 mM); B, Co(NH₃)₄AMP (5.2 mM); Rib-5-P (6.1 mM); C, Co(NH₃)₄ATP (R) (28.3 mM); D, Co(NH₃)₄ATP (Δ) (35.4 mM); E, AMP (27.8 mM); F, AMP (23.2 mM); G, AMP (23.3 mM); Rib-5-P (29.2 mM); H, P present as potassium salt (40 mM) in A through G. The site concentration of PP-Rib-P synthetase was 80 µM. The pH was 7.0 and T = 15°C. D₂O (20%) was present. Measurements were carried out at 145.8 MHz except for E which was at 40.5 MHz.

![Diagram](image)

**TABLE III**

Bound-state 31P relaxation rates of Mn²⁺ - E-ligand complexes and Mn²⁺ - phosphorus distances

| Complex and nucleus | 1/T₁P₂ | 1/T₂P₂ | r | A (Å) |
|---------------------|--------|--------|---|------|
| Mn - E - Co(NH₃)₄AMPCPP - Rib-5-P |
| Pₚ | 6.9 10^-3 | 1.1 10^-3 | 6.5 |
| Pₚ | 8.3 10^-3 | 1.2 10^-3 | 6.4 |
| 5-P | 3.0 | 10 | 7.5 |
| Mn - E - Co(NH₃)₄AMPCPP |
| Pₚ | 24.6 | 18 | 5.3 |
| Pₚ | 14.9 | 16 | 5.8 |
| Mn - E - Co(NH₃)₄ATP (R)′ |
| Pₚ | 16.2 | 16 | 5.7 |
| Pₚ | 15.4 | 16 | 5.7 |
| Pₚ | 24.3 | 18 | 5.3 |
| Mn - E - Co(NH₃)₄ATP (Δ)′ |
| Pₚ | 16.2 | 16 | 5.7 |
| Pₚ | 17.0 | 16 | 5.6 |
| Pₚ | 24.6 | 17 | 5.3 |
| Mn - E - AMP (R) - Rib-5-P |
| Pₚ | 19.2 | 198 | 5.5 |
| 5-P | 24.3 | 191 | 5.3 |
| Mn - E - AMP (Δ) |
| Pₚ | 20.5 | 223 | 5.5 |
| Mn - E - AMP |
| Pₚ | 94.0 | 207 | 4.6 |

* At 145.8 MHz.
* Obscured by Pₚ.
* Separated Δ isomer of Co(NH₃)₄ATP.
* At 40.5 MHz.

The estimated error in the bound state relaxation rates is 20% and in the distances <10%. pH 7.0, T = 15°C.

FIG. 3. Effects of Mn²⁺ on the 1H longitudinal relaxation rates of Co(NH₃)₄ATP (15.4 mM) in the presence of PP-Rib-P synthetase (73 µm sites) and P (40 mM). Solutions were in D₂O, pH 7.0. T = 15°C, 250 MHz.

![Diagram](image)

**TABLE IV**

Relaxation rates of P bound to PP-Rib-P synthetase complexes

| Complex | 1/T₁P₂ | 1/T₂P₂ |
|---------|--------|--------|
| Mn - E - Co(NH₃)₄AMPCPP - Rib-5-P | 0.9 | 0.4 |
| Mn - E - Co(NH₃)₄ATP | 3.0 | 4.0 |
| Mn - E - Co(NH₃)₄ATP (R) | 2.5 | 3.8 |
| Mn - E - Co(NH₃)₄ATP (Δ) | 2.7 | 4.0 |
| Mn - E - AMP (R) - Rib-5-P | 4.4 | 4.8 |
| Mn - E - AMP | 6.3 | 4.2 |
| Mn - E - AMP | 7.3 | 4.0 |

The estimated error in the bound state relaxation rates is 20% and in the distances <10%. pH 7.0, T = 15°C.

**TABLE V**

Bound-state 1H relaxation rates of Mn²⁺ - E - Co(NH₃)₄ATP and Mn²⁺ - proton distances

| Complex | 1/T₁P₂ | 1/T₂P₂ |
|---------|--------|--------|
| Hₖ | 11.3 | 5.1 |
| H₄ | 2.0 | 6.8 |
| H₈ | 0.9 | 7.6 |
| H₉ | 1.2 | 7.4 |
| H₇ | 0.7 | 8.2 |
| H₆₉ | 1.5 | 7.2 |

| Nucleus | 1/T₁P₂ | 1/T₂P₂ |
|---------|--------|--------|
| Hₖ | 11.3 | 5.1 |
| H₄ | 2.0 | 6.8 |
| H₈ | 0.9 | 7.6 |
| H₉ | 1.2 | 7.4 |
| H₇ | 0.7 | 8.2 |
| H₆₉ | 1.5 | 7.2 |

The estimated error in the bound state relaxation rates is 20% and in the distances <10%. pH 7.0, T = 15°C, 250 MHz.

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**Kinetic Properties of the Enzyme-Nucleotide Complexes**

The largest values of $1/T_{1\text{p}}$ (Table III) set lower limits to $k_{\text{on}}$, the first order rate constant for the dissociation of the substrates from the enzyme complex. For Co(NH$_3$)$_2$ATP and Co(NH$_3$)$_2$AMPCPP, $k_{\text{on}}$ values of $\geq 1.7 \times 10^9$ s$^{-1}$ are observed. From these values and from the $K_a$ value of Co(NH$_3$)$_2$ATP (1.7 × 10$^{-4}$ M) (3), which in this case is of the same order of magnitude as the true dissociation constant (3-5), we estimate $k_{\text{off}}$ values of $\geq 1 \times 10^6$ M$^{-1}$ s$^{-1}$ for both of these complexes. In the case of AMP and AMPaS, larger limiting values of $k_{\text{on}}$ are observed (≥2 × 10$^9$ s$^{-1}$). However, these and from their $K_a$ values (which were calculated from the $K_{\text{a}}^{\text{pp}}$ (Table VIII)) using the relation $K_{\text{a, slow}} = K_{\text{a, prop}} (1 + [ATP]/K_a)^{-1}$, which assumes simple competition of these products with ATP), similar $k_{\text{on}}$ values of $\geq 1 \times 10^9$ M$^{-1}$ s$^{-1}$ and $\geq 0.6 \times 10^9$ M$^{-1}$ s$^{-1}$ are estimated for AMP and AMPaS, respectively. Hence, all nucleotide substrates and analogs appear to have similar $k_{\text{on}}$ values. The nucleotide exchange rates measured by NMR are significantly greater than $k_{\text{on}}$ measured with MnATP (22 s$^{-1}$, Table VII) indicating that the complexes detected by NMR are kinetically competent to function in catalysis.

Kinetic studies with ATP and ATPaS as substrates and AMP and AMPaS as inhibitors using Mg$^{2+}$ and Cd$^{2+}$ as activators were previously reported (4). In order to relate such studies to the present work in which Mn$^{2+}$ was used, these kinetic measurements were repeated, under similar conditions, with Mn$^{2+}$ as the activator (Tables VII and VIII). With ATP as substrate, the rate observed with Mn$^{2+}$ as activator is intermediate between those found with Mg$^{2+}$ and Cd$^{2+}$ (Table VII). While Mg$^{2+}$ activates only with the A-isomer of ATPaS and Cd$^{2+}$ activates weakly with both isomers, Mn$^{2+}$ also activates with both isomers but at higher rates than Cd$^{2+}$ (Table VII). The concentration of AMP which is required for 50% inhibition is essentially independent of the activating metal (Table VIII). However, AMPaS shows a slightly higher affinity (factor of ~2) in the presence of Mn$^{2+}$ relative to Mg$^{2+}$, and a much higher affinity when Cd$^{2+}$ is the activator metal (Table VIII).

**Binding Sites of P$_r$**—The presence of P$_r$ (≥20 mM) in PP-Rib-P synthetase solutions is essential both for activity and to prevent irreversible denaturation of the enzyme (1). The longitudinal relaxation rates ($1/T_{1\text{p}}$) of P$_r$ (Fig. 2H) show significant decreases when substrates with greater numbers of phosphoryl groups are added. Thus, $1/T_{1\text{p}}$ decreases when NTP replaces NMP or when Rib-5-P is also bound (Table IV). While such effects could in principle result from changes in the affinity for P$_r$ or from Mn$^{2+}$ or from changes in the $r_1$ values, these mechanisms are unlikely in the present case in view of the saturating level of P$_r$ and the high levels of enzyme used in all of the studies, and the directly measured $r_1$ values which showed no significant changes. Hence, these observations led to the hypothesis that in addition to an activating site on the enzyme, P$_r$ may also bind at two substrate sites, i.e.

![Fig. 4. Conformation of enzyme-bound βγ-bidentate Co(NH$_3$)$_2$ATP on PP-Rib-P synthetase. This conformation, consistent with the indicated distances from the enzyme-bound Mn$^{2+}$ as determined by NMR (Tables III and V), was obtained by a search procedure (13, 15). Two views (A and B) are shown.](image)

![Fig. 5. Effect of Rib-5-P binding on the conformation of enzyme-bound Co(NH$_3$)$_2$AMPCPP. The solid bonds denote the conformation in the absence of Rib-5-P, while the dashed bonds denote the conformation in the presence of Rib-5-P. These conformations were determined using the enzyme-bound Mn$^{2+}$ as a paramagnetic reference point as discussed in the text. Note that the conformation change occurs only in the triphosphate chain.](image)
the 5-P site of Rib-5-P and the P site of ATP, when these sites are unoccupied. Indeed, the relaxation data could be well occupied, the respective substrate sites.

The present results establish that the metal-ATP substrate, upon binding to PP-Rib-P synthetase, undergoes a small but significant conformational change in the adenine-ribose region, resulting in at least a 20° increase in the torsional angle at the glycosidic bond to a higher anti conformation (\(\chi = 62 \pm 5^\circ\)) (Fig. 4). Another possibility, consistent with the measured errors, but far less likely on energetic grounds, is a 230° change in \(\chi\) to a syn conformation. Conformational changes of 40 to 50° about the glycosidic bond have also been found by NMR to accompany the binding of nucleotide substrates to protein kinase (12), DNA polymerase (14), and RNA polymerase (15) enzymes which, like PP-Rib-P synthetase, show a high substrate base specificity. Pyruvate kinase, an enzyme with low nucleotide base specificity (16), produces no significant change in \(\chi\) when ATP is bound (17). Hence, those enzyme-nucleotide interactions that are strong enough to alter \(\chi\) may also be responsible for high nucleotide base specificities of enzymes (18).

Upon binding of Rib-5-P to PP-Rib-P synthetase, no further change occurs in the adenine-ribose portion as indicated by the absence of changes in the Mn\(^{2+}\)-proton distances, but a significant conformational change occurs in the polypolyphosphate chain of the bound metal-ATP substrate as manifested by 1.2 Å and 0.6 Å increases in the distances from the bound Mn\(^{2+}\) to the P\(_{\text{enolpyruvate}}\) and P\(_{\text{Rib-5-P}}\) atoms, respectively (Table III, Fig. 5). This conformational change occurs only when a triphosphate chain is present on the nucleotide, since no corresponding change in distance is observed with AMPoS upon the binding of Rib-5-P (Table III). The conformational change may better align the P\(_{\text{enolpyruvate}}\)—O—P\(_{\text{Rib-5-P}}\) portion of the bound ATP for nucleophilic attack with inversion at P\(_{\text{Rib-5-P}}\) by the α-1-OH of Rib-5-P (3, 5). A conformational change when both ATP and Rib-5-P are bound is also suggested from the fact that AMPcPP binds appreciably more tightly to PP-Rib-P synthetase when Rib-5-P is present. The reaction mechanism of Fig. 6 has been drawn to be consistent with the conformation of ATP in the Mn\(^{2+}\)-Co(NH\(_3\))\(_3\)ATP-Rib-5-P complex, with previously determined distances from the nucleotide-bound metal to the C-1 protons and 5-P atoms of Rib-5-P (5), and with distances from both metals to the essential P\(_{\text{Rib-5-P}}\) activator. Two additional constraints that have been incorporated into the mechanism (Fig. 6) are an appropriate (180°) alignment between the entering and leaving groups for inversion about P\(_{\text{Rib-5-P}}\), and the minimal distance (3.8 Å) previously measured between the entering oxygen and the attacked P\(_{\text{Rib-5-P}}\) of the ATP (5). The leaving AMP is not coordinated to the enzyme-bound Mn\(^{2+}\) activator but rather forms a predominantly second sphere complex, as do all of the phosphoryl groups of the metal-ATP substrate and its analog (Table III). The ~1 Å smaller Mn\(^{2+}\)—P\(_{\text{Rib-5-P}}\) distance in the enzyme-Mn\(^{2+}\)-AMP complex, as compared with this distance in all of the other enzyme-Mn\(^{2+}\)-nucleotide complexes studied, suggests either a more closely packed second sphere AMP complex, or the averaging of a small amount (≤5%) of an inner sphere AMP complex with a distance of 2.9 Å from the Mn\(^{2+}\). Similar second sphere distances were found on pyruvate kinase from the essential enzyme-bound divalent cation activator to the phosphorus atoms of the metal-ATP (17) and P-enolpyruvate substrates (19). Direct coordination of a metal-ATP substrate by an additional enzyme-bound divalent cation (on protein kinase)

### TABLE VI

| Complex                  | 1/\(T_1\) \(\mu s\) | No. of P sites | Site 1 | Site 2 | Site 3 |
|--------------------------|----------------------|----------------|--------|--------|--------|
| Mn\(^{2+}\)-NTP-Rib-5-P | 0.9                  | 1              | 6.3    | 5.6    | 5.0    |
| Mn\(^{2+}\)-NTP-ATP     | 2.7                  | 2              | 6.3    | 5.6    | 5.0    |
| Mn\(^{2+}\)-NTP-Rib-5-P | 4.4                  | 2              | 6.3    | 5.6    | 5.0    |
| Mn\(^{2+}\)-NTP-MNP     | 6.3                  | 3              | 6.3    | 5.6    | 5.0    |

*These are the measured average bound state paramagnetic relaxation rates. 1/\(T_1\) = \(\sum_{i=1}^{n} 1/\(T_1\)\(_{i}\) = \(1/\(T_1\)\(_{\text{intrinsic}}\)\). 1/\(T_1\)\(_{\text{intrinsic}}\) is the intrinsic relaxation rate in the site. n may vary between 1 and 3.

* Mn\(^{2+}\)-P distances.

### TABLE VII

| Nucleotide | Metal ion | \(V_{\text{in}}\) \(\mu M\) | Reference |
|------------|-----------|-----------------------------|-----------|
| ATP        | Mg\(^{2+}\) | 117                         | 4         |
| ATPoS (A)  | Mg\(^{2+}\) | 111                         |           |
| ATPoS (B)  | Mg\(^{2+}\) | <0.004                      |           |
| ATP        | Mn\(^{2+}\) | 44                          | This work |
| ATPoS (A)  | Mn\(^{2+}\) | 42                          | This work |
| ATPoS (B)  | Mn\(^{2+}\) | 8.6                         | This work |
| ATP        | Cd\(^{2+}\) | 11                          | 4         |
| ATPoS (A)  | Cd\(^{2+}\) | 1.8                         | 4         |
| ATPoS (B)  | Cd\(^{2+}\) | 2.2                         | 4         |

### TABLE VIII

| Metal ion | \(K_{i/2}\) | pH | Reference |
|-----------|------------|----|-----------|
| Mg\(^{2+}\) | 900       | 1700 | 8        | 4        |
| Mn\(^{2+}\) | 1000     | 650  | 8        | This work |
| Cd\(^{2+}\) | 900      | 50   | 8        | 4        |
| Mg\(^{2+}\) | 450      | 1200 | 7        | This work |
| Mn\(^{2+}\) | 450      | 620  | 7        | 2        |

* Concentration required for 50% inhibition at 50 μM ATP and 5 mM Rib-5-P. T = 37°C.
results in marked inhibition of the enzyme-catalyzed reaction (12).

The finding that Cd\(^{2+}\), but not Mg\(^{2+}\), activates with the B-isomer of ATP\(\alpha\)S, while both Cd\(^{2+}\) and Mg\(^{2+}\) activate with the A-isomer, were interpreted to indicate a coordination of the \(\beta,\gamma\)-bidentate metal ATP substrate by the enzyme-bound divalent cation (4). This view was plausible since \(\beta,\gamma\)-bidentate Co(NH\(_3\))\(\alpha\)ATP (A isomer) was found to be a substrate (3) while \(\alpha,\beta,\gamma\)-tridentate Cr\(\alpha\)ATP was at least 10-fold less active, indicating that a coordination by the nucleotide-bound metal was not essential but actually inhibitory (5). The present NMR studies, which fail to detect coordination at P\(_3\) by the enzyme-bound Mn\(^{2+}\) with either the substrate or product forms of the nucleotide (including AMP\(\alpha\)S), argue strongly against such an interaction with Mn\(^{2+}\) in any of the complexes examined, but can be rationalized with the kinetic data in several ways. Labile \(\alpha,\beta,\gamma\) coordination of ATP by Cd\(^{2+}\) or Mn\(^{2+}\), although inhibitory, could partially relax the complete inhibition caused by an incorrectly oriented bulky thiol group. A second alternative is that the enzyme-bound Cd\(^{2+}\), due to its extremely high affinity for sulfur, may allow some activity with the B-isomer of ATP\(S\) due to direct coordination of sulfur as previously proposed (4), but Mn\(^{2+}\), which activates more effectively with this isomer, may do so via a second sphere complex, as detected by NMR. Direct coordination between Cd\(^{2+}\) only and the thio-nucleotide is consistent with the finding that the \(K_{\text{app}}\) of AMP is largely independent of the nature of the divalent cation activator (Mg\(^{2+}\) ~ Mn\(^{2+}\) ~ Cd\(^{2+}\)), while the \(K_{\text{app}}\) of AMP\(\alpha\)S is significantly smaller with Cd\(^{2+}\) (Mg\(^{2+}\) ~ Mn\(^{2+}\) ~ Cd\(^{2+}\)) (Table VII), and with the NMR studies (Table III) which show only second sphere nucleotide complexes of the enzyme-bound Mn\(^{2+}\). Thirdly, transient \(\alpha\)-phosphoryl coordination by the enzyme-bound (or nucleotide-bound) metal could occur only in the transition state before bond breaking to the leaving AMP group is complete. Finally, Co(NH\(_3\))\(\alpha\)ATP, although a substrate, may interact somewhat differently with the enzyme-bound cation than ATP or ATP\(\alpha\)S. Further kinetic studies with stable metal complexes of ATP\(\alpha\)S may distinguish among these possibilities.

A self-consistent location for the activating phosphate ion, which is required by all known PP-Rib-P synthetases, has been found, based on distances from two paramagnetic reference points, the enzyme-bound (Table VI) and the nucleotide-bound metal activators (5). The location of P\(_3\) near the bound substrates (Fig. 6) further supports our previous suggestion that it may be directly involved in catalysis, possibly functioning as the general base which deprotonates the \(\alpha\)-OH group of Rib-5-P (5). In addition to binding at this site in all complexes, P\(_3\) also appears to bind weakly at the 5-P site of Rib-5-P and at the P\(_3\) site of ATP on the enzyme when these sites are not occupied by the substrates. Independent evidence for the binding of approximately three phosphate anions at the active site of PP-Rib-P synthetase has previously been obtained in kinetic studies of the protection by P\(_3\) of the enzyme against inactivation by permanganate oxidation of an essential thiol group (20). The dependence of P\(_3\) protection on the P\(_3\) concentration raised to the 2.7th power led to the suggestion that "as many as three P\(_3\) anions may associate with the active site to protect it against KMnO\(_4\)" (20). Binding of P\(_3\) at these sites on PP-Rib-P synthetase, and on a number of other ATP utilizing enzymes, may constitute a physiological mechanism for stabilization of such enzymes, and by simple competition, may regulate their affinity for phosphorylated substrates in cells.

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