# Determination of the Absolute Configurations of the Isomers of Triamminecobalt(III) Adenosine Triphosphate

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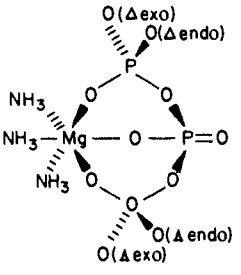
**Abstract:** $Co^{III}(NH_3)_3ATP$ exists as four tridentate complexes, which can be separated on columns of cross-linked cycloheptaamylose or by reversed-phase HPLC. The absolute configurations at the $\alpha$- and $\beta$-phosphates for each isomer were determined from the size of $^{18}O$-induced shifts in $^{31}P$ NMR peaks when a mixture of ATP and either $(R_P)$-[$\alpha$-$^{18}O$]ATP or $(S_P)$-[$\beta$-$^{18}O$]ATP was used to form the complexes. Isomers **1**–**4**, in order of elution from cycloheptaamylose, were Λ endo, Δ endo, Δ exo, and Λ exo (the order of elution with reversed-phase HPLC was **3**, **1**, **4**, **2**). Endo isomers gave $^{31}P$ NMR chemical shifts relative to 85% $H_3PO_4$ of −0.81 (Λ) or −0.92 (Δ), −7.7, and +5.2 for $\alpha$-, $\beta$- and $\gamma$-phosphates, while the exo isomers gave −0.27 (Λ) or −0.56 (Δ), −7.5, and +4.8. The $\alpha$–$\beta$ coupling constants for the endo isomers were several Hertz higher than those for the exo isomers, or for the $\beta$–$\gamma$ coupling in all isomers. CD spectra show positive ellipticity at 590 nm and negative elliplicity at 530 nm for Λ isomers and the opposite for Δ isomers, but the endo isomers have higher ellipticities at 530 nm and the exo isomers higher ones at 590 nm. These tridentate isomers should prove useful as probes of substrate specificity for enzymes that use tridentate MgATP as a substrate. Thus, creatine kinase is preferentially inhibited by the Λ exo isomer with $K_i$ = 2.3 mM (the Λ endo isomer shows $K_i$ = 6.7 mM, while the Δ isomers do not inhibit at 10 mM). The desulfurization of chiral ADP$\alpha$S and ATP$\beta$S with $Br_2$ occurs with better than 95% inversion when the initial pH is 3 or less, making this a convenient method for synthesizing chiral $^{18}O$-labeled nucleotides.

Inert chromium(III) and cobalt(III) complexes of nucleotides have proven to be a useful tool in deducing the substrate specificity and mechanisms of enzymes where MgATP or similar rapidly equilibrating metal ion complexes are the substrates.[3,4] Thus, a number of kinases use $\beta,\gamma$ bidentate $Co(NH_3)_4ATP$ or $Cr(H_2O)_4ATP$ as substrates, with half of them using the Λ and half the Δ isomers.[5] Four tridentate isomers are predicted for a facial octahedral metal complex of ATP, with adenosine attached to the indicated oxygens:




Creatine kinase is thought to use the Λ exo isomer of MgATP as substrate.[6-8] While acid treatment converts $Cr(H_2O)_4ATP$ into a different form that may be tridentate,[9] isolation of pure isomers and structural characterization are lacking (as is proof that the complexes are still mononuclear). $Co(NH_3)_3ATP$ exists solely as four mononuclear tridentate complexes,[10] and in this paper we will describe the separation and proof of structure of these complexes, which should prove to be useful structural probes of enzymes where tridentate MgATP is the substrate.

## Experimental Section

**ADP$\alpha$S.**[11,12] A solution of 1.08 mmol of AMPS in 250 mL of 0.1 M Hepes, pH 8, was incubated with 25 units/mL pyruvate kinase, 38.5 units/mL myokinase, 15 mM phosphoenolpyruvate, 0.5 mM ATP, 25 mM acetate, 0.1 M KCl, 1 mM dithiothreitol, and 12 $\mu$M EDTA for 6 h at room temperature, with the release of pyruvate followed in aliquots with a lactate dehydrogenase, NADH assay. When reaction was complete, 250 mL of 0.1 M triethylamine bicarbonate (redistilled triethylamine diluted with water and sparged with $CO_2$ at 4 °C until the pH was 7.4) was added and the solution applied to a 2.5 × 60 cm column of DEAE Sephadex A 25 containing the same buffer. The column was eluted with a linear gradient of 3.5 L each of 0.1 and 0.7 M triethylamine bicarbonate, yielding 1.04 mmol of $(S_P)$-ATP$\alpha$S (98%).

The ATP$\alpha$S was concentrated by rotary evaporation, and 1.00 mmol was incubated in 250 mL of 0.16 M Hepes, pH 8.0, with 40 mM glucose, 32 mM magnesium acetate, 20 $\mu$M EDTA, and 20 units/mL yeast hexokinase for 1 h, with the formation of glucose 6-phosphate followed with a glucose 6-phosphate dehydrogenase, NADP assay. When reaction was complete, 250 mL of 0.1 M triethylamine bicarbonate was added, and the solution applied to the DEAE Sephadex column. Elution with a linear gradient of 3.5 L each of 0.1 and 0.6 M triethylamine bicarbonate gave 98% of $(S_P)$-ADP$\alpha$S.

**$(R_P)$-[$\alpha$-$^{18}O$]ADP.**[13] ADP$\alpha$S (0.32 mmol) was evaporated to dryness three times with methanol in a rotary evaporator and dissolved in 0.5 mL of 90% $H_2{}^{18}O$. The pH was adjusted to 2.5 with concentrated HCl (~0.1 mL), 0.1 mL of $Br_2$ was added, and the reaction was stirred for 4 min. Solid sodium bisulfite was added until the solution was colorless, and triethylamine was added until pH 7.0. The solution was diluted to 20 mL and applied to a 1.4 × 18 cm column of DEAE Sephadex, which was eluted with a linear gradient of 700 mL each of 0.05 M and 0.45 M triethylamine bicarbonate. Of the starting material 33% was recovered as ADP, 30% as AMP, and 35% as adenine as the result of the pH being lowered below zero during the reaction. We did not attempt to optimize the yields in this reaction; when the reaction was carried out under more dilute conditions (~20 mM in ADP$\alpha$S and 1% $Br_2$ by volume), a 70% yield of ADP was obtained. To obtain the highest incorporation of $^{18}O$, concentrated $H_2SO_4$ or dry HCl gas should be used to adjust the pH

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(3) Cleland, W. W.; Mildvan, A. S. *Adv. Inorg. Biochem.* **1979**, *1*, 163.
(4) Cleland, W. W. *Methods Enzymol.* **1982**, *87*, 159.
(5) Dunaway-Mariano, D.; Cleland, W. W. *Biochemistry* **1980**, *19*, 1506. In the screw-sense nomenclature used here,[15] a line through the metal ion perpendicular to the chelate ring (the $\beta$-$\gamma$ one for a tridentate complex) and the bond to the rest of the ligand (the P–O bond from the $\beta$-phosphorus to AMP for tridentate complexes) defines the helical sense. For purposes of this analysis the chelate ring is considered planar, although in reality it is of course puckered into a twist configuration. In tridentate complexes, exo designates complexes in which adenosine and the $\gamma$-phosphate are on opposite sides of the $\alpha$–$\beta$ chelate ring and endo designates ones where they are on the same side.
(6) Burgers, P. M. J.; Eckstein, F. *J. Biol. Chem.* **1980**, *255*, 8229.
(7) Leyh, T. S.; Goodhart, P. J.; Nguyen, A. C.; Kenyon, G. L.; Reed, G. H. *Biochemistry* **1985**, *24*, 308.
(8) Jarori, G. K.; Ray, B. D.; Nageswara Rao, B. D. *Biochemistry* **1985**, *24*, 3487.
(9) Dunaway-Mariano, D.; Cleland, W. W. *Biochemistry* **1980**, *19*, 1496.
(10) Cornelius, R. D.; Hart, P. A.; Cleland, W. W. *Inorg. Chem.* **1977**, *16*, 2799.
(11) Sheu, K.-F. R.; Frey, P. A. *J. Biol. Chem.* **1977**, *252*, 4445.
(12) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1979**, *254*, 10839.
(13) Lowe, G.; Sproat, B. S.; Tansley, G.; Cullis, P. M. *Biochemistry* **1983**, *22*, 1229.

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initially. Addition of $K_2SO_4$ as a buffer and incubation for shorter times should also decrease hydrolysis and thus improve the yield.

The product contained $^{18}O$ only in the $\alpha$-phosphate on the basis of $^{31}P$ NMR, while mass spectrometric analysis showed 75% $^{18}O$ in the $\alpha$-phosphate and less than 3% in the $\beta$-phosphate. This reaction is reported to go with 93% inversion and 7% retention of configuration when the initial pH was not acidic;[13] starting at a lower pH appears to have reduced the degree of label scrambling in the product.

**($R_P$)-[$\alpha$-$^{18}O$]ATP.**[11] In 25 mL of 0.1 M Hepes, pH 8.0, were incubated 4 mM [$\alpha$-$^{18}O$]ADP, 16 mM phosphoenolpyruvate, 25 mM magnesium acetate, 0.1 M KCl, 1 mM dithiothreitol, 12 $\mu$M EDTA, and 24 units/mL pyruvate kinase. Reaction was over in a few seconds, and the reaction mixture was diluted with an equal volume of 0.1 M triethylamine bicarbonate and chromatographed on a 1.4 × 18 cm column of DEAE Sephadex with a linear gradient of 600 mL each of 0.2 and 0.8 M triethylamine bicarbonate. [$\alpha$-$^{18}O$]ATP was recovered in 98% yield, and integration of the $\alpha$-phosphate region of the $^{31}P$ NMR spectrum was used to calculate the amount of ATP to add to bring the $^{18}O$ content to 50%.

**$Co(NH_3)_3ATP$.** This complex was prepared in ~55% yield by keeping a solution 10 mM each in $Na_2ATP$ and $[Co(NH_3)_3(H_2O)Cl_2]Cl$[14] at 80 °C for 8 min by dipping it in and out of a boiling water bath.[10] The reaction was then chilled in ice, and subsequent steps were carried out at 4 °C. Unreacted triamminecobalt was removed by passage through a column of Chelex 100 (10-mL column for a 0.12-mmol preparation) and washing with water. The eluant was applied to a column of Dowex 50-X2 ($H^+$, 200–400 mesh) (5-mL column for this size preparation), and elution with water removed unreacted ATP, which could be reused when it was $^{18}O$ labeled. Elution with 0.1 M lithium formate, pH 3.6, focused the lavender complex into a sharp band, which was adjusted to pH 5.6 and applied directly to a 1.4 × 190 cm column of cross-linked cycloheptaamylose.[15] Elution with 10 mM Mes, pH 5.6, separates the four isomers of the complex with ~80% recovery, although rechromatography would be necessary to prepare pure fractions (that is, fractions containing only isomers **1** and **4** or **2** and **3**; the equilibration of each pair of isomers makes it impractical to obtain completely pure individual isomers by slow chromatography on cycloheptaamylose). The isomers can also be separated (or further purified) by chromatography on a reversed-phase C-18 column with 10 mM potassium methanesulfonate, pH 2.5, as the eluting buffer;[16] this is probably the best way to obtain highest purity individual isomers for specificity studies.

When $^{18}O$-labeled ATP was used, the eluant from the Dowex 50 column had to be concentrated by rotary evaporation (below 25 °C) before chromatography on cycloheptaamylose, and the eluted fractions (selected by their CD spectra) were also concentrated for NMR analysis to ~0.1 M.

**($S_P$)-[$\beta$-$^{18}O$]ATP.** ($R_P$)-ATP$\beta$S was prepared by reaction of 10 mM ADP$\beta$S, 100 mM acetyl phosphate, 1 mM dithiothreitol, and 100 units/mL acetate kinase in 20 mL of 0.1 M Hepes, pH 7.6, for 26 h.[17] The reaction mixture was diluted with 20 mL of 0.15 M triethylamine bicarbonate and applied to a 4 × 22 cm column of DEAE Sephadex. Elution with a linear gradient of 1.4 L each of 0.3 and 0.7 M triethylamine bicarbonate gave 60% of ATP$\beta$S and 40% ADP$\beta$S, which was used for a second preparation. The combined fractions of ATP$\beta$S (0.15 mmol) were evaporated to dryness and dissolved in 0.5 mL of 95% $H_2{}^{18}O$. After addition of 50 mg of $K_2SO_4$, the pH was adjusted to 3 with concentrated $H_2SO_4$. Fifty microliters of $Br_2$ was added and the reaction stirred for 3 min. Sufficient solid sodium bisulfite was added to decolorize the solution, 2 mL of water was added, and the pH was adjusted to 7 with triethylamine after the temperature was lowered to 4 °C. The reaction was diluted to 20 mL and applied to a 1.4 × 20 cm column of DEAE Sephadex, which was eluted with a linear gradient of 700 mL each of 0.1 and 0.5 M triethylamine bicarbonate. The [$\beta$-$^{18}O$]ATP isolated in 45% yield contained $^{18}O$ only in the $\beta$-phosphate on the basis of its $^{31}P$ NMR spectrum and was diluted with sufficient ATP to contain 50% $^{18}O$. This material was converted to triamminecobalt ATP and separated by cycloheptaamylose chromatography as described above. Fractions containing mixtures of isomers that were not used for NMR analysis were adjusted to pH 7 and heated at 65 °C for 30 min with 1 mM dithiothreitol to reduce Co(III) to Co(II). The resulting solution was filtered and the recovered [$\beta$-$^{18}O$]ATP isolated by chromatography on DEAE Sephadex.

**$Co(NH_3)_4$[$\beta$-$^{18}O$]ATP.** ($S_P$)-[$\beta$-$^{18}O$]ATP was converted to the Na form and the pH adjusted to 3.3 with HCl. Heating at 80 °C for 8 min with $[Co(NH_3)_4CO_3]NO_3$ that had been titrated to pH 3 with HCl (10 mM of each reactant), followed by elution from Dowex 50 ($H^+$) by 0.1 M lithium formate, pH 3.5, gave this bidentate complex, which was concentrated by rotary evaporation, desalted by Sephadex G-10 chromatography, and further concentrated to 0.2 mL. A sample of 18 $\mu$mol of this complex plus 20 $\mu$mol of glucose was placed in 0.9 mL of 50% $D_2O$ containing 0.1 M Pipes, pH 6.5, and 5 mM EDTA and the $^{31}P$ NMR spectrum taken. Hexokinase (1000 units) was added, and further spectra were taken until reaction was complete.

**$^{31}P$ NMR Spectra of $^{18}O$-Containing Compounds.** Proton-decoupled $^{31}P$ NMR spectra were obtained at 25 °C with a Nicolet NT-200 Fourier transform spectrometer operating at 80.99 MHz in the quadrature detection mode. The pH(D) was 5.8 (pH meter reading) unless otherwise noted. The spectra were the sum of 100–3000 scans of ±1000 Hz with 4K data points and were externally referenced to 100 mM $H_3PO_4$. The data were routinely zero filled to 8K and resolution enhanced by a double-exponential multiplication (DM = 1 in the Nicolet software) of the free induction decay prior to Fourier transformation. Samples were concentrated to 0.1 M if possible (although some were as low as 10 mM) and made 50% in $D_2O$. They were passed through a small column of Chelex 100 and made 5–10 mM in EDTA just before the spectra were taken to minimize line broadening by Co(II) or other paramagnetic impurities. No evidence was seen in the NMR spectra for hydrolysis of the coordinated ATP.

**$\alpha$-Phosphate Interconversion.** The rate of conversion of isomer **1** to isomer **4** was determined by CD spectroscopy. Freshly isolated solutions of isomer **1** were adjusted to the appropriate pH with KOH. The ionic strength of the solution was maintained at 0.2 M with $KNO_3$. The solutions were maintained at constant temperature with a circulating water bath. Data were collected at pH 2.3, 3.8, 5.65, 7.19, and 9.11 at 26 °C. The temperature dependence of the interconversion was determined at pH 2.3 by measuring the rate at 4, 17, 26, 36, and 48 °C.

**Creatine Kinase Kinetics.** Creatine and pyruvate kinases, lactate dehydrogenase, NADH, and phosphoenolpyruvate were from Boehringer. Assays were run at 25 °C with 100 mM morpholinoethanesulfonate buffer, pH 6.5. ADP production was followed with a pyruvate kinase, lactate dehydrogenase coupled assay with the disappearance of NADH monitored at 340 nm. Competitive inhibition data were fitted to the equation

$$v = VA/[K(1 + I/K_i) + A]$$

to determine the inhibition constant. $Co(NH_3)_3ATP$ isomers were used in inhibition experiments immediately after elution from the cycloheptaamylose column, with isomeric purity assessed by CD assay. The concentrations were determined from the absorbance at 259 nm ($\epsilon$ = 15 400).

## Results

**$Co(NH_3)_3ATP$ Isomers.** $Co(NH_3)_3ATP$ was formed in 55% yield by heating $[Co(NH_3)_3(H_2O)Cl_2]Cl$ with $Na_2ATP$ at 80 °C for 8 min. Uncomplexed triamminecobalt was removed by passage through a Chelex column, and the product was adsorbed on a column of Dowex 50 ($H^+$) and eluted in a sharply focused band with 0.1 M lithium formate, pH 3.5. This material is lavender and shows a visible spectrum with peaks at 390 ($\epsilon$ = 48) and 552 nm ($\epsilon$ = 60). These transitions are assigned, using an octahedral approximation, as $^1A_{1g} \rightarrow {}^1T_{2g}$ and $^1A_{1g} \rightarrow {}^1T_{1g}$, respectively. The absorption maxima are consistent with the presence of three strong-field ammonia ligands in the inner coordination sphere. This material was separated into the four diastereomeric isomers on a column of cross-linked cycloheptaamylose.

On the basis of CD spectra of the fractions, the four isomers were present in roughly equal amounts. Two isomers have positive ellipticity at 530 nm and negative ellipticity at 590 nm, while the other two have opposite shapes (Figure 1). Because the isomers are diastereomers, no two spectra are exact mirror images, however. The molar ellipticities and $^{31}P$ NMR chemical shifts are in Table I. The size of the chemical shifts shows that all three phosphates of ATP are coordinated to cobalt (this causes an ~10 ppm downfield shift). The proton NMR spectra show the expected protons of adenine and ribose with no evidence for coordination of cobalt to either. The $NH_3$ proton peak is a broad single band at 0.18 ppm, consistent with a facial arrangement of ligands.[10]

**Interconversion of Isomers.** The isolated $Co(NH_3)_3ATP$ isomers were not totally inert, and isomers **1** and **4** (numbered in order of elution from the cycloheptaamylose column), as well as isomers **2** and **3**, equilibrated with each other as the result of apparent epimerization at the $\alpha$-phosphate. The rate of conversion from

(14) Schlessinger, G. *Inorg. Synth.* **1960**, *6*, 180.
(15) Cornelius, R. D.; Cleland, W. W. *Biochemistry* **1978**, *17*, 3279.
(16) Gruys, K. J.; Schuster, S. M. *Anal. Biochem.* **1982**, *125*, 66.
(17) Sheu, K.-F.; Ho, H.-T.; Nolan, L. D.; Markovitz, P.; Richard, J. B.; Utter, M. F.; Frey, P. A. *Biochemistry* **1984**, *23*, 1779.

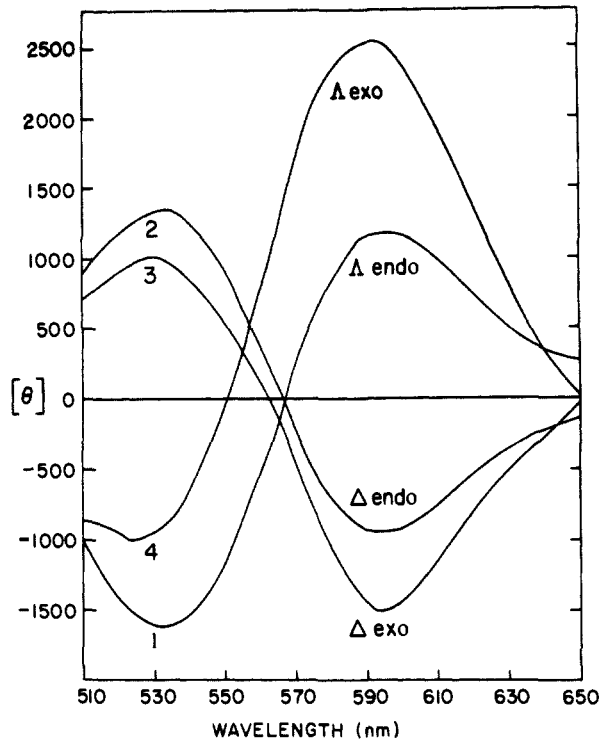


**Figure 1.** CD spectra of $Co(NH_3)_3ATP$ isomers, numbered in order of elution from cycloheptaamylose columns. The units of $[\theta]$ are deg $cm^2$ $dmol^{-1}$.

**Table I.** $^{31}P$ NMR and CD Spectral Properties of $Co(NH_3)_3ATP$ Isomers

| | isomer[a] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| chem shift,[b] ppm | | | | |
| $P_\alpha$ | −0.815 | −0.923 | −0.563 | −0.268 |
| $P_\beta$ | −7.78 | −7.74 | −7.54 | −7.53 |
| $P_\gamma$ | +5.24 | +5.23 | +4.83 | +4.81 |
| coupling const, Hz | | | | |
| $\alpha$–$\beta$ | 18.4 | 18.5 | 16.8 | 15.9 |
| $\beta$–$\gamma$ | 16.8 | 16.7 | 16.4 | 16.7 |
| mol ellipt, deg $cm^2$ $dmol^{-1}$ | | | | |
| 530 nm | −1600 | +1360 | +1000 | −1000 |
| 590 nm | +1160 | −960 | −1500 | +2500 |
| configuration | Λ endo | Δ endo | Δ exo | Λ exo |

[a] Numbered in order of elution from cycloheptaamylose columns. From reversed-phase HPLC columns, the order is **3**, **1**, **4**, **2**. [b] Relative to external standard of 85% $H_3PO_4$ at pH 5.8, 50% $D_2O$. The $\alpha$- (when proton decoupled, as here) and $\gamma$-phosphate peaks are doublets, and the $\beta$ one is a doublet of doublets.

isomer **1** to **4** at 26 °C (measured by change in the CD spectrum at 530 nm) was nearly pH independent from pH 2.3 (0.14 $h^{-1}$) to 5.65 (0.19 $h^{-1}$) and showed an activation energy of 17 ± 2 kcal/mol (log $A$ = 16 ± 4). This activation energy is consistent with a mechanism requiring Co–O bond cleavage to form a five-coordinate intermediate. The rate at 4 °C, pH 2.3, was 0.035 $h^{-1}$, corresponding to a half-life of 20 h, while the half-life at 25 °C was 4 h. At pH 7 or above the interconversion rate increased somewhat (to 0.45 $h^{-1}$ at pH 9, 25 °C), and epimerization at the $\beta$-phosphate occurred as well (that is, the ellipticity went to zero after 20 h, instead of reaching a plateau value, as was the case at lower pH values). Thus, these isomers must be kept cold and used as soon as they are prepared to avoid isomerization. They may also be separated on a reversed-phase HPLC column with 10 mM potassium methanesulfonate, pH 2.5, as eluant[16] (elution order: **3**, **1**, **4**, **2**), and final purification in this fashion may be the most practical way to obtain really pure isomers for specificity studies.

**Absolute Configuration at the $\alpha$-Phosphate.** ($R_P$)-[$\alpha$-$^{18}O$]ATP was prepared by treatment of ($S_P$)-ADP$\alpha$S with $Br_2$ at pH 3 in $H_2{}^{18}O$, followed by phosphorylation. This material was diluted with ATP to give ~50% $^{18}O$ labeling and reacted to give $Co(NH_3)_3ATP$ in the usual way. Separation of isomers by cycloheptaamylose chromatography and examination of the $^{31}P$ NMR spectra readily identified which oxygen was coordinated (~0.02 ppm separation between $^{16}O$- and $^{18}O$-containing molecules when the $^{18}O$ was coordinated and ~0.03 ppm when it was not[18]). A representative spectrum showing all four isomers is shown in Figure 2, and the data for the $^{18}O$-induced shifts are in Table II. These experiments show that isomers **1** and **4** (and also **2** and **3**) are epimers at the $\alpha$-phosphate, as one would expect on the basis of their interconversion.

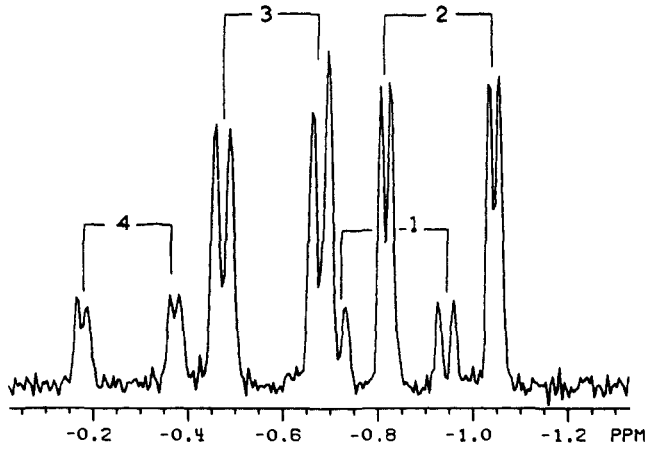


**Figure 2.** $^{31}P$ NMR spectra of the $\alpha$-phosphate doublets (0.2 ppm splitting by the $\beta$-phosphate) from a mixture of $Co(NH_3)_3ATP$ isomers, showing the upfield shifts (apparent splittings of 0.02 or 0.03 ppm) caused by 50% $^{18}O$ labeling in the $\alpha$-phosphate. The complex was formed from ($R_P$)-[$\alpha$-$^{18}O$]-ATP. The isomers are numbered in order of elution from cycloheptaamylose columns; for the absolute configurations, see Table I.

**Table II.** $^{18}O$-Induced Shifts in $^{31}P$ NMR Peaks ($\Delta^a$ ppm) of $Co(NH_3)_3ATP$ Isomers

| | isomer[b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| $P_\alpha$ | 0.0325 | 0.020 | 0.032 | 0.0195 |
| $P_\beta$ | 0.023 | 0.033 | 0.032 | 0.021 |

[a] Upfield shift caused by $^{18}O$ substitution in ATP at the $\alpha$ ($R_p$) or $\beta$ ($S_p$) position (pH 5.8, 50% $D_2O$). [b] Numbered in order of elution from cycloheptaamylose columns.

**Absolute Configuration at the $\beta$-Phosphate.** [$\beta$-$^{18}O$]ATP was prepared by treatment of ($R_P$)-ATP$\beta$S with $Br_2$ at low pH in $H_2{}^{18}O$. Since $^{18}O$ was found only in the $\beta$-phosphate by $^{31}P$ NMR, the low pH apparently prevents internal attack by the $\gamma$- or $\alpha$-phosphate to give cyclic intermediates whose subsequent hydrolysis would have scrambled the label.[19] Since the stereochemistry of this desulfurization has not been reported, we converted the [$\beta$-$^{18}O$]ATP to $Co(NH_3)_4ATP$, the Λ isomer of which is a substrate for yeast hexokinase[15] (these isomers cannot be separated by chromatography; thus, enzymatic resolution is the only way to prepare a pure isomer in which the $^{18}O$-induced splitting can be accurately observed). Table III shows the NMR data for this experiment. By adding yeast hexokinase, we converted the Λ isomer initially to $Co(NH_3)_4ADP$–glucose 6-phosphate, which decomposed slowly during the course of the reaction, but not to materials whose chemical shifts interfered with the analysis. After this reaction, it was possible to see clearly the 0.029 ppm shift caused by $^{18}O$ in the $\beta$-phosphate peaks of the residual Δ isomer. The 0.022 ppm shift in the Λ isomer was established by observing this splitting in the peaks corresponding to the $\beta$-phosphate of the $Co(NH_3)_4ADP$–glucose 6-phosphate product.

(18) Coderre, J. A.; Gerlt, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 6594.
(19) Iyengar, R.; Ho, H.-T.; Sammons, R. D.; Frey, P. A. *J. Am. Chem. Soc.* **1984**, *106*, 6038.

Table III. $^{31}P$ NMR Parameters for $Co(NH_3)_4ATP$ Isomers Prepared from $(S_P)$-[$\beta$-$^{18}O$]-ATP

| | isomer[a] | |
|---|---|---|
| | Λ | Δ |
| chem shift,[b] ppm | | |
| $P_\alpha$ | −11.16 | −11.28 |
| $P_\beta$ | −10.58 | −10.55 |
| $P_\gamma$ | +3.85 | +3.85 |
| coupling const, Hz | | |
| $\alpha$–$\beta$ | 17.7 | 17.8 |
| $\beta$–$\gamma$ | 16.0 | 16.0 |
| Δ chem shift,[c], ppm | | |
| $P_\beta$ | 0.022[d] | 0.029[e] |

[a] Based on substrate activity of the Λ isomer with yeast hexokinase.[15] [b] Relative to external standard of 85% $H_3PO_4$ (pH 6.5, 50% $D_2O$). [c] Upfield shift caused by $^{18}O$ substitution. [d] Observed in the $Co(NH_3)_4ADP$–glucose 6-phosphate product of the enzymatic reaction with glucose and hexokinase. [e] Observed in residual Δ isomer after reaction of the Λ isomer with glucose and hexokinase.

These data show that the [$\beta$-$^{18}O$]ATP was $(S_P)$ and that the desulfurization went, as expected, with inversion.

The $(S_P)$-[$\beta$-$^{18}O$]ATP was converted to $Co(NH_3)_3ATP$ in the usual fashion, and Table II shows the NMR data. While these spectra were more difficult to interpret because of the small chemical shift difference between the endo and exo isomers, at least one of the $\beta$-phosphate peaks was always in a position where the $^{18}O$-induced shift could be measured. The data in Table II show that isomers **1** and **4** are Λ and **2** and **3** are Δ, and the knowledge of which $\alpha$-phosphate oxygen is coordinated allows us to identify isomers **1**–**4** as Λ endo, Δ endo, Δ exo, and Λ exo.

**Inhibition of Enzymes.** $Co(NH_3)_3ATP$ isomers do not inhibit yeast hexokinase at levels of 500 $\mu$M, which is consistent with the fact that the substrate for hexokinase is Λ bidentate MgATP and that the configuration of the triphosphate chain is thought to be extended.[20] Creatine kinase uses Λ exo tridentate MgATP as substrate.[6-8] This assignment is confirmed by inhibition studies with $Co(NH_3)_3ATP$ isomers. The Λ exo $Co(NH_3)_3ATP$ (isomer 4) was a competitive inhibitor of creatine kinase with $K_i$ = 2.3 mM. The Λ endo isomer, which has the proper $\beta$-phosphate configuration but the wrong $\alpha$-phosphate orientation, was not as effective ($K_i$ = 6.7 mM). Neither of the Δ isomers inhibited creatine kinase at levels of 10 mM. The $CF_1$ ATPase from spinach chloroplasts is believed to use tridentate MgATP as a substrate,[21] and $Co(NH_3)_3ATP$ isomers also competitively inhibited this enzyme.[22] $K_i$ values were 170 and 280 $\mu$M for isomers 1 and 4, respectively, while neither of the Δ isomers was an effective inhibitor ($K_i$ values of 900 $\mu$M for isomer 3 and $\gg$700 $\mu$M for isomer 2). Thus, this enzyme exhibits a slight preference for the Λ endo configuration.

## Discussion

**$Co(NH_3)_3ATP$ Isomers.** The complexes characterized in the present work are the first tridentate mononuclear ATP complexes to have their absolute configurations determined. Now that the configurations are known, these complexes may be used as probes of enzyme active sites for enzymes that use tridentate MgATP as a substrate. They thus should prove to be a useful addition to bidentate chromium and cobalt ATP and ADP complexes whose absolute configurations are also known.[15] Note that while triamminecobalt ATP exists only as tridentate complexes, the corresponding triamminechromium ATP complexes are solely bidentate,[23] and efforts to prepare tridentate $Cr(NH_3)_3ATP$ have so far been unsuccessful. Bidentate $Cr(H_2O)_4ATP$ is converted by acid treatment into a material that may be tridentate and appears to exist in at least four or more isomeric forms; the structures of these are not known, and they may not be mononuclear complexes.[9] Rhodium ATP exists as a mixture of two bidentate and four tridentate complexes, but the configurations of the latter at the $\alpha$-phosphate have not been determined (the screw senses at the $\beta$-phosphate are known for all six isomers).[24] The reason why tridentate complexes are more stable than bidentate ones for cobalt, equally stable for rhodium, and less stable for chromium is not clear.

The $^{31}P$ NMR parameters in Table I suggest that chemical shifts may be a guide to structure in tridentate complexes. The $\alpha$-phosphate values are considerably farther upfield for endo than for exo isomers, while the $\beta$-phosphate values are slightly upfield and the $\gamma$ ones, by contrast, somewhat downfield. Only the $\alpha$-phosphate chemical shift is affected by the screw sense (that is, the configuration at the $\beta$-phosphate), with the Δ isomer farther upfield than the Λ one for both the endo and exo configurations. This also occurs in bidentate $Co(NH_3)_4ATP$ (Table III), although the shift is smaller. The $\alpha$–$\beta$ coupling constant for the endo isomers is higher than that of the exo ones or than any of the $\beta$–$\gamma$ values, which are all similar. These patterns will probably be seen in any tridentate cobalt or rhodium complexes (chromium complexes cannot be examined by NMR methods because of the paramagnetism of the chromium) and thus may serve as a guide to the absolute configurations.

The CD spectra show an interesting pattern as well. All bidentate chromium and cobalt complexes with nucleotides or polyphosphates that are chiral show CD spectra where the longest wavelength major band has positive ellipticity for the Λ isomer and negative for the Δ one.[9] This empirical rule continues to hold true for tridentate $Co(NH_3)_3ATP$ complexes, although the ratio of molar ellipticities of the major bands at 590 and 530 nm is greater than unity for the exo isomers and less than unity for the endo ones. This latter pattern is also seen in bidentate $Cr(H_2O)_4ATP$ isomers, where the pseudoaxial ring conformers have a lower ratio of molar ellipticities at 640 and 575 nm (0.3–0.4) than do the pseudoequatorial ones (ca. unity).[9] Thus, a more extended configuration (exo tridentate or pseudoequatorial bidentate) leads to lower ellipticity in the longer wavelength band than in the shorter one. It will be intriguing to see whether these patterns are fortuitous or have some solid structural basis. For the moment, no theory exists that can adequately explain CD spectra of chiral octahedral complexes in a quantitative manner. This is especially true in systems where there is more than one chiral center associated with the transition-metal complex. However, sufficient experimental data are now available so that development of such a theory may be practical.

The electronic transitions associated with each CD band can be assigned as follows, since the facial ammine arrangement lowers the point group symmetry of $Co(NH_3)_3ATP$ from pseudooctahedral to pseudo-$C_3$. An empirical rule for assigning the absolute configuration of $d^3$, $d^8$, and low-spin $d^6$ complexes in $C_3$ symmetry states that, for complexes with a Λ configuration, the transitions with $E$ and $A_2$ symmetry have positive and negative CD curves, respectively.[25] Since we know the absolute configurations of the four diastereomers of $Co(NH_3)_3ATP$, we can, if we assume that this empirical rule applies, assign the absorbance at 590 nm to the $^1A_1 \rightarrow {}^1E_a$ transition and the band at 530 nm to the $^1A_1 \rightarrow {}^1A_2$ transition. Both of these bands arise from the $^1A_{1g} \rightarrow {}^1T_{1g}$ manifold in octahedral symmetry.

**Desulfurization of Sulfur-Substituted Nucleotides with $Br_2$ in $H_2{}^{18}O$.** This procedure for preparing chiral $^{18}O$-labeled nucleotides was first used by Lowe and co-workers,[13] but they found that it worked only with ADP$\alpha$S and only if the pH was not buffered but was allowed to fall during the reaction. With ATP$\alpha$S, or ADP$\alpha$S when the pH was buffered in the neutral pH range, attack by the ionized and thus nucleophilic terminal phosphate during sulfur release, as opposed to attack by water, led to cyclic phosphate triesters that hydrolyzed with scrambling of the $^{18}O$ label into the terminal phosphate as well as the $\alpha$ one. These

(20) Shoham, M.; Steitz, T. A. *J. Mol. Biol.* **1980**, *140*, 1.
(21) Frasch, W. D.; Selman, B. R. *Biochemistry* **1982**, *21*, 3636.
(22) Frasch, W. D.; Pecoraro, V. L., manuscript in preparation.
(23) Pecoraro, V. L.; Cleland, W. W., unpublished experiments.
(24) Lin, I.; Knight, W. B.; Ting, S. J.; Dunaway-Mariano, D. *Inorg. Chem.* **1984**, *23*, 988.
(25) Hawkins, C. J. *Absolute Configuration of Metal Complexes*; Wiley-Interscience: New York, 1971; p 212.

workers reported 93% inversion and 7% retention at the α-phosphate of ADP.[13] At least some of this scrambling of label probably occurred at the start of the reaction before the pH dropped. By starting at pH 3, we have reduced the degree of label scrambling so that no $^{18}O$ was found in the β-phosphate of ADP by $^{31}P$ NMR after the reaction. Mass spectrometric analysis showed less than 3% $^{18}O$ in the β-phosphate, which suggests that the reaction went with ~97% inversion. With ATPβS we also found $^{18}O$ only in the β-phosphate and none in the α- or γ-phosphates. The $^{18}O$ shifts reported in Table II show that this reaction also went with inversion. Here again, the initial low pH keeps the γ-phosphate protonated so that it is too poor a nucleophile to compete with water and produce cyclic triesters whose hydrolysis would scramble the label. Thus, desulfurization by $Br_2$ with the initial pH kept at 3 or below is a rapid and efficient method for preparing chiral $^{18}O$-labeled nucleotides. The low pH results in some hydrolysis, but the yields could probably be increased above those reported here by careful adjustment of temperature, time of incubation, pH during the reaction, and level of $Br_2$ present.

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# Comparison of the Properties of Binary and Ternary Metal Ion Complexes of 1,$N^6$-Ethenoadenosine 5′-Triphosphate (ε-ATP) and Adenosine 5′-Triphosphate (ATP), Including Macrochelate and Purine–Indole Stack Formation

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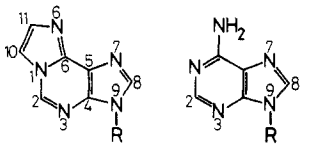
**Abstract:** As ε-ATP can undergo self-association, the experimental conditions for the potentiometric pH titrations, used to determine the acidity constants of $H_2(\varepsilon\text{-ATP})^{2-}$ (the first proton is released from the base residue as spectrophotometric measurements reveal) and the stability constants of several metal ion ($M^{2+}$ = $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$) complexes of $H(\varepsilon\text{-ATP})^{3-}$ and $\varepsilon\text{-ATP}^{4-}$, were chosen such that the ligand was present in its monomeric form. The stabilities of the $Mg^{2+}$ complexes of $\varepsilon\text{-ATP}^{4-}$ and $ATP^{4-}$ are quite similar, but the stabilities of $Mn(\varepsilon\text{-ATP})^{2-}$ and $Zn(\varepsilon\text{-ATP})^{2-}$ are clearly larger than those of the corresponding $M(ATP)^{2-}$ complexes. On the basis of the stabilities of complexes formed with pyrimidine nucleoside 5′-triphosphates (PNTP), in which the base moiety does not participate in complex formation, the extent of macrochelate formation (i.e., the simultaneous coordination of a metal ion to the phosphate and the base residues) is evaluated for monomeric $M(\varepsilon\text{-ATP})^{2-}$ complexes and compared with the $M(ATP)^{2-}$ species; the percentage of the macrochelated isomer is always larger for $M(\varepsilon\text{-ATP})^{2-}$. When the chemical shifts of H-8 and H-11 at infinite dilution ($\delta_0$) for $Mg(\varepsilon\text{-ATP})^{2-}$ and $Zn(\varepsilon\text{-ATP})^{2-}$ are compared, the macrochelate formation in the $Zn^{2+}$ complex is confirmed. Intramolecular aromatic-ring stacking occurs in the ternary 2,2′-bipyridyl complexes $Cu(bpy)(PNTP)^{2-}$, $Cu(bpy)(ATP)^{2-}$, and $Cu(bpy)(\varepsilon\text{-ATP})^{2-}$; the percentage of the stacked isomer increases within this series from about 50 to 90%. $^1H$ NMR shift measurements of the ternary complexes formed between $Mg^{2+}$ or $Zn^{2+}$, $\varepsilon\text{-ATP}^{4-}$ or $ATP^{4-}$, and L-tryptophanate prove that in all four complexes intramolecular purine–indole stacks are formed; an estimate gives about 40% of the stacked isomer in all cases, thus giving evidence that in these ternary complexes the stacking tendencies of the adenine and the ε-adenine moieties, as well as the coordination spheres of $Mg^{2+}$ and $Zn^{2+}$, are quite alike. Several points are outlined that should be considered if ε-ATP is employed as a probe for ATP in enzymic systems.

Metal ion activated processes of nucleotide-depending enzyme systems are playing a prime role in biology,[1] and, consequently, nucleotide–metal ion as well as nucleic acid–metal ion interactions are intensively studied.[2-5] Among the nucleotides, ATP[6] is especially prominent: One-sixth of all known enzymes requires ATP or a related adenine-containing cofactor.[7] It has been estimated[8]

**Chart I**




(1) (a) Sigel, H. Ed. *Met. Ions Biol. Syst.* **1979**, *8*. (b) Spiro, T. G., Ed. *Met. Ions Biol.* **1980**, *1*. (c) Eichhorn, G. L.; Marzilli, L. G., Eds. *Adv. Inorg. Biochem.* **1981**, *3*.

(2) (a) Cooperman, B. S. *Met. Ions Biol. Syst.* **1976**, *5*, 79–125. (b) Mildvan, A. S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1979**, *49*, 103–126. (c) Eichhorn, G. L. *Met. Ions Biol. Syst.* **1980**, *10*, 1–21. (d) Mildvan, A. S.; Loeb, L. A. *Adv. Inorg. Biochem.* **1981**, *3*, 103–123; cf. ref 1c. (e) Wu, F. Y.-H.; Wu, C.-W. *Met. Ions Biol. Syst.* **1983**, *15*, 157–192.

(3) (a) Martin, R. B.; Mariam, Y. H. *Met. Ions Biol. Syst.* **1979**, *8*, 57–124 (cf. ref 1a). (b) Martin, R. B. *Acc. Chem. Res.* **1985**, *18*, 32–38.

(4) (a) Martin, R. B. *Rev. Port. Quim.* **1985**, *27*, 84. (b) Chottard, J.-C. *Rev. Port. Quim.* **1985**, *27*, 85–86. (c) Reedijk, J. *Rev. Port. Quim.* **1985**, *27*, 86–87. (d) Lippard, S. J. *Rev. Port. Quim.* **1985**, *27*, 87–88. (e) Eichhorn, G. L.; Clark, P.; Shin, Y. A.; Butzow, J. J.; Rifkind, J. M.; Pillai, R. P.; Chuknyiski, P. P.; Waysbort, D. *Rev. Port. Quim.* **1985**, *27*, 88–90. (f) Andronikashvili, E. L.; Kharatishvili, M.; Esipova, N. *Rev. Port. Quim.* **1985**, *27*, 90–91. (g) See also: Xavier, A. V., Ed. *Frontiers in Bioinorganic Chemistry*; VCH: Weinheim, 1986.

(5) Barton, J. K. *Comments Inorg. Chem.* **1985**, *3*, 321–348.

(6) Abbreviations and definitions: A, adenine derivative; Ado, adenosine; ε-Ado, ε-adenosine = 1,$N^6$-ethenoadenosine; AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphate; ε-AMP, ε-ADP, and ε-ATP, ε-adenosine 5′-mono-, di-, and triphosphate; bpy, 2,2′-bipyridyl; CTP, UTP, and TTP, cytidine, uridine, and thymidine 5′-triphosphate; $M^{2+}$, bivalent metal ion; NP, nucleotide; Ns, nucleoside; NTP, nucleoside 5′-triphosphate; phen, 1,10-phenanthroline; PNTP, pyrimidine nucleoside 5′-triphosphate; Trp, L-tryptophan. The phosphate groups in NTP are labeled as α, β, and γ, where the latter refers to the terminal phosphate group.

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