

The results show that $(OP)_2Cu^+$ reacts with β -hydroxyl radicals to form intermediates with copper-carbon σ -bonds, which decompose via β -hydroxyl elimination reactions. It will be very interesting to find out whether $(OP)_2Cu^+$ reacts also with free radicals formed in biological systems to produce transients with copper-carbon σ -bonds. If this is the case, one of the routes for biological damage may occur through such intermediates.

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Contribution from the Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

Preparation and Configurational Analysis of the Stereoisomers of β,γ -Bidentate $Rh(H_2O)_4ATP$ and α,β,γ -Tridentate $Rh(H_2O)_3ATP$. A New Class of Enzyme Active Site Probes^{†,1}

Zichun Lu, Andrew L. Shorter,[†] Irene Lin,[†] and Debra Dunaway-Mariano*

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Reaction of $[Rh(H_2O)_6](ClO_4)_3$ with adenosine 5'-triphosphate (ATP) at pH 3 and 80 °C generated a mixture of β,γ -bidentate $Rh(H_2O)_4ATP$ and α,β,γ -tridentate $Rh(H_2O)_3ATP$ in a 1:1 ratio. Purification of the two structural forms was accomplished on a Sephadex-G10 gel filtration column (yield of each purified isomer \approx 10%). The two β -P epimers (Δ and Λ) of the β,γ -bidentate $Rh(H_2O)_4ATP$ complex were resolved on a reverse-phase HPLC column as were the four α,β,γ -tridentate $Rh(H_2O)_3ATP$ diastereoisomers (β -P Δ , α -P endo; β -P Δ , α -P exo; β -P Λ , α -P endo; β -P Λ , α -P exo). β,γ -Bidentate $Rh(H_2O)_4ATP$ isomer **1** (the first of the two isomers to elute from the HPLC column) was found to be a substrate for pyruvate kinase but not to be a substrate for hexokinase. Isomer **2**, on the other hand, served as a substrate for hexokinase, but it was not a substrate for pyruvate kinase. On the basis of the known stereochemical specificities of these two enzymes, isomer **1** was assigned the Δ configuration and isomer **2**, the Λ configuration. The Δ -bidentate isomer displayed a (-) Cotton effect at the 425-nm λ_{max} ($\theta = -380 \text{ deg cm}^2 \text{ dmol}^{-1}$), while the Λ isomer displayed a (+) Cotton effect ($\theta = +180 \text{ deg cm}^2 \text{ dmol}^{-1}$) at this wavelength. The configuration of the β -P in the four α,β,γ -tridentate $Rh(H_2O)_3ATP$ isomers was determined by separately converting the Δ and Λ bidentate isomers to the corresponding pairs of tridentate α -P epimers. The Δ isomer gave rise to tridentate isomers **1** and **4**, and the Λ isomer gave rise to tridentate isomers **2** and **3**. So to determine the α -P configuration in the four tridentate isomers, α,β,γ -tridentate $Rh(H_2O)_3((R_p)-[\alpha-^{16}O,^{18}O]ATP)$ was prepared and the magnitudes of the ^{18}O -induced chemical shifts of the α -P resonances observed in the ^{31}P NMR spectrum of the mixture of stereoisomers were measured. On the basis of these measurements the isomer configurations were shown to be as follows: isomer **1**, β -P Δ , α -P exo; isomer **2**, β -P Λ , α -P exo; isomer **3**, β -P Λ , α -P endo; isomer **4**, β -P Δ , α -P endo. Both tridentate isomers having the Δ - β -P configuration showed negative Cotton effects at the 450-nm λ_{max} (for **1** -340 and for **4** -500 $\text{deg cm}^2 \text{ dmol}^{-1}$) and positive Cotton effects at the 400-nm λ_{max} (for **1** +1400 and for **4** +980 $\text{deg cm}^2 \text{ dmol}^{-1}$), while the tridentate isomers having the Λ - β configuration displayed positive Cotton effects at the 450-nm λ_{max} (for **2** +250 and for **3** +450 $\text{deg cm}^2 \text{ dmol}^{-1}$) and negative Cotton effects at the 400-nm λ_{max} (for **2** -960 and for **3** -480 $\text{deg cm}^2 \text{ dmol}^{-1}$). The bidentate and tridentate $Rh(H_2O)_nATP$ isomers were found to be most stable at low pH and low temperature. At 80 °C the conversion of the bidentate form to the tridentate form occurred significantly faster than did epimerization at the β -P of either structural isomer ($t_{1/2}$ of bidentate $Rh(H_2O)_4ATP$ at pH 3 is \sim 5 min). At higher pH (e.g. pH 6-7) solutions of bidentate or tridentate $Rh(H_2O)_nATP$ were found to equilibrate comparatively faster (at pH 6 and 25 °C $t_{1/2}$ of bidentate $Rh(H_2O)_4ATP$ is \sim 1 h and $K_{eq} \approx$ 1). However, at low temperature the isomerization rate was found to be slow enough to allow the utilization of the individual isomers of the $Rh(H_2O)_nATP$ complexes as structural and biochemical probes of MgATP.

Introduction

Exchange-inert Co(III) and Cr(III) complexes of polyphosphates (e.g. ATP and ADP) have proven to be useful probes of the structural and biochemical properties of naturally occurring Mg^{II} (polyphosphate) complexes.² Because these complexes are stable in aqueous solution, they can be used to determine the structure and stereochemistry of the kinetically labile Mg^{II} (polyphosphate) complex that serves as the natural substrate for a given enzyme. The exchange-inert complexes have also been successfully used in the study of the kinetic mechanisms, metal ion cofactor requirements, and the substrate and cofactor binding

sites of Mg^{II} (polyphosphate) processing enzymes.²

However, the application of the Cr(III) and Co(III) complexes as structural and biochemical probes does have some serious limitations. For example, NMR methods cannot be used to characterize Cr^{III} (polyphosphate) probes or their enzymic products owing to the paramagnetic nature of the Cr(III) metal. The Co^{III} (polyphosphate) complexes on the other hand are diamagnetic and are therefore easily characterized by NMR techniques, but the redox properties of the metal require that the Co^{III} (polyphosphate) complexes also be coordinated to nitrogen-containing ligands such as NH_3 . In many cases the NH_3 ligands interfere with tight and productive binding of the $Co^{III}(NH_3)_n$ (polyphosphate) complex to the enzyme and for this reason use of these systems as biochemical probes is restricted.

* To whom correspondence should be addressed.

[†] Current address: I.L., Development Center for Biotechnology, 81 Chang Hing St., Taipei, Taiwan; A.L.S., Smith Klein and French Co., Philadelphia, PA 19101.

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- (1) Abbreviations: adenosine 5'-triphosphate, ATP; adenosine 5'-diphosphate, ADP; adenosine 5'-phosphate, AMP; potassium 2-morpholinoethanesulfonate, K(MES); mixture of β,γ -bidentate $Rh(H_2O)_4ATP$ and α,β,γ -tridentate $Rh(H_2O)_3ATP$, $Rh(H_2O)_nATP$; nuclear magnetic resonance, NMR; high-pressure liquid chromatography, HPLC; circular dichroism, CD.
- (2) For recent reviews see: Cleland, W. W. *Methods Enzymol.* **1982**, *87*, 159. Cleland, W. W. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1985; pp 141-148. Dunaway-Mariano, D. *Ibid.* pp 149-164.

The current investigation was prompted by our desire to create a new class of exchange-inert metal polyphosphate complexes which contain a metal ion that is both diamagnetic and redox stable. We envisaged that, by use of Rh(III) in place of either Cr(III) or Co(III) in the metal polyphosphate complexes, problems associated with structural characterization (as experienced with the Cr(III) systems) would be avoided. In addition, we anticipated that the Rh(III) complexes would be more biochemically active than the $\text{Co}^{\text{III}}(\text{NH}_3)_n$ (polyphosphate) complexes. In an earlier publication³ we described a general method that could be used for the preparation of Rh(III) complexes of pyrophosphate, triphosphate, ADP, and ATP. In the present paper the preparation, properties, and configurational analysis of the stereoisomers of β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ are described.⁴

Experimental Section

Methods. Proton-decoupled ³¹P NMR spectra were recorded at 25 °C by using either an IBM WP 200 SY (operating at 81.02 MHz) or Bruker AM 400 (operating at 162.04 MHz) NMR spectrometer. NMR samples contained 1.5 mM EDTA in 20% D₂O. Chemical shifts are reported in ppm downfield (+) or upfield (-) from a 0.1 M D₃PO₄ external standard. CD spectra were recorded with a JASCO 500-C spectropolarimeter (equipped with a microcell), and UV/visible absorption spectra were recorded with a Perkin-Elmer 552 spectrophotometer. Concentrations of the solutions of the Rh^{III}ATP complexes were determined at pH 5 by measuring solution absorption at 260 nm ($\epsilon = 15\,400\text{ M}^{-1}\text{ cm}^{-1}$). High-pressure liquid chromatography was carried out by using a Beckman 332 HPLC equipped with a Hitachi 100-10 variable-wavelength detector, an Altex C-18 reverse-phase analytical column (4.6 mm \times 25 cm, 1 mL/min flow rate), or a Du Pont C-18 reverse-phase preparative column (21.2 mm \times 25 cm, 4 mL/min flow rate) and 0.01–0.10 M potassium methanesulfonate at pH 2.2 as an isocratic eluant. All enzymes, nucleotides, buffers, and chromatography resins were obtained from Sigma Chemical Co. Rhodium chloride was purchased from Aldrich Chemical Co.

Synthesis of β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$. Fifty milliliters of 20 mM $[\text{Rh}(\text{H}_2\text{O})_6](\text{ClO}_4)_3$ at pH 3 was combined with 50 mL of 20 mM ATP at pH 3, and the resulting solution was heated at 80 °C for 15 min. After the solution was cooled to 0 °C, the pH was adjusted to 2 with 6 N HCl. This solution was then absorbed onto a 1.7 \times 40 cm column of Dowex 50-X2 (H⁺) resin. The $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}/\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ mixture was slowly eluted from the column at 4 °C with deionized water. The $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}/\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ -containing fractions were combined, concentrated in vacuo (25 °C) to 5 mL, adjusted to pH 4, and chromatographed on a 1.7 \times 150 cm G-10 Sephadex column at 4 °C. Ten millimolar 1,4-bis(2-hydroxyethyl)piperazine, at pH 4.5, was used as eluant. The elution profile (A^{260} vs fraction) obtained showed two partially resolved, equal-sized peaks. The fractions of the first peak contained β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$, while the fractions of the second peak were enriched with α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$. AMP eluted in the fractions comprising the back quarter of the second peak. Fractions containing mainly bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and fractions containing mainly tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ were separately combined, concentrated in vacuo, and rechromatographed on the Sephadex G-10 column. Fractions containing pure bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ or pure tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ were separately combined and concentrated in vacuo to give 20 mM solutions of the complexes. The proton-decoupled ³¹P NMR data measured at pH 2.5 are as follows: β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$, +7.7 (d, $J = 20.5$ Hz, γ -P), -8.0 (dd, β -P), -11.2 (isomer 1), -11.0 ppm (isomer 2) (d, $J = 17.0$ Hz, α -P); α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$, +8.1 (d, $J = 21.0$ Hz, γ -P isomer 1), +8.0 (d, $J = 20.3$ Hz, γ -P isomer 2), +7.7 (d, $J = 20.0$ Hz, γ -P isomer 4), +7.6 (d, $J = 20.2$ Hz, γ -P isomer 3), +1.5 (d, $J = 19.4$ Hz, α -P isomer 3), +1.2 (d, $J = 20.0$ Hz, α -P isomer 4), +0.73 (d, $J = 22.0$ Hz, α -P isomer 1), +0.70 (d, $J = 22.4$ Hz, α -P isomer 2), -7.0 ppm (m, β -P isomers 1–4).

Separation of β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ Stereoisomers. Reverse-Phase HPLC Method. The diastereoisomers of purified β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ were separated for analysis by using an Altex

C-18 reverse-phase analytical HPLC column (4.6 mm \times 25 cm) and 100 mM potassium methanesulfonate (pH 2.2) eluant (1 mL/min flow rate). Under these conditions the retention times of the two bidentate isomers were 7.2 and 7.5 min and the retention times of the four tridentate isomers were 6.0, 7.0, 7.5, and 9.5 min. Preparative-scale separation of the bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ stereoisomers was carried out by using a Dupont C-18 reverse-phase preparative column (21.2 mm \times 25 cm) and 100 mM potassium methanesulfonate (pH 2.2) (for bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$) or 10 mM potassium methanesulfonate (pH 2.2) (for tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$) as eluant (4 mL/min flow rate).

Dowex 50 (H⁺) Method. Chromatography of a roughly equal mixture of β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers (10 mL, 20 mM, pH 2) was carried out on a 1.5 \times 60 cm Dowex 50-2X (H⁺) column with deionized water (4 °C) as eluant. The $\text{Rh}(\text{H}_2\text{O})_n\text{ATP}$ complexes chromatographed through the column as a broad yellow band. Five-milliliter fractions were collected and analyzed by using analytical reverse-phase (C-18) HPLC techniques (100 mM potassium methanesulfonate, pH 2.2).

Configurational Analysis of the β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ Stereoisomers. The configurations of the purified bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ β -P epimers were determined by testing the substrate activities of the two isomers with yeast hexokinase and rabbit muscle pyruvate kinase. Reaction mixtures (250 μL) containing one of the two $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers at a concentration of 100 μM and either 2 mM [¹⁴C]glycolate, 50 mM KCl, 5 mM MnCl₂, 100 mM K(MES) (pH 6), and 1000 units/mL of rabbit muscle pyruvate kinase or 1 mM [¹⁴C]glucose, 1000 units/mL of yeast hexokinase, and 100 mM K(MES) (pH 6) were incubated for 8 min at 0 °C. The reactions were terminated by the addition of 25 μL of concentrated HClO₄, diluted to a volume of 1 mL with deionized water, and filtered through a glass wool plug in a disposable pipet. The reaction solution was then absorbed onto a column of 4.5 mL of Dowex 50-X2 (H⁺) resin in a 10-mL volumetric pipet. The unreacted glucose and glycolic acid were eluted from the column with 50 mL of 10 mM HCl, and the product complexes, $\text{Rh}(\text{H}_2\text{O})_4(\text{glucose-6-P})(\text{ADP})$ and $\text{Rh}(\text{H}_2\text{O})_4(\text{glycolate-P})(\text{ADP})$, were eluted with 50 mL of 1 N HCl. The amount of ¹⁴C contained in each fraction was determined by using liquid scintillation counting techniques.

Configurational Analysis of the α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ Stereoisomers. Conversion of β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ to α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$. Potassium methanesulfonate solutions (pH 2.5) of the pure α,β -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers were heated at 80 °C. The isomer composition of each reaction was examined at varying conversion by injection of aliquots of the solutions onto an Altex C-18 analytical reverse-phase HPLC column.

³¹P NMR Analysis of α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{18}\text{O},^{16}\text{O}]\text{ATP})$ Isomers. $(R_P)-[\alpha\text{-}^{18}\text{O}]\text{ATP}$ was prepared according to the method of Speckhard et al.⁵ and mixed with 1 molar equiv of $[\alpha\text{-}^{16}\text{O}]\text{ATP}$. The reaction of $[\text{Rh}(\text{H}_2\text{O})_6](\text{ClO}_4)_3$ with the $(R_P)-[\alpha\text{-}^{18}\text{O}]\text{ATP}/[\alpha\text{-}^{16}\text{O}]\text{ATP}$ mixture was carried out on a 10-mL scale (see above). The reaction mixture was chromatographed on a 1.5 \times 30 cm Dowex 40 (H⁺) column with deionized H₂O and then analyzed by using ³¹P NMR techniques.

Results and Discussion

Preparation and Configurational Analysis of the β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ Stereoisomers. β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ was formed, along with α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ by heating a solution of $[\text{Rh}(\text{H}_2\text{O})_6](\text{ClO}_4)_3$ and ATP at pH 3 and 80 °C. The β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}/\alpha,\beta,\gamma$ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ mixture ($\text{Rh}(\text{H}_2\text{O})_n\text{ATP}$) was separated from reactants and byproducts present in the reaction solution by using Dowex 50-X2 (H⁺) ion-exchange chromatography. $\text{Rh}(\text{H}_2\text{O})_n\text{ATP}$ obtained in this manner was essentially pure except for contamination by a small amount of AMP (ca 15% of the total nucleotide present) that was formed during the course of the reaction. AMP was removed from $\text{Rh}(\text{H}_2\text{O})_n\text{ATP}$ by using G-10 Sephadex column chromatography. Because of its larger size, $\text{Rh}(\text{H}_2\text{O})_n\text{ATP}$ elutes from the column at a faster rate than does the AMP. Separation of the bidentate and tridentate structural isomers was also accomplished by the gel filtration chromatography. Because of its more compact shape, the tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ complex is retained by the resin more efficiently than is the bidentate complex. Rechromatography

(3) Lin, I.; Knight, W. B.; Ting, S.-J.; Dunaway-Mariano, D. *Inorg. Chem.* **1984**, *23*, 988.

(4) The absolute charge carried by these complexes is pH dependent. Above pH 4 the complexes have a -1 charge ($N\text{-}1\text{ p}K_a \sim 3.8$), between pH 2 and 3 they are neutral and below pH 2 they have a +1 charge ($\gamma\text{-P secondary p}K_a \sim 2$).

(5) Speckhard, D. C.; Pecoraro, V. L.; Knight, W. B.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 4167; corrections in *J. Am. Chem. Soc.* **1988**, *110*, 2349, that reassign $(\text{NH}_3)_3\text{ATP}$ isomers 1–4 as Δ exo, Δ exo, Δ endo, and Λ endo.

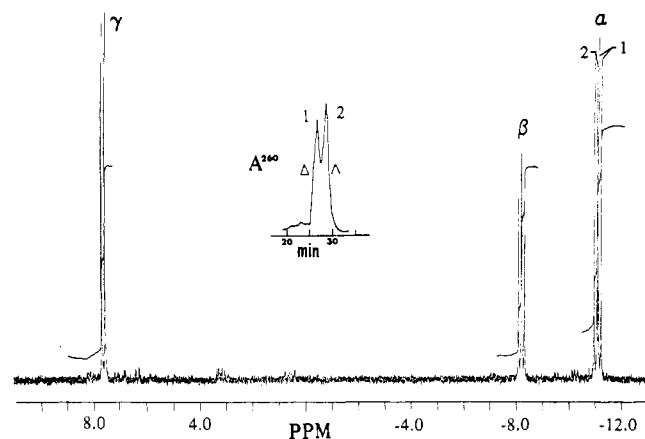


Figure 1. Proton-decoupled ^{31}P NMR spectrum of β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ measured at 162.04 MHz, pH 2.5, and 25 °C. The inset is the elution profile of the two $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers chromatographed on the Du Pont C-18 reversed-phase HPLC preparative column (see Experimental Section). The isomers are numbered according to their relative position of elution from the HPLC column. The α -P resonances are assigned accordingly.

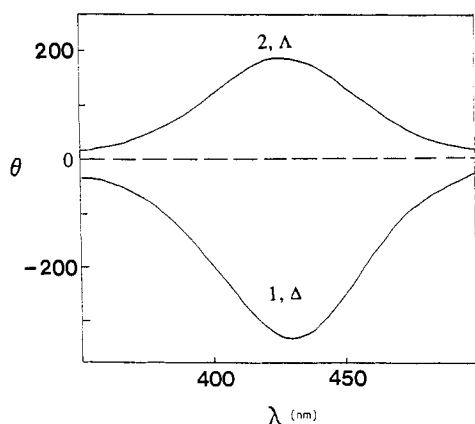


Figure 2. Circular dichroism spectra of the β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers at pH 2.5. The isomer designation is based upon the observed order of isomer elution from the reversed-phase HPLC column.

of bidentate enriched fractions and tridentate enriched fractions separately on a second G-10 Sephadex column produced pure samples of the two structural isomers in 10% and 8% overall yields, respectively.

After purification of the bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ complexes, the next task was to separate their stereoisomers. The β -P of β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ is chiral, and consequently, the bidentate complex exists as two β -P epimers. By convention,⁶ these epimers are designated as Δ and Λ screw sense isomers (Chart I). The α,β,γ -tridentate complex, on the other hand, is chiral at the α -P as well as at the β -P. Thus, four diastereoisomers exist, and these are designated as follows: β -P Δ , α -P endo; β -P Δ , α -P exo; β -P Λ , α -P endo; β -P Λ , α -P exo (Chart I).⁶

The ^{31}P NMR spectrum of the β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ complex (Figure 1) reveals the presence of both screw sense isomers. The ^{31}P NMR spectra of the HPLC purified isomers (numbered on the basis of their order of elution from the HPLC column) provided for the assignments of the α -P resonances made in Figure 1. The CD spectra of the pure β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers are shown in Figure 2. Isomer 1 displays a (-) Cotton effect at the 425-nm λ_{max} ($\theta = -380 \text{ deg cm}^2 \text{ dmol}^{-1}$) while isomer 2 displays a (+) Cotton effect ($\theta = +180 \text{ deg cm}^2 \text{ dmol}^{-1}$) at this wavelength.

The configuration at the β -P of each of the two bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers was determined by testing the purified isomers as substrates for the enzymes yeast hexokinase and rabbit

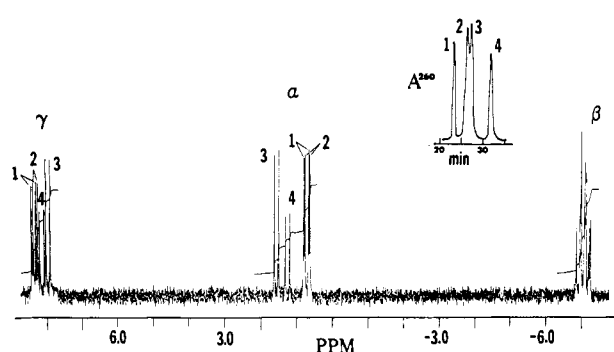


Figure 3. Proton-decoupled ^{31}P NMR spectrum of α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ measured at 162.04 MHz, pH 2.5, and 25 °C. The inset is the elution profile of the four $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers chromatographed on the Du Pont C-18 reversed-phase HPLC preparative column (see Experimental). The isomers are numbered according to their relative elution position. The γ -P and α -P resonances are assigned accordingly.

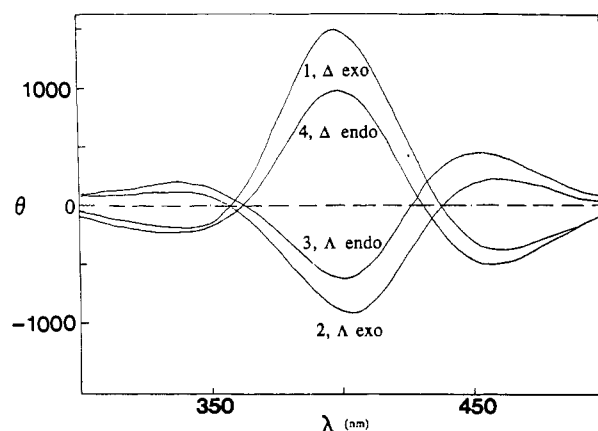


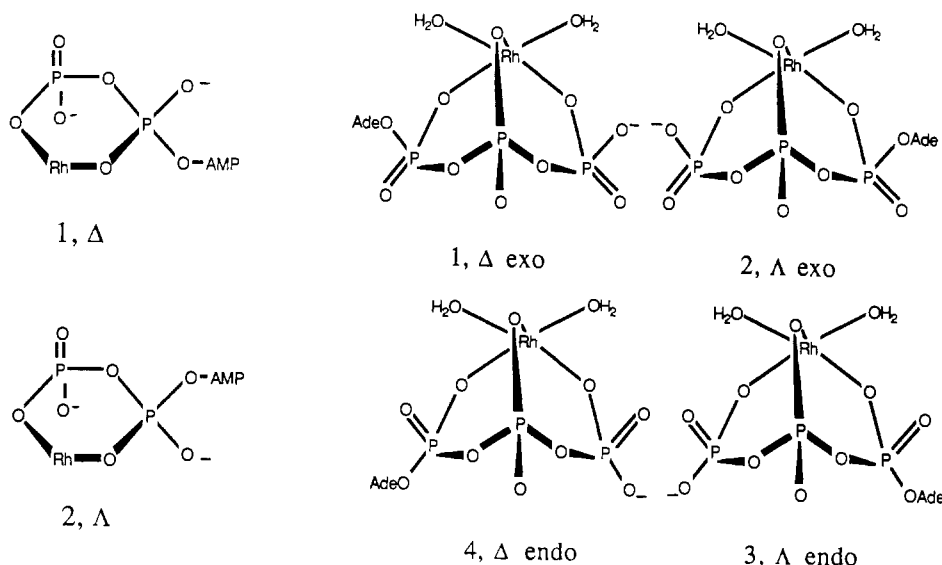
Figure 4. Circular dichroism spectra of the α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers at pH 2.5. The isomer designation is based upon the order of isomer elution from the reversed-phase HPLC column.

muscle pyruvate kinase. Previous studies carried out with exchange-inert Co(III) and Cr(III) complexes of ATP have shown that hexokinase is specific for the Λ isomer of the β,γ -bidentate complex while pyruvate kinase is specific for the Δ isomer.^{6,7} Independent reaction of the purified Λ - and Δ - β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers with glucose in the presence of hexokinase (see Methods for reaction conditions) produced 113 mmol of $\text{Rh}(\text{H}_2\text{O})_4(\text{glucose 6-P})(\text{ADP})$ in 10 min from isomer 2 and 5 mmol of this product from isomer 1. Reaction of bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomers 1 and 2 with glycolate in the presence of pyruvate kinase produced 10 nmol of $\text{Rh}(\text{H}_2\text{O})_4(\text{glycolate-P})(\text{ADP})$ from isomer 1 and less than 1 nmol of product from isomer 2. On the basis of these results, we conclude that the isomer that displays the shorter of the two retention times on an HPLC column (isomer 1) has the Δ configuration while the other isomer (isomer 2) has the Λ configuration.

The ^{31}P NMR spectrum of the mixture of the four tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers is shown in Figure 3. ^{31}P NMR spectra obtained for the HPLC-purified isomers (numbered on the basis of their order of elution from the HPLC column) were used to make the isomer assignments depicted in Figure 3. The CD spectra of the four individual tridentate isomers are presented in Figure 4. Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers 1 and 4 show negative Cotton effects at the 450-nm λ_{max} (-340 and $-500 \text{ deg cm}^2 \text{ dmol}^{-1}$, respectively) and positive Cotton effects at the 400-nm λ_{max} ($+1400$ and $+980 \text{ deg cm}^2 \text{ dmol}^{-1}$, respectively) while isomers 2 and 3 display positive Cotton effects at 450 nm ($+250$ and $+450 \text{ deg cm}^2 \text{ dmol}^{-1}$, respectively) and negative Cotton effects at 400

(6) Cornelius, R. D.; Cleland, W. W. *Biochemistry* 1978, 17, 3279.

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Chart I. Representation of the Two β,γ -Bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ Isomers and Four α,β,γ -Tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ Isomers

nm (-960 and -480 deg cm^2 dmol^{-1} , respectively).

The configuration at the β -P of each of the four tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ isomers was determined by first converting each of the β,γ -bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ β -P epimers having known configuration (see above) to the corresponding pairs of tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ α -P epimers. The Δ bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomer (pH 2.5) heated at 80°C produced tridentate isomers **1** and **4**, whereas the Λ bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ isomer yielded tridentate isomers **2** and **3**. On the basis of these results, tridentate isomers **1** and **4** were assigned the Δ β -P configuration and isomers **2** and **3** the Λ β -P configuration.

The stereochemistry at the α -P of the four tridentate isomers was determined by using an NMR-based approach. The α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{16}\text{O},^{18}\text{O}]\text{ATP})$ complex was prepared, and the magnitudes of the ^{18}O -induced chemical shifts of the α -P resonances corresponding to the four isomers were measured. Previous ^{31}P -NMR analysis of $\text{Co}(\text{III})$ and $\text{Rh}(\text{III})$ complexes of ^{18}O -labeled nucleotides have shown that substitution of an ^{18}O atom at a nonbridge position on the phosphorus atom (viz. $\text{P}=\text{O}$) causes an approximate 0.03 ppm upfield shift of the phosphorus resonance, while substitution of ^{18}O at a bridge position (viz. $\text{P}-\text{O}-\text{metal}$) causes only a 0.015 ppm shift.^{5,8} Thus, by measurement of the magnitude of the ^{18}O -induced chemical shifts of the α -P resonances arising from the four α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{16}\text{O},^{18}\text{O}]\text{ATP})$ isomers (Figure 5), the position of $\text{Rh}(\text{III})$ coordination at the α -phosphoryl moiety of each isomer could be determined. Accordingly, tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{16}\text{O},^{18}\text{O}]\text{ATP})$ isomers **1** and **3**, which were previously shown to have the Δ and Λ configurations at the β -P, respectively displayed a 0.015 ppm isotopic shift and were therefore assigned the β -P Δ , α -P exo and β -P Λ , α -P endo configurations, respectively. Likewise, tridentate isomers **2** and **4**, having the respective Λ and Δ configurations at the β -P, both showed an isotopic shift of 0.03 ppm and were thus assigned the β -P Λ , α -P exo and the β -P Δ , α -P endo configurations, respectively.

The CD and ^{31}P NMR spectral data for the RhATP complexes presented in Figures 1–4 of this paper were compared to the spectral data reported for the CoATP and CrATP complexes in earlier publications.^{5,6,9,10} Basic trends were observed that allow correlation of the coordination stereochemistry to the CD and ^{31}P NMR patterns. For instance, all bidentate and tridentate com-

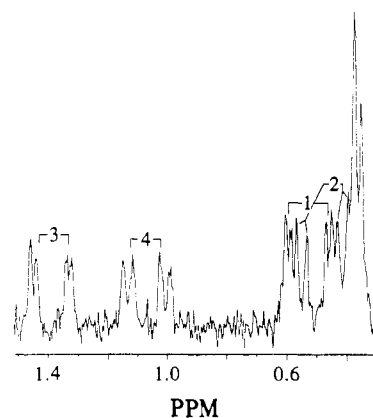


Figure 5. α -P resonances of the proton-decoupled ^{31}P NMR spectrum of a 1:1 mixture of α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{16}\text{O}]\text{ATP})$ and α,β,γ -tridentate $\text{Rh}(\text{H}_2\text{O})_3((R_P)-[\alpha\text{-}^{18}\text{O},^{18}\text{O}]\text{ATP})$ at pH 2. The isomer designation is based upon the order of isomer elution from the reversed-phase HPLC column. The two resonances appearing furthest upfield in the spectrum derive from $(R_P)-[\alpha\text{-}^{18}\text{O}]\text{AMP}$ and $(R_P)-[\alpha\text{-}^{16}\text{O}]\text{AMP}$.

plexes having the Δ configuration at the β -P display a negative Cotton effect at the longest wavelength absorption band, while isomers having the Λ β -P configuration show a positive Cotton effect in this region. A more subtle correlation is seen in the CD spectra of the tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ and $\text{Co}(\text{NH}_3)_3\text{ATP}$ isomers. Specifically, the ratios of the ellipticities at the long λ_{max} (460 nm for $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ and 590 nm for $\text{Co}(\text{NH}_3)_3\text{ATP}$) and the short λ_{max} (400 nm for $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ and 530 nm for $\text{Co}(\text{NH}_3)_3\text{ATP}$) are significantly closer to 1:1 for the α -P endo isomers than for the α -P exo isomers.

It can be seen from the ^{31}P NMR spectra of the β -P epimers of the bidentate $\text{Rh}(\text{H}_2\text{O})_4\text{ATP}$ and $\text{Co}(\text{NH}_3)_4\text{ATP}$ complexes that the α -P resonance of the Δ β -P isomer is shifted further upfield than is the α -P resonance of the Λ isomer (the γ -P and β -P resonances are not well resolved). The stereoisomers of the tridentate $\text{Rh}(\text{H}_2\text{O})_3\text{ATP}$ and $\text{Co}(\text{NH}_3)_3\text{ATP}$ complexes are characterized by their relative γ -P and α -P resonance chemical shifts. The γ -P resonances of the α -P endo isomers appear furthest upfield in the γ -P resonance region. Of the two endo isomers, that having the Λ β -P configuration shows the greatest upfield shift. The chemical shifts of the γ -P resonances of the exo isomers are similar. The α -P endo isomers characteristically show α -P resonances that are further downfield from those of the exo isomers. In this case the β -P Λ , α -P endo resonance is furthest downfield and the chemical shifts of the resonances of the two exo isomers are nearly identical.

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Kinetic and Thermodynamic Properties of the Rh(III) Complexes of ATP. ³¹P NMR and HPLC techniques were used to assess the stabilities of the β,γ -bidentate Rh(H₂O)₄ATP and α,β,γ -tridentate Rh(H₂O)₃ATP complexes in aqueous solution as a function of pH and temperature. We found that at low pH (~ 2) and temperature (4 or -80 °C) stereoisomerization and structural isomerization occurred so slowly that purified isomers could be stored for at least 2 weeks without any isomer interconversion or degradation. At 80 °C where the isomerization process was easily observed, the half-life of the bidentate complex was found to be ca. 5 min. Examination of the reaction (80 °C, pH 2) of the individual bidentate screw sense isomers at varying conversion showed that insertion of the α -P into the coordination sphere of the Rh(III) took place significantly more rapidly than did epimerization at the β -P. Thus, each bidentate Rh(H₂O)₄ATP isomer converted selectively to the corresponding pair of tridentate α -P epimers. Continued heating of the isomer solutions beyond a 5-min period did, however, lead to the eventual appearance of the tridentate α -P epimers having β -P configurations opposite that of the bidentate precursor.

At higher pH (e.g. pH 6–7) solutions of bidentate or tridentate Rh(H₂O)_nATP were found to equilibrate comparatively faster to an equilibrium mixture in which the ratio of bidentate to tridentate complex was ca. 1:1. At pH 6 and 25 °C, for example, the half-life measured for the bidentate complex was only 1 h. At lower temperatures, however, the isomerization process was suppressed. Bidentate or tridentate isomers can be incubated at pH 6 for up to 1 h at 0 °C without detectable levels of isomerization (<5%). We therefore recommend that studies of individual isomers that must be carried out at or near neutral pH should utilize short incubation periods at the lowest temperature possible.

It is interesting to note that the structural isomer composition of the exchange-inert metal-ATP complexes prepared from Co(III), Cr(III), and Rh(III) are quite different. Reaction of ATP with Co(NH₃)₃(H₂O)₃³⁺ produces α,β,γ -tridentate Co(NH₃)₃ATP,^{9,5} while reaction of ATP with Cr(NH₃)₃(H₂O)₃³⁺ or Cr(H₂O)₆³⁺ yields β,γ -bidentate Cr(NH₃)₃(H₂O)ATP⁵ and Cr(H₂O)₄ATP,¹⁰ respectively. Reaction of ATP with Rh(H₂O)₆³⁺, on the other hand, produces an equal mixture of β,γ -bidentate and α,β,γ -tridentate isomers. In the case of the Co^{III}ATP and Rh^{III}ATP complexes, the isomer population observed undoubtedly reflects the relative stability of the tridentate vs bidentate forms. In contrast, the bidentate Cr(NH₃)₃(H₂O)ATP and Cr(H₂O)₄ATP complexes appear to be unable to overcome the kinetic barrier to insertion of the α -P under "normal" reaction conditions (viz. pH 3 and 80 °C). Specifically, prolonged heating of the bidentate Cr^{III}ATP complexes fails to convert them to tridentate complexes. However, exposure of the bidentate Cr(H₂O)₄ATP complex to strong acid at 4 °C for a 48-h period produces a new Cr^{III}ATP complex (stoichiometry of Cr(III) to ATP is 1:1) which displays properties that are consistent with the tridentate structure.^{10,11} Among these is the chromatographic behavior of the complex on Dowex 50 (H⁺) columns that are eluted with H₂O. During the chromatography of bidentate Cr(H₂O)₄ATP on a Dowex 50 (H⁺) column, the putative tridentate complex is formed. Half of this newly formed complex elutes from the column just prior to the unconsumed bidentate complex, while the other half elutes immediately after it.¹¹ The CD spectra measured for fractions constituting the front halves and back halves of each of the "tridentate" peaks indicate the bidentate Cr(H₂O)₄ATP-

derived complex consists of four isomers. Cleland¹¹ proposed that the first peak eluting from the column is comprised of the two partially resolved α -P endo isomers of the tridentate Cr(H₂O)₃ATP complex and the second peak is comprised of two partially resolved isomers having the α -P exo configuration. Cleland reasoned that the pK_a of the γ -P phosphoryl group in the endo isomers would be lower since, unlike the case of the two exo isomers, the protonated N-1 of the adenine ring comes in close contact with the γ -phosphoryl group. Because ionization of the γ -phosphoryl promotes the elution of the complex from the column, the two α -P endo isomers may be expected to elute before the two α -P isomers. The putative Cr(H₂O)₃ATP has not yet been crystallized for X-ray analysis, and consequently, definitive structural identification of this material has not been made.

Although the exact structure of the species generated from the bidentate Cr(H₂O)₄ATP in acid is not known, this complex has been used as a probe in numerous biochemical studies.^{2,11} Because the structure of the bidentate Cr(H₂O)₄ATP-derived material was of both practical and theoretical interest to us, we set out to explore Cleland's postulate first by determining the chromatographic behavior of a mixture of bidentate and tridentate Rh(H₂O)_nATP isomers on Dowex 50 (H⁺) columns. We found that tridentate Rh(H₂O)₃ATP isomers 3 and 4, both of which have the α -P endo configuration, did indeed elute first from the column, followed closely by bidentate Rh(H₂O)₄ATP and then by the α -P exo tridentate isomers 1 and 2. In a separate study¹² the inhibition constants for the tridentate Rh(H₂O)₃ATP complex serving as a competitive inhibitor of kinases were measured for comparison to those obtained earlier⁷ for the putative tridentate Cr(H₂O)₃ATP complex. The results obtained show that the binding affinities of the two complexes toward kinases are quite close. This suggests that the two complexes have similar structures. Thus, we judge Cleland's postulate to be correct and suggest that the acid product of bidentate Cr(H₂O)₄ATP is the tridentate complex. However, it is still unclear why conversion of the bidentate Cr(H₂O)₄ATP complex to the tridentate Cr(H₂O)₃ATP complex does not occur under conditions known to induce isomerization of the bidentate isomers to the tridentate isomers in the Co^{III}ATP and Rh^{III}ATP series.^{9,10} It is possible that in the case of the Cr^{III}ATP complex α -P insertion can only occur via a dissociative mechanism. The acid catalyst that is required may serve to protonate a water ligand of the Cr(H₂O)₄ATP complex, thereby inducing the dissociation of that water ligand from the Cr(III) center.

In conclusion, we have prepared and assigned the configurations to the two β,γ -bidentate Rh(H₂O)₄ATP stereoisomers and to the four α,β,γ -tridentate Rh(H₂O)₄ATP stereoisomers. In an independent study¹² we have evaluated the properties of these complexes as biochemical probes and have found them to be superior models of the naturally occurring complex MgATP. In this paper we have reported interesting differences that exist between the kinetic and thermodynamic properties of the Rh^{III}ATP complex and those of the Co^{III}ATP and Cr^{III}ATP complexes. In future studies the basis for these differences will be closely examined. Finally, by comparing properties of the tridentate Rh(H₂O)₃ATP complex to those of the bidentate Cr(H₂O)₄ATP acid product complex, we have provided supporting evidence that this complex is α,β,γ -tridentate Cr(H₂O)₃ATP. Through the use of methods that have allowed us to purify the tridentate Rh(H₂O)₃ATP stereoisomers, we hope to be able to resolve the tridentate Cr(H₂O)₃ATP stereoisomers for configurational analysis.

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