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Hydrolysis and Decomposition of Chromium(III) and Cobalt(III) Complexes of Adenosine 5′-Triphosphate[1]

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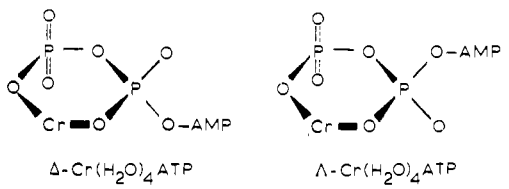
The β,γ-bidentate and α,β,γ-tridentate complexes of Cr(III) and Co(III), which are useful kinetic and spectroscopic probes of enzymes catalyzing phosphoryl-transfer reactions, have been found to decompose at elevated pH and temperature to either free ATP or the products of ATP hydrolysis, ADP and, to a lesser extent, AMP. The decomposition of these complexes was examined by means of thin-layer chromatographic analysis and ^{31}P NMR spectroscopy as well as by coupled enzymatic assays. Decomposition of $Cr(H_2O)_4ATP$ proceeds predominantly with release of free ATP, producing lesser amounts of ADP, while tridentate $Cr(H_2O)_3ATP$ produces exclusively ADP. $Co(NH_3)_4ATP$ decomposes more slowly to yield levels of ATP and ADP that are lower than those produced with the analogous $Cr(H_2O)_4ATP$. By contrast, the breakdown of tridentate $Co(NH_3)_3ATP$ is rapid and produces high levels of free ATP and lesser amounts of ADP. The initial rates of these decompositions are rapid, and the initial rate constants for hydrolysis are 100–5000 times larger than those for uncomplexed ATP. The dependence of these decomposition processes on pH and the nature of the metal chelation are discussed, particularly in relation to previous studies of metal catalysis of nucleotide hydrolysis.

Introduction

Adenosine triphosphate (ATP[4]) plays a crucial role in the energy metabolism of all living cells. The enzyme-catalyzed hydrolysis of ATP to adenosine diphosphate (ADP) provides the metabolic energy for such functions as biosynthesis, active transport, and muscular contraction. Since the enzymes catalyzing these phosphoryl transfers always require a divalent metal (usually Mg^{2+} or Mn^{2+}), and since ATP has a high affinity for metal ions, much work has been directed toward understanding the function of the divalent metal. Perhaps the best way to study the role of the metal in these systems is through the use of model systems.

Cr(III) and Co(III) complexes of ATP have been shown to be very good analogues of MgATP.[5a-c] The chromium complexes were originally prepared by DePamphilis and Cleland,[5d] and the cobalt complexes by Cornelius, Hart and Cleland.[5e] These complexes, which are kinetically inert with respect to ligand exchange, have seen widespread use as probes of enzyme active sites.[6-8] The diamagnetic cobalt complexes are complementary to the paramagnetic chromium complexes and thus facilitate a wide range of NMR and EPR experiments with enzyme systems. The suitability of these complexes as analogues of MgATP is supported by the fact that bidentate CrATP is a substrate for all 9 kinases tested by Dunaway-Mariano and Cleland.[5b] While not as thoroughly tested, bidentate $Co(NH_3)_4ATP$ is also a substrate for hexokinase.[5c] The tridentate isomers of either cobalt or chromium have not been shown to be substrates for any enzyme.

Because these chromium and cobalt complexes are kinetically inert with respect to ligand exchange, they exhibit chirality, which enhances their utility as enzyme probes. In a β,γ-bidentate complex of ATP, the γ-phosphate is not asymmetric but only prochiral. However, the β-phosphate is asymmetric and there are at least two possible isomers of the complex determined by the position of the AMP moiety as indicated:



Δ-$Cr(H_2O)_4ATP$ Λ-$Cr(H_2O)_4ATP$

Cleland devised the nomenclature illustrated, which defines the screw sense of the coordination to the metal.[5c] This nomenclature eliminates any ambiguity possible with the conventional *R,S* notation.

With some exceptions, most studies using these cobalt and chromium nucleotides have only dealt with their enzymatic properties. Dunaway-Mariano and Cleland observed the nonenzymatic epimerization of the isomers of bidentate CrATP.[9] This base-catalyzed process is biphasic and consists of at least two competing reactions, epimerization and hydrolysis. Epimerization is the faster of the two processes and was studied by following the change in ellipticity by circular dichroism. The hydrolysis was studied by chromatographing the results of an incubation at pH 7 on a cycloheptaamylose column. The primary product was identified as $Cr(H_2O)_4(ADP)(P_i)$. There was no significant breakdown to form free nucleotides.

For several reasons a better understanding of the nonenzymatic chemistry of these complexes is needed. One of the problems involved in studying the role of metal ions in the hydrolysis of ATP lies in the uncertainty in the actual structure of the active metal–nucleotide complex. Since the structures of these cobalt and chromium complexes are known, the effect of metal on ATP may be easier to understand. In addition to the role of the metal in catalysis, more information on the general characteristics and stability of these complexes is needed. As these compounds see wider use in various enzymatic systems, it will become important to ensure that these

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(4) Abbreviations used were as follows: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; Caps, 3-(cyclohexylamino)propanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MK, myokinase; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; P_i, inorganic phosphate; PEP, phosphoenolpyruvate; PGK, phosphoglycerate kinase; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); PK, pyruvate kinase; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.
(5) (a) Cleland, W.; Mildvan, A. *Adv. Inorg. Biochem.* **1979**, *1*, 163–191. (b) Dunaway-Mariano, D.; Cleland, W. *Biochemistry* **1980**, *19*, 1506–1515. (c) Cornelius, R.; Cleland, W. *Ibid.* **1978**, *17*, 3279–3286. (d) DePamphilis, M.; Cleland, W. *Ibid.* **1973**, *12*, 3714–3724. (e) Cornelius, R.; Hart, P.; Cleland, W. *Inorg. Chem.* **1977**, *16*, 2799–2805.
(6) Gupta, R.; Fung, C.; Mildvan, A. *J. Biol. Chem.* **1976**, *251*, 2421–2430.
(7) Li, T.; Mildvan, A.; Switzer, R. *J. Biol. Chem.* **1978**, *253*, 3918–3923.
(8) Grisham, C. *J. Inorg. Biochem.* **1981**, *14*, 45–57.
(9) Dunaway-Mariano, D.; Cleland, W. *Biochemistry* **1980**, *19*, 1496–1505.

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complexes are not misused, leading to erroneous data and interpretations.

In the course of our investigations, we observed breakdown of these complexes that was temperature and pH sensitive. This paper describes the systematic incubation of several Cr(III) and Co(III) complexes of ATP at elevated temperatures and at various alkaline pHs. The buffered solutions were examined for the presence of free AMP, ADP, and ATP at specified time intervals. The goal of this work was to determine correlations between the structure and the rate of breakdown and hydrolysis of the metal–nucleotide complexes.

A second, related goal of this work was to establish a reliable method for the quantitative determination of free AMP, ADP, and ATP in solutions containing the original metal–nucleotide complexes. Previous studies of the nonenzymatic hydrolysis of ATP often used a phosphate assay to determine the amount of hydrolysis. However, in this work it was necessary to know the amount of free ATP released from the complexes as well as the extent of any hydrolysis. We describe here three enzymatic assays that are sensitive to low concentrations of nucleotides yet are not compromised by the presence of the Cr(III) and Co(III) complexes themselves.

Experimental Procedures

Materials. Myokinase, pyruvate kinase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, ATP, ADP, AMP, Pipes, Caps, Ches, Tris, Tes, NADH, phosphoenolpyruvate, and glycerate 3-phosphate were purchased from the Sigma Chemical Co. The free radical 2,2,6,6-tetramethylpiperidinyl-1-oxy was purchased from Aldrich. Other chemicals were of reagent grade or the highest purity commercially available.

Borate, Caps, Ches, Pipes, and Tes buffers were sodium salts. Tris buffer was the hydrochloride.

Syntheses. The bidentate and tridentate complexes of CrATP were prepared according to the general procedures of Dunaway-Mariano and Cleland.[9] $Co(NH_3)_4ATP$ was prepared according to the procedures of Cornelius, Hart, and Cleland.[5e] Several attempts to prepare tridentate $Co(NH_3)_3ATP$ according to published procedures[5e] were unsuccessful. Due to the simplicity of the reaction that forms the final metal–nucleotide complex, it was believed that the problem must lie with the starting material, which was originally prepared according to a procedure of Schlessinger.[10,11] Therefore, an alternate method was found to prepare the starting material. The two-step procedure of Siebert[12] was successfully used to form the starting material *trans*-$[Co(NH_3)_3H_2O(Cl)_2]Cl$. The final product, $Co(NH_3)_3ATP$, was then formed from *trans*-$[Co(NH_3)_3H_2O(Cl)_2]Cl$ and ATP as described;[5e] the purification, however, was somewhat different. The reaction product was absorbed onto a 20 × 2.5 cm column of Dowex 50-X2 resin (H^+ form) and eluted with 0.1 M lithium formate, pH 3.5. The pH of the eluate was adjusted to pH 2.0 with HCl. The complex was then absorbed onto a fresh 5 × 2.5 cm column of Dowex 50-X2 (H^+ form). The product was eluted with 0.5 M $HClO_4$. The solution was adjusted to pH 3 with saturated $KHCO_3$, and the precipitate was removed.

Incubation of Nucleotides. In all experiments the alkaline incubation took place in a buffered solution. The buffers and concentrations used for each experiment are given in the Results section. In spite of high buffer to nucleotide ratios, the pH of the solutions usually drifted toward neutrality with time. The pH was kept as close to the initial pH as possible with the addition of small amounts of dilute NaOH. The drift was usually kept to within 0.07 pH unit of the initial pH.

Test tubes containing the incubation solution were kept in a water bath at the appropriate temperature. Aliquots of the incubation solution were removed by pipet as needed. The test tubes were kept covered as much as possible during the course of an incubation to minimize evaporation.

Coupled Enzymatic Assays. Coupled enzymatic assays were used to determine the amount of free ATP, ADP, and AMP present in solutions of the ATP–metal complexes. Each nucleotide is determined separately by following the disappearance of NADH as reflected by the change in absorbance at 340 nm in a dual-beam spectrophotometer,

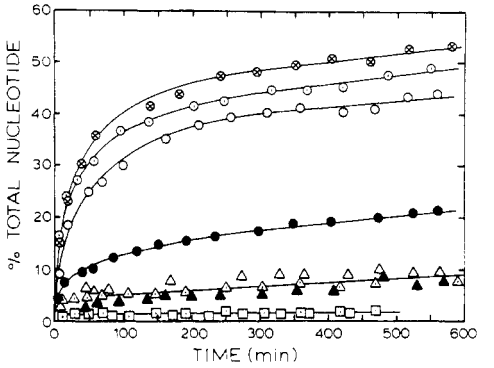


Figure 1. Decomposition of β,γ-bidentate $Cr(H_2O)_4ATP$ as measured by coupled enzymatic assay. The incubation of 25 mM $Cr(H_2O)_4ATP$ was carried out at 37 °C in 225 mM Ches (pH 9.0, ●, ▲, ■) or 225 mM Caps (pH 10.0, ○, △, □; pH 11.0, ⊙, ▲, ⊡). The data for ATP are given as circles, those for ADP as triangles, and those for AMP as squares. Data in the presence of EDTA at pH 11 are given by ⊗.

with the assumption of a molar extinction coefficient of 6220 M^{-1} cm^{-1}. The assay for ATP involved the coupling of 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase as shown in Scheme I. The final 3-mL assay mixture contains 150 mM sodium Pipes buffer (pH 7.0), 6.2 mM 3-phosphoglycerate, 2 mM $MgCl_2$, 0.2 mM NADH, 25 units of PGK and 18 units of GAPDH. For most measurements, 25 μL of the incubation solution that is originally 25 mM in metal nucleotide is an appropriate amount of sample.

Scheme I

$$\text{3-phosphoglycerate} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{PGK}} \text{1,3-diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-diphosphoglycerate} + \text{NADH} + H^+ \xrightarrow{\text{GAPDH}} \text{glyceraldehyde 3-phosphate} + NAD^+ + P_i$$

The assay for ADP uses a coupled assay containing pyruvate kinase (PK) and lactate dehydrogenase (LDH) and the coupling reagent phosphoenolpyruvate as shown in Scheme II. The final 3-mL assay mixture contains 150 mM sodium Pipes (pH 7.0), 0.5 mM phosphoenolpyruvate (PEP), 2.5 mM $MgCl_2$, 0.1 mM KCl, 0.2 mM NADH, 18 units of LDH and 20 units of PK. For most measurements, 25 μL of the incubation sample is added to the assay mixture.

Scheme II

$$\text{ADP} + \text{PEP} \xrightarrow[Mg^{2+}, K^+]{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + H^+ + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

The assay for AMP uses a coupled assay involving the enzymes myokinase (MK), pyruvate kinase, and lactate dehydrogenase as shown in Scheme III. The final 3-mL assay mixture contains 150 mM sodium Pipes (pH 7.0), 0.3 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM KCl, 0.5 mM PEP, 0.2 mM NADH, 18 units of LDH, 20 units of PK, and 27 units of MK. This assay is a two-step process. First, any free ADP is consumed in the absence of myokinase. This step is identical with the assay described for ADP above; therefore, a value for ADP can be determined at this point. After all of the ADP has been consumed, excess ATP is added to the assay mix followed by myokinase.

Scheme III

$$\text{ATP} + \text{AMP} \xrightarrow[Mg^{2+}]{\text{MK}} 2\text{ADP}$$

$$2\text{ADP} + 2\text{PEP} \xrightarrow[Mg^{2+}]{\text{PK}} 2\text{ATP} + 2(\text{pyruvate})$$

$$2(\text{pyruvate}) + 2\text{NADH} + 2H^+ \xrightarrow{\text{LDH}} 2(\text{lactate}) + 2NAD^+$$

Results

Decomposition of Bidentate $Cr(H_2O)_4ATP$. The breakdown of β,γ-bidentate $Cr(H_2O)_4ATP$, as determined by coupled enzymatic assays, is shown in Figure 1. The decomposition

(10) Schlessinger, G. *Inorg. Synth.* **1960**, *6*, 180–182.
(11) Schlessinger, G. *Inorg. Synth.* **1960**, *6*, 189–191.
(12) Siebert, V. *Z. Anorg. Allg. Chem.* **1978**, *441*, 47–57.

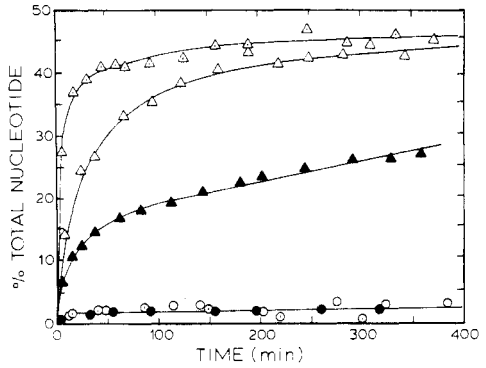


Figure 2. Decomposition of α,β,γ-tridentate $Cr(H_2O)_3ATP$ as measured by coupled enzymatic assay. Incubation of 25 mM $Cr(H_2O)_4ATP$ was carried out at 37 °C in 225 mM Ches (pH 9.0) or 225 mM Caps (pH 10.0 and 11.0). Symbols used were according to the scheme in Figure 1.

yields primarily free ATP, with small amounts of ADP produced and even lower levels of AMP. The amounts of AMP and ADP produced are essentially independent of pH, but the amount of ATP produced is strongly pH dependent. No significant breakdown is observed at pH 7 (data not shown), but the amount of free ATP produced by a 9-h incubation at 37 °C ranges from 20% of the total nucleotide at pH 9 to 50% at pH 11. In order to separate the catalytic effects of free metal (formed by the breakdown of $Cr(H_2O)_4ATP$) from that of the Cr(III) in the native complex, we compared the decomposition products formed in the presence of 25 mM EDTA with those in the absence of chelator (Figure 1). Under these conditions, no significant differences are observed. In fact, the rate of release of ATP is slightly increased rather than decreased, suggesting that the initial release of chromium does not then catalyze further breakdown of bidentate $Cr(H_2O)_4ATP$.

Decomposition of Tridentate $Cr(H_2O)_3ATP$. In contrast to bidentate $Cr(H_2O)_4ATP$, tridentate $Cr(H_2O)_3ATP$ decomposes predominantly with hydrolysis to yield large amounts of ADP and only small amounts of ATP. Figure 2 shows the breakdown of $Cr(H_2O)_3ATP$ with time at several values of pH. Above pH 9, the breakdown to ADP is closely pH sensitive, but little difference is observed between pH 8 and 9 (data not shown). The yield of ADP at high pH approaches 45–50% of the total nucleotide, and this process is essentially complete after 60 min at 37 °C. On the other hand, over the range of pH values studied, the nucleotide released as ATP never exceeded 4% of the total nucleotide, and no AMP was detected in any of these studies. As was the case with the bidentate $Cr(H_2O)_4ATP$, the presence of EDTA has little or no effect on the decomposition of tridentate $Cr(H_2O)_3ATP$.

Decomposition of Bidentate $Co(NH_3)_4ATP$. The decomposition of bidentate $Co(NH_3)_4ATP$ is similar in several respects to that of bidentate $Cr(H_2O)_4ATP$. First of all, the breakdown produces predominantly ATP, with much lower levels of ADP and even lower levels of AMP. Second, the breakdown is greatly accelerated at high pH. As shown in Figure 3, negligible amounts of AMP, ADP, and ATP are produced upon incubation of bidentate $Co(NH_3)_4ATP$ at pH 7.5 and 25 °C. However, at 37 °C at pH 9, substantial amounts of ATP and ADP are produced (Figure 3). Moreover, as the pH is increased from pH 9 to pH 11, the amount of ATP released increases significantly, whereas the level of ADP produced remains essentially constant over this range of pH. The rate of breakdown and the levels of ATP liberated from bidentate $Co(NH_3)_4ATP$ are substantially lower than those observed for bidentate $Cr(H_2O)_4ADP$. The effects of EDTA on the breakdown of $Co(NH_3)_4ATP$ were also examined, and the results are presented in Figure 4. At pH 9 and

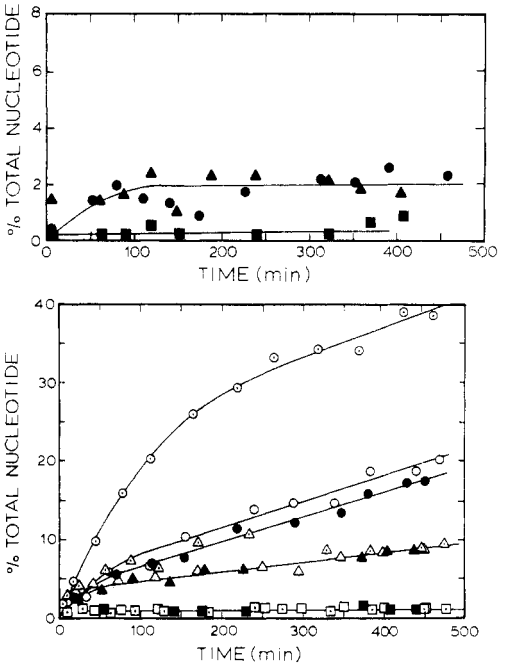


Figure 3. Decomposition of β,γ-bidentate $Co(NH_3)_4ATP$: top, pH 7.5, temperature 25 °C; bottom, temperature 37 °C. Conditions and symbols used are the same as those of Figure 1.

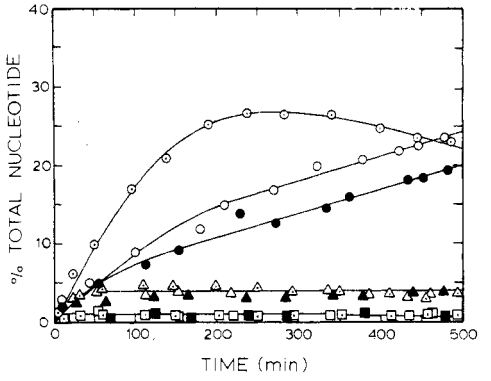


Figure 4. Decomposition of β,γ-bidentate $Co(NH_3)_4ATP$ in the presence of 25 mM EDTA. The solutions contained 225 mM Ches (pH 9.0, ●, ■, ▲) or 225 mM Caps (pH 10.0, ○, □, △; pH 11.0, ⊙, ⊡, ◬). The data for ATP are given in circles, those for ADP as triangles, and those for AMP as squares.

10, no effect of EDTA was observed on the formation of ATP. The levels of ADP, however, were somewhat reduced under these conditions. On the other hand, at pH 11, the effect of EDTA in the incubation was to decrease the final levels of ATP produced. The levels of ATP were unaffected at incubation times up to 120–150 min. Beyond this point, however, the level of ATP in the incubation mixture began to decline, suggesting the formation of a species distinct from free ATP, ADP, or AMP.

Decomposition of Tridentate $Co(NH_3)_3ATP$. The decomposition of tridentate $Co(NH_3)_3ATP$ proceeded with the release (Figure 5) of both ATP and substantial amounts of ADP. In this respect, it provides a case that is intermediate to those of bidentate $Co(NH_3)_4ATP$ and tridentate $Cr(H_2O)_3ATP$. It is unusual, too, in the fact that the largest amounts of ATP and ADP are formed at pH 10. At pH 11, the levels of both ATP and ADP are lower than at pH 10. The decomposition of $Co(NH_3)_3ATP$ is clearly more rapid than that of the other three complexes studied here. At pH 10 and 11, the decomposition is complete after approximately 100 min. The total of the three products yielded at pH 10 is approximately 72–75% of the total nucleotide. The fate of the rest of the

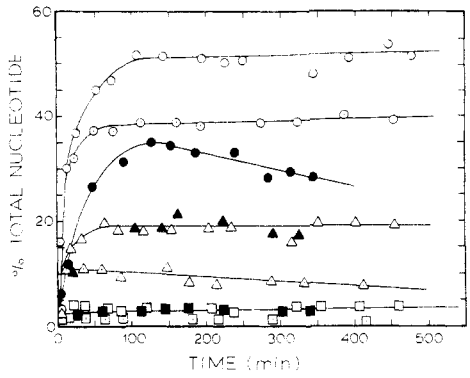


Figure 5. Decomposition of α,β,γ-tridentate $Co(NH_3)_3ATP$. Conditions and symbols used are the same as those of Figure 1.

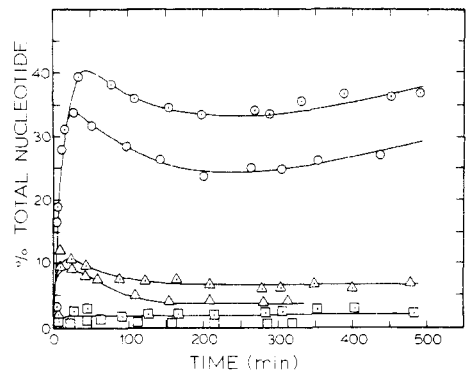


Figure 6. Decomposition of α,β,γ-tridentate $Co(NH_3)_3ATP$ in the presence of 25 mM EDTA. Conditions and symbols are the same as those of Figure 4.

original $Co(NH_3)_3ATP$ is not known, but reports of polymerization of these complexes have appeared.[5d]

The effect of EDTA on the decomposition of $Co(NH_3)_3ATP$ is shown in Figure 6. These interesting multiphasic plots suggest that the nucleotides, once released from the Co(III) complex, are recomplexing in some fashion. It is beyond the goals and scope of the present study to characterize these products, but the effects of metal chelation by EDTA leave open the possibility that, in this case at least, the Co(III) produced in the original decomposition may prevent recomplexation by some mechanism.

Comparison of Methods for Determining Free Nucleotides in the Presence of Cr(III) and Co(III) Complexes. In addition to the enzymatic assay described above, two other techniques were used to quantitatively determine the amount of free nucleotide present in solutions of Cr(III) and Co(III) nucleotides: (1) thin-layer chromatography and (2) ^{31}P nuclear magnetic resonance. While thin-layer chromatography (TLC) yielded promising results in some cases, several limitations soon became apparent. For example, both tridentate $Co(NH_3)_3ATP$ and bidentate $Co(NH_3)_4ATP$ decompose rapidly on the TLC plate and cause severe streaking. On the other hand, good results were obtained with TLC analysis of the tri- and bidentate CrATP complexes. Figure 7 shows the decomposition of bidentate $Cr(H_2O)_4ATP$ upon incubation at 45 °C at pH 10, as monitored by TLC. Despite the good quality of the data in studies such as these, the collection of data by this method was tedious, and the long times required to run the TLC plates and to carry out the nucleotide analysis led us to look at other methods.

Phosphorus-31 NMR was examined as a method for analyzing mixtures of the Co(III) nucleotides. The paramagnetism of Cr(III), however, made such measurements impossible for solutions of the chromium nucleotides. For the cobalt nucleotides, ^{31}P NMR methods did yield reasonable

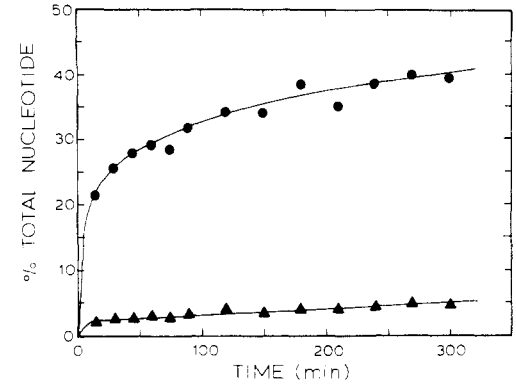


Figure 7. Decomposition of bidentate $Cr(H_2O)_4ATP$ as measured by the thin-layer chromatographic separation method. The incubation of 25 mM $Cr(H_2O)_4ATP$ was carried out at 45 °C in 225 mM borate buffer, pH 9.0. The data for ATP are given as circles while those for ADP are given as triangles. AMP could not be measured due to streaking on the TLC plates. Thin-layer chromatography was carried out with plastic-backed polyethylenimine cellulose plates (Brinkmann). Elution with 0.75 M LiCl was followed by visualization by UV lamp. The relevant spots were cut out, and the nucleotides were extracted with 0.7 M $MgCl_2$ in 20 mM (Tris)Cl, pH 7.4. Optical densities were read at 260 nm. A molar extinction of 15 400 M^{-1} cm^{-1} was used for all free nucleotides.

Table I. Comparison of ^{31}P NMR and Enzymatic Methods for Determination of Free Nucleotides in Solutions of Co(III) Complexes[a]

	% total nucleotide		% free nucleotide	
	NMR	enzymatic	NMR	enzymatic
	450-Min Incubation			
ATP	41.6	30.6	72.1	71.7
ADP	16.3	14.0	28.2	25.3
AMP	...	1.3	...	3.2
$Co(NH_3)_4ATP$	42.1	...		
	510-Min Incubation			
ATP	48.6	31.8	79.0	72.4
ADP	13.8	10.7	22.4	25.3
AMP	...	1.0	...	2.3
$Co(NH_3)_4ATP$	37.7	...		

[a] Incubation solutions included 25 mM $Co(NH_3)_4ATP$ and 250 mM Caps buffer at pH 11. Solutions were incubated at 37 °C for the times indicated. The total nucleotide percentages exceed 100% in some cases due to errors in calculating contributions from overlapping resonances from different species.

results in some cases. Due to the long (10–11 s) values of T_1 for ^{31}P in diamagnetic solutions of nucleotides, the NMR spectra were collected in the presence of 90 mM 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy to shorten the T_1 times and permit more rapid data accumulation. Under these conditions, the T_1 times of the relevant nuclei were reduced to 0.17–0.46 s, with virtually no effect on the observed line widths. Also, in order to quantitate ADP levels in the presence of ATP, it was necessary to measure the ATP levels by using the β-P resonance and then to calculate the ADP levels by subtracting this same peak intensity from the α-P signals of ATP (since these peaks contain the α-P and β-P of ADP, respectively).

$Co(NH_3)_4ATP$ was incubated at pH 11 in 150 mM Caps buffer, and the NMR results were compared with data from the enzymatic assays. The results of incubations of 7.5 and 9.5 h are shown in Table I. While no AMP was detected in the NMR spectra, a weak α-P signal could have been obscured by the resonances of $Co(NH_3)_4ATP$. Good agreement was obtained for the determinations of ADP and ATP between the NMR and enzymatic methods under these conditions. On the

other hand, the time required to accumulate and analyze the NMR spectra made this method much less convenient than the enzymatic method.

Discussion

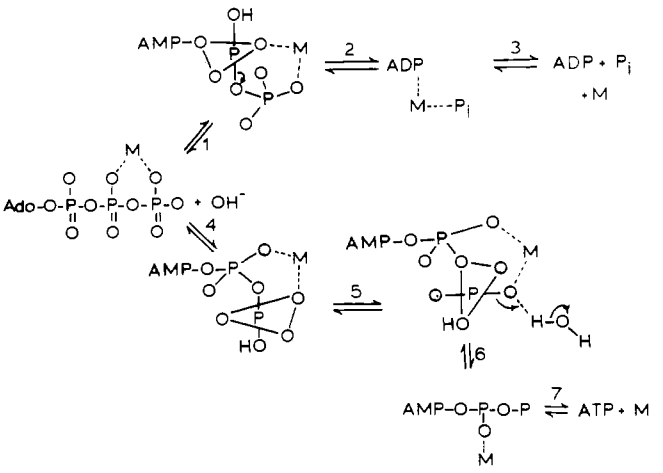
The coupled enzymatic assays used here are particularly suited for the measurement of free nucleotides in the presence of the bidentate or tridentate Cr(III) and Co(III) nucleotide complexes. It has been established that the bidentate Cr(III) and Co(III) complexes are relatively poor substrates for a variety of nucleotide-requiring enzymes,[5a] with apparent values of V_{max} that are typically 2 orders of magnitude lower than those for ATP. Furthermore, the tridentate complexes of ATP with Cr(III) and Co(III) have not been found to be substrates for any ATP-requiring enzyme. Thus it is a straightforward matter to measure the levels of free nucleotides in the presence of the Cr(III) and Co(III) complexes with use of the enzymatic methods described here. Very small amounts of material can be used with these methods, with 0.6 μmol of total nucleotide being typical and less than 5 nmol of free nucleotide being detectable in this manner.

By comparison, the thin-layer chromatographic method and the determinations by ^{31}P NMR were less accurate, required more material (at least in the latter case), and were far more time consuming. The preparation of the TLC plates, the development of the plates, and the subsequent nucleotide analyses were quite tedious when a large number of points were to be measured. In addition, the decomposition of the complexes on the TLC plates and the resultant streaking made the TLC method much less attractive than the other methods, particularly with $Co(NH_3)_3ATP$. The long times required for the NMR accumulations essentially precluded the continuous monitoring of a decomposition such as was possible with the enzymatic methods. Of course, as mentioned above, the NMR method could only be used with the (diamagnetic) Co(III) complexes. The principal value of the TLC and NMR methods in the present study was to provide a means of confirming the results of the enzymatic assays. The comparison (see, for example, Table I) indicates good agreement between the two methods.

The decomposition behavior observed for these complexes was interesting, particularly so in light of recent studies of metal catalysis of ATP hydrolysis.[13-16] Bidentate $Cr(H_2O)_4ATP$ yielded predominantly free ATP with only small amounts of hydrolysis to ADP occurring. Tridentate $Cr(H_2O)_3ATP$, on the other hand, decomposes via a hydrolysis mechanism, yielding large amounts of ADP and only insignificant amounts of free ATP. The results with the Co(III) complexes were much less clear-cut. The breakdown of $Co(NH_3)_4ATP$ is slower than that of the analogous $Cr(H_2O)_4ATP$, and the level of ADP produced was higher for the tridentate $Co(NH_3)_3ATP$. The rates of hydrolysis observed in these studies ranged from 1×10^{-5} (for $Co(NH_3)_4ATP$) to 5×10^{-4} s^{-1} (for $Cr(H_2O)_4ATP$). Also hydrolysis is not the dominant mechanism for breakdown of $Co(NH_3)_3ATP$, which yields substantially more ATP than ADP (or of course AMP).

Nevertheless, it is significant that substantial amounts of ADP are rapidly produced in all these cases. It has long been known that hydrolysis of ATP is promoted by metal ions.[13-16] However, the rate enhancements observed in many cases are not particularly large, and a number of metals (Ni^{2+}, Mn^{2+}, and Mg^{2+}) show no significant effect on the hydrolysis of

Scheme IV



ATP.[13-15] The rate constant obtained here for hydrolysis of $Cr(H_2O)_3ATP$ ($\sim 5 \times 10^{-4}$ s^{-1}) at pH 11 and 37 °C (Figure 2) is approximately 5000 times the rate constant obtained by Sigel and Amsler for free nucleotides at 50 °C in alkaline solution.[13] By comparison, the best enhancement observed in the latter study was a factor of 300 for Cu^{2+} at pH 5.5.

Of interest also with respect to the hydrolysis observed here are (1) the pH dependence of the hydrolysis, (2) the differences between the results obtained with the bidentate and tridentate complexes, and (3) the fact that these decompositions only proceed to 50–70% of completion. The last phenomenon may be due to the formation of polymeric metal–nucleotide species. The formation of such species by Cr(III) and Co(III) nucleotides at pH values above 7 has been described previously by DePamphilis and Cleland.[5d] The increased rates and levels of hydrolysis observed at increasing pH suggest a role for OH^- in these processes. However, Sigel and Amsler[13] report decreasing rates of hydrolysis with increasing pH from pH 7 to pH 10 both for uncomplexed ATP and for the complexes of ATP with Cu^{2+} and Zn^{2+}, while Ni^{2+} complexes show only a slight increase in hydrolysis with increasing pH in the range of 7–10. The increased hydrolysis observed with the tridentate complexes of Co(III) and Cr(III) relative to that for the bidentate cases is also distinctly different from results obtained with other metals. Thus Ni^{2+} and Mn^{2+}, which coordinate to the α-, β-, and γ-phosphates of ATP, show low rates of hydrolysis[13] relative to Cu^{2+} and Zn^{2+}, which coordinate only to the β- and γ-phosphates.[17,18] While the reason for this discrepancy is not immediately obvious, one can formulate a mechanism that reconciles the behavior observed here and the studies of Sigel and Amsler. The pH dependence of the decomposition of the Co(III) and Cr(III) complexes of ATP and the increased yield of ADP from the tridentate complexes are consistent with a mechanism involving nucleophilic attack of OH^- on the phosphates of ATP with an additional acceleration resulting from strain in the chelate ring in the case of the tridentate complexes. Nucleophiles attack mainly the P_γ and P_α electrophilic centers of nucleoside 5'-triphosphates under catalysis by a variety of enzymes, including the ATPases,[19] kinases,[20] and related enzymes.[21,22] On the other hand, it is difficult to rule out nucleophilic attack at the P_β position, at

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Table II. Comparison of Metal-Oxygen Bond Lengths[a]

metal	M-O bond lengths, Å range	av	ref
Mn(II)	2.125-2.369	2.208	25
Ni(II)	2.048-2.081	2.065	26
Co(III)	1.936-1.953	1.944	27
Cr(III)	1.940-1.980	1.965	28

[a] The references given are examples from the recent literature. Many other reports exist for each of these metals, with the values shown being typical.

least in the case of nonenzymatic hydrolysis. Following the oxyphosphorane intermediate model of Ramirez et al.,[23,24] we can write a simple mechanism that can give rise to either breakdown of the metal-nucleotide complex to give free ATP or hydrolysis to ADP (Scheme IV).

While this mechanism is written for the β,γ-bidentate metal-nucleotide complex, it could apply equally well to the tridentate Co(III) and Cr(III) complexes. In the latter case, chelate ring strain due to the coordination of the α-phosphorus could make steps 1, 2, and 3 (the hydrolysis pathway) more likely relative to steps 4, 5, and 6 (decomposition to free ATP). This induced strain model also provides an explanation for the failure to observe substantial hydrolysis in the putative tridentate complexes of Mn(II) and Ni(II).[13] From model-building studies it is apparent that increasing the metal-oxygen bond lengths in the tridentate metal-nucleotide complex reduces the inherent chelate ring strain. As shown in Table II, Cr(III)-O and Co(III)-O bond lengths are substantially shorter than those of Mn(II) and Ni(II). Further support for this model is found in the fact that Ni(II), with a bond length to oxygen intermediate between that of Co(III) and Cr(III) on the one hand and Mn(II) on the other, shows a low level of hydrolysis in 1:1 Ni:ATP systems that is increased sixfold as the pH is increased from pH 7 to pH 10. Mn(II), on the other hand, with the longest metal-oxygen bond length in Table II, shows a very low level of hydrolysis in 1:1 complexes with ATP that is insensitive to pH between pH 5 and pH 9.5.

The notion of nucleophilic attack on phosphorus facilitated by ring strain has been put forth in a slightly different manner by Martin and Mariam[29] to explain the enhancement of hydrolysis of ATP by Cu(II) in weakly acidic and neutral solutions. In this case it is proposed that the metal ion may induce strain by chelating two oxygens bound to the γ-phosphorus. Several crystal structures in fact show a metal ion chelating two oxygens of a single phosphate to produce a (strained) four-membered ring.[30-32] The resulting reduction of the OPO bond angle facilitates the conversion from tetrahedral to trigonal-bipyramidal geometry in forming the pentacovalent phosphorus intermediate. The strain produced by the formation of the tridentate Co(III) and Cr(III) complexes examined here can produce much the same effect and can set the stage for release of free nucleotides and hydrolysis of bound ATP, both of which will relieve this strain.

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Registry No. $Co(NH_3)_4ATP$, 63915-26-4; $Co(NH_3)_3ATP$, 83214-28-2; $Cr(H_2O)_4ATP$, 58682-54-5; $Cr(H_2O)_3ATP$, 83214-29-3; ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; ATP, Cr salt, 69381-95-9; ATP, Co salt, 78738-35-9.

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Asymmetric Transformation of α-Amino Acids Promoted by Optically Active Cobalt(III) Complexes. 3.[1] Importance of Side-Chain Intramolecular Interligand Hydrogen Bonding to Stereoselectivity

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The Λ-β_2-$[Co(aa)(N_4)]^{n+}$ complexes, where aa refers to aspartate, asparaginate, and glutamate and N_4 to chiral derivatives of 3,7-diazanonane-1,9-diamine (2,3,2-tet), were prepared, and the hydroxide ion catalyzed epimerizations of these complexes were examined at pH 11.2 in water or pH 12.3 in water-methanol (1/1), to give rise to equilibrium mixtures of diastereomers (Λ-*R* and Λ-*S*). The equilibrated isomeric ratios for Λ-*S*/Λ-*R* ranged from 80/20 to 91/9 except for that of the glutamato complex (68/32). Higher stereoselectivity was observed in water-methanol than in water. Hydrogen bonding between the side-chain β-carboxylate or β-amide group and the secondary amine nitrogen explains the preference for the (*S*)-amino acidato isomer in the examined systems.

Introduction

Metal ions increase the reactivity of the α-proton of chelated α-amino acids.[2] It has been known that chelated α-amino acidates in chiral cobalt(III) complexes undergo hydroxide ion catalyzed epimerization,[1,3,4] which is considered as an example of a "first-order asymmetric transformation"[5] of racemic α-amino acids.[1,4] No remarkable stereoselectivity has been observed in the case of the C-substituted tetraamine complexes

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