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Synthesis and Stereochemical Analysis of Exchange-Inert Chromium(III) Complexes of Adenosine 5′-*O*-(2-Thiodiphosphate), Adenosine 5′-*O*-(2-Thiotriphosphate), and Adenosine 5′-*O*-(3-Thiotriphosphate). Generation of a New Class of Chiral Enzyme Active Site Probes[1]

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Abstract: Exchange-inert chromium(III) complexes of the thionucleotides adenosine 5′-(2-thiodiphosphate) (ADPβS), adenosine 5′-(2-thiotriphosphate) (ATPβS), and adenosine 5′-(3-thiotriphosphate) (ATPγS) were prepared for use as stereochemical probes of enzyme active sites. α,β-Bidentate $Cr(H_2O)_4ADP\beta S$, β,γ-bidentate $Cr(H_2O)_4ATP\beta S$, and β,γ-bidentate $Cr(H_2O)_4ATP\gamma S$ were prepared in 20%, 60%, and 50% respective yields by reaction of $Cr(H_2O)_6^{3+}$ with the corresponding nucleotides. The stereoisomers of each complex were separated with reversed-phase HPLC and subjected to stereochemical analysis. The configurations of the α-P of the $Cr(H_2O)_4ADP\beta S$ isomers and of the β-P of the $Cr(H_2O)_4ATP\gamma S$ isomers were identified by converting the isomers to the respective $Cr(H_2O)_4ADP$ and $Cr(H_2O)_4ATP$ isomers with bromine. The γ-P configurations of the $Cr(H_2O)_4ATP\gamma S$ isomers were identified by excising the chiral P^1, P^2-bidentate $Cr(H_2O)_4$(thiopyrophosphate) ($Cr(H_2O)_4PPS$) unit with nucleotide pyrophosphatase and comparing it with independently prepared (from the (R_P)- and (S_P)-$Cr(H_2O)_4ADP\alpha S$ isomers) $Cr(H_2O)_4PPS$ enantiomers having known configuration. Accordingly, $Cr(H_2O)_4ATP\gamma S$ isomers 1–4 (isomer number is based upon the order of elution from the reversed-phase HPLC column) were assigned the (γ*R*,β*S*), (γ*S*,β*R*), (γ*R*,β*R*), and (γ*S*,β*S*) configurations, respectively. $Cr(H_2O)_4ADP\beta S$ isomers 1 and 3 were found to have the Λ-(*R*)-α-P configuration and isomers 2 and 4 the Δ-(*S*)-α-P configuration. Attempts to excise $Cr(H_2O)_4PPS$ from the $Cr(H_2O)_4ADP\beta S$ isomers failed. The $Cr(H_2O)_4ATP\beta S$ complex prepared from (R_P)-ATPβS was found to exist as two Λ-β-P screw-sense isomers (1, 4), while the $Cr(H_2O)_4ATP\beta S$ complex prepared from (S_P)-ATPβS was found to exist as two Δ-β-P screw-sense isomers (2, 3). By analogy to the two sets of β-P configurational isomers observed for $Cr(H_2O)_4ATP$, $Cr(H_2O)_4ATP\beta S$ isomers 1 and 4 and isomers 2 and 3 are thought to be chelate ring conformers. Bromine-induced desulfurization of the $Cr(H_2O)_4ATP\beta S$ isomers was shown to proceed with retention of β-P configuration and with retention of chelate ring conformation. On the basis of comparisons made between the isomeric compositions of $Cr(H_2O)_4ATP$, $Cr(H_2O)_4ATP\beta S$, and $Cr(H_2O)_4ATP\gamma S$ the chelate ring conformations are tentatively assigned. $Cr(H_2O)_4ATP$ isomers 1 and 2 have the AMP-pseudoaxial conformation, and isomers 3 and 4 have the AMP-pseudoequatorial conformation; $Cr(H_2O)_4ATP\beta S$ 1 and 2 have the AMP-pseudoequatorial conformation, and isomers 3 and 4 have the AMP-pseudoaxial conformation; $Cr(H_2O)_4ATP\gamma S$ isomers 1–4 have the S-pseudoequatorial conformation; isomers 1 and 2 have the AMP-pseudoaxial conformation, and isomers 3 and 4 have the AMP-pseudoequatorial conformation.

Enzymes that catalyze phosphoryl transfer from polyphosphates (e.g., ATP)[2] by associative-type mechanisms are likely to do so by delocalizing electron density at the phosphoryl group undergoing transfer and at the phosphoryl group being displaced. In such cases electropositive active site amino acid side chains and/or divalent metal ion cofactors can act as electron sinks.

In recent years four different methodologies have been developed for the purpose of identifying sites on the enzyme-bound polyphosphate substrate at which metal cofactors bind. These include the Mn(II)–polyphosphate NMR T_1 method,[3] the Mg(II)/Cd(II) phosphorothioate method,[4] the Mn(II)–[^{17}O]–polyphosphate EPR method,[5] and the exchange-inert metal–polyphosphate complex method.[6] These techniques, particularly when used in combination, have proved to be quite useful for identifying metal ion–substrate contacts that take place during catalysis. This has enhanced our understanding of the role(s) of the metal ion cofactor in catalysis.

Aside from X-ray crystallographic analysis of enzyme–substrate or enzyme–inhibitor complexes, techniques for defining sites of contact between enzyme active site residues and the phosphoryl oxygen atoms of the bound substrate have yet to be developed. With this goal in mind we have used exchange-inert complexes of the substrate to define the structure and stereochemistry of the active metal–substrate complex. On the basis of these results, the corresponding exchange-inert metal complexes were prepared from substrate analogues in which a phosphoryl oxygen atom has been replaced with a sulfur atom. The sulfur atom is both larger and "softer" than the oxygen atom that it replaces. Thus, if the oxygen atom that we have substituted has the correct spatial disposition to interact with an amino acid side chain or bound metal ion cofactor during catalysis, the kinetic constants (viz. V_m/K_m, V_m) determined for the polyphosphorothioate complex should differ substantially from those of the oxygen analogue.

In the present study we have prepared exchange-inert Cr(III) complexes of ATPγS, ATPβS, and ADPβS and have begun to use them to study kinase–MgATP and kinase–MgADP complexes. In this paper we describe the synthesis and configurational analysis of these Cr(III)–thionucleotide complexes. In addition, the isomeric composition of the β,γ-bidentate $Cr(H_2O)_4ATP\gamma S$ and

(1) A communication on the preliminary results from a portion of this work has been published: Lin, I.; Dunaway-Mariano, D. *J. Am. Chem. Soc.* **1984**, *106*, 6074.

(2) Abbreviations: adenosine 5′-triphosphate, ATP; adenosine 5′-(2-thiodiphosphate, ADPβS; adenosine 5′-(2-thiotriphosphate), ATPβS; adenosine 5′-(1-thiodiphosphate), ADPαS; adenosine 5′-(3-thiotriphosphate), ATPγS; adenosine 5′-diphosphate, ADP; thiopyrophosphate, PPS, pyrophosphate, PP; adenosine 5′-monophosphate, AMP; circular dichroism, CD; nuclear magnetic resonance, NMR; electron paramagnetic resonance, EPR; high-pressure liquid chromatography, HPLC; 2-(*N*-morpholino)ethanesulfonic acid, MES; cycloheptaamylose, CHpA.

(3) See: Mildvan, A. S.; Gupta, R. K. *Methods Enzymol.* **1979**, *49*, 322. Granot, J. G.; Mildvan, A. S.; Bramson, H. N.; Kaiser, E. T. *Biochemistry* **1980**, *10*, 3537.

(4) For a review see: Eckstein, F. *Annu. Rev. Biochem.* **1985**, *54*, 367.

(5) For a review see: Reed, G. H.; Markham, G. D. *Biol. Magn. Reson.* **1984**, *6*, 73.

(6) For a review see: Cleland, W. W. *Methods Enzymol.* **1982**, *87*, 159.

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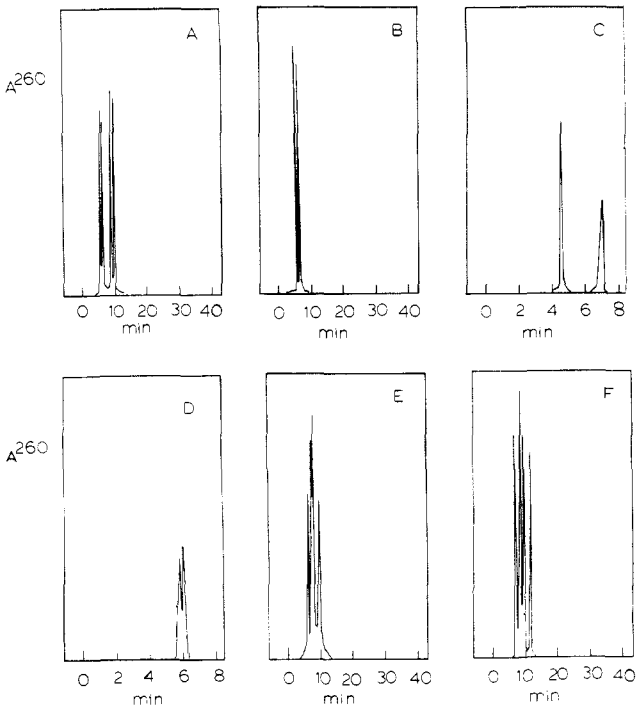

Figure 1. Reversed-phase HPLC (C-18 reversed-phase analytical column; 0.1 M potassium methanesulfonate, pH 2.2; 1 mL/min) elution profiles: A, $Cr(H_2O)_4ADP\beta S$; B, $Cr(H_2O)_4ADP$; C, Λ-$Cr(H_2O)_4ATP\beta S$; D, Δ-$Cr(H_2O)_4ATP\beta S$; E, $Cr(H_2O)_4ATP\gamma S$; F, $Cr(H_2O)_4ATP$.

$Cr(H_2O)_4ATP\beta S$ complexes is correlated with that of the parent complex β,γ-bidentate $Cr(H_2O)_4ATP$. On the basis of this correlation, conformations for the four previously identified Cr-$(H_2O)_4ATP$ chelate ring conformers[7] are proposed.

Results and Discussion

Preparation and Purification of the Diastereoisomers of α,β-Bidentate $Cr(H_2O)_4ADP\beta S$, β,γ-Bidentate $Cr(H_2O)_4ATP\gamma S$, and β,γ-Bidentate $Cr(H_2O)_4ATP\beta S$. The reaction of $Cr(H_2O)_6^{3+}$ with ADPβS, ATPβS (R_P or S_P isomer), or ATPγS followed by ion-exchange chromatography provided partially purified preparations of $Cr(H_2O)_4ADP\beta S$ (20% yield), $Cr(H_2O)_4ATP\beta S$ (60% yield), and $Cr(H_2O)_4ATP\gamma S$ (50% yield). The isomeric compositions of these preparations and the preparations of α,β-bidentate Cr-$(H_2O)_4ADP$ and β,γ-bidentate $Cr(H_2O)_4ATP$ were examined by using analytical C-18 reversed-phase HPLC.[8] As indicated by the HPLC elution profiles shown in Figure 1, each complex can be resolved into four isomers with the exception of the Cr-$(H_2O)_4ADP$ complex, which separates into two isomers.[9,10] The diastereoisomers of each complex were purified by using preparative reversed-phase HPLC and assigned a number (1–4) on the basis of the order in which they eluted from the HPLC column. The visible CD spectra of the diastereomers (excluding the Cr-$(H_2O)_4ADP$ diastereoisomers) are shown in Figure 2.

The four diastereoisomers of α,β-bidentate $Cr(H_2O)_4ADP\beta S$ result from chirality at the α-P and β-P. The visible CD spectra of isomers 3 and 4 are mirror images and are close facsimilies of the visible CD spectra reported for the α-P epimers of α,β-bidentate $Cr(H_2O)_4ADP$ and α,β-bidentate $Cr(H_2O)_4ADP\alpha S$.[7,9] The visible CD spectra of the $Cr(H_2O)_4ADP\beta S$ isomers 1 and 2 are also mirror images, but they are quite different in appearance from the CD spectra of isomers 3 and 4.

The four β,γ-bidentate $Cr(H_2O)_4ATP\gamma S$ isomers result from chirality at the β-P and γ-P. The visible CD spectra of the four isomers are close matches for the CD spectra of the four Cr-

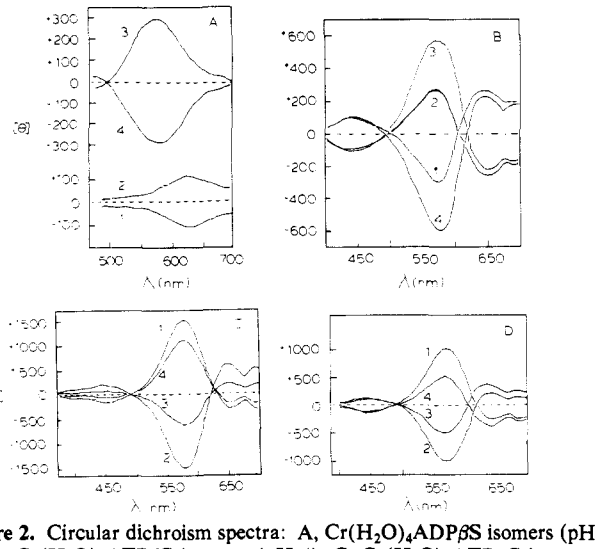

Figure 2. Circular dichroism spectra: A, $Cr(H_2O)_4ADP\beta S$ isomers (pH 5); B, $Cr(H_2O)_4ATP\beta S$ isomers (pH 4); C, $Cr(H_2O)_4ATP\gamma S$ isomers (pH 4); D, $Cr(H_2O)_4ATP$ (pH 4). The designation of isomers as 1–4 is based on their order of elution from reversed-phase HPLC columns as depicted in Figure 1.

$(H_2O)_4ATP$ isomers (Figure 2). The $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 4 and the $Cr(H_2O)_4ATP$ isomers 1 and 4 show positive Cotton effects at the λ_{max}, while isomers 2 and 3 of each complex show negative Cotton effects. In addition, isomers 3 and 4 of the $Cr(H_2O)_4ATP$ and $Cr(H_2O)_4ATP\gamma S$ complexes have ca. 50% of the λ_{max} ellipticity that isomers 2 and 1 have.

Unlike the $Cr(H_2O)_4ATP\gamma S$ complex, $Cr(H_2O)_4ATP$ is chiral at only one phosphorus center, the β-P. The four stereoisomers observed for $Cr(H_2O)_4ATP$ are thought to derive from two stable ring conformations for the β-P epimers.[7] The observation of four rather than eight $Cr(H_2O)_4ATP\gamma S$ stereoisomers is consistent with availability of only a single stable ring conformation for each configurational isomer. This point is considered in greater detail below.

The $Cr(H_2O)_4ATP\beta S$ complex, like the $Cr(H_2O)_4ATP$ complex, is chiral at the β-P and exists in four rather than two stereoisomeric forms. The visible CD spectra of the four Cr-$(H_2O)_4ATP\beta S$ isomers closely match those of the four Cr-$(H_2O)_4ATP$ isomers. In this case the CD spectra of Cr-$(H_2O)_4ATP\beta S$ isomers 1 and 2 matched those of $Cr(H_2O)_4ATP$ isomers 3 and 4, respectively, and the CD spectra of Cr-$(H_2O)_4ATP\beta S$ isomers 3 and 4 matched those of $Cr(H_2O)_4ATP$ isomers 1 and 2, respectively (Figure 1 and 2).

Configurational Analysis of the β,γ-Bidentate $Cr(H_2O)_4ATP\beta S$, α,β-Bidentate $Cr(H_2O)_4ADP\beta S$, and β,γ-Bidentate Cr-$(H_2O)_4ATP\gamma S$ Diastereoisomers. A summary of the configurations of the chiral centers of these complexes is provided in Table I.

β,γ-Bidentate $Cr(H_2O)_4ATP\beta S$. The two β,γ-bidentate Cr-$(H_2O)_4ATP\beta S$ isomers formed from (R_P)-ATPβS elute at positions 1 and 4 from the reversed-phase HPLC column, while the two isomers formed from (S_P)-ATPβS elute at positions 2 and 3 (Figure 1). Since Cr(III) coordinates to the nonbridging oxygen atom and not to the sulfur atom[9] of the β-P of ATPβS, Cr-$(H_2O)_4ATP\beta S$ isomers 1 and 4 must be Λ β-P screw-sense isomers and isomers 2 and 3 must be Δ screw-sense isomers. Bromine treatment of $Cr(H_2O)_4ATP\beta S$ isomer 1 produced $Cr(H_2O)_4ATP$ isomer 3 in quantitative yield, while analogous reactions of Cr-$(H_2O)_4ATP\beta S$ isomer 2 produced $Cr(H_2O)_4ATP$ isomer 4, that of $Cr(H_2O)_4ATP\beta S$ isomer 3 gave $Cr(H_2O)_4ATP$ isomer 1, and that of $Cr(H_2O)_4ATP\beta S$ isomer 4 yielded $Cr(H_2O)_4ATP$ 2 (Table I). These correlations were made by comparing the retention times and CD spectral properties of the $Cr(H_2O)_4ATP\beta S$-derived Cr-$(H_2O)_4ATP$ isomers with those of independently prepared Cr-$(H_2O)_4ATP$ isomers.[7] Since $Cr(H_2O)_4ATP$ isomers 1 and 4 have the Δ (or *S*) β-P configuration and isomers 2 and 3 have the Λ (or *R*) β-P configuration, it is evident that the desulfurization of

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Table I. Properties of Tetraaquachromium(III) Complexes of ADP, ADPαS, ADPβS, ATP, ATPβS, and ATPγS

complex	isomer[a]	CD λ_{max}, mm	$[\theta]^b$	Br_2 product	config ($[\theta]$) of excised $Cr(H_2O)_4PPS$	phosphoryl phosphorus config	chelate ring conform[e]
$Cr(H_2O)_4ADP$	1	580	+195[c]			αR (Λ)	
	2	580	−100[c]			αS (Δ)	
$Cr(H_2O)_4ADP\alpha S$	1	590	+700[d]		S (+50)	αS (Λ)	
	2	600	−550[d]		R (−50)	αR (Δ)	
$Cr(H_2O)_4ADP\beta S$	1	625	−120	$Cr(H_2O)_4ADP$ (1)		αR (Λ)	
	2	625	+120	$Cr(H_2O)_4ADP$ (2)		αS (Δ)	
	3	590	+300	$Cr(H_2O)_4ADP$ (1)		αR (Λ)	
	4	590	−300	$Cr(H_2O)_4ADP$ (2)		αS (Δ)	
$Cr(H_2O)_4ATP$	1	575	+1000[c]			βS (Δ)	AMP(a)
	2	575	−1000[c]			βR (Λ)	AMP(a)
	3	575	−550[c]			βR (Λ)	AMP(e)
	4	575	+550[c]			βS (Δ)	AMP(e)
$Cr(H_2O)_4ATP\beta S$	1	575	−250	$Cr(H_2O)_4ATP$ (3)		βR (Λ)	S(a), AMP(e)
	2	575	+250	$Cr(H_2O)_4ATP$ (4)		βS (Δ)	S(a), AMP (e)
	3	575	+550	$Cr(H_2O)_4ATP$ (1)		βS (Δ)	S(e), AMP(a)
	4	575	−550	$Cr(H_2O)_4ATP$ (2)		βR (Λ)	S(e), AMP(a)
$Cr(H_2O)_4ATP\gamma S$	1	575	+1500	$Cr(H_2O)_4ATP$ (1)	R (−50)	γR,βS (Δ)	S(e), AMP(a)
	2	575	−1500	$Cr(H_2O)_4ATP$ (2)	S (+50)	γS,βR (Λ)	S(e), AMP(a)
	3	575	−650	$Cr(H_2O)_4ATP$ (3)	R (−50)	γR,βR (Λ)	S(e), AMP(e)
	4	575	+1000	$Cr(H_2O)_4ATP$ (4)	S (+50)	γS,βS (Δ)	S(e), AMP(e)

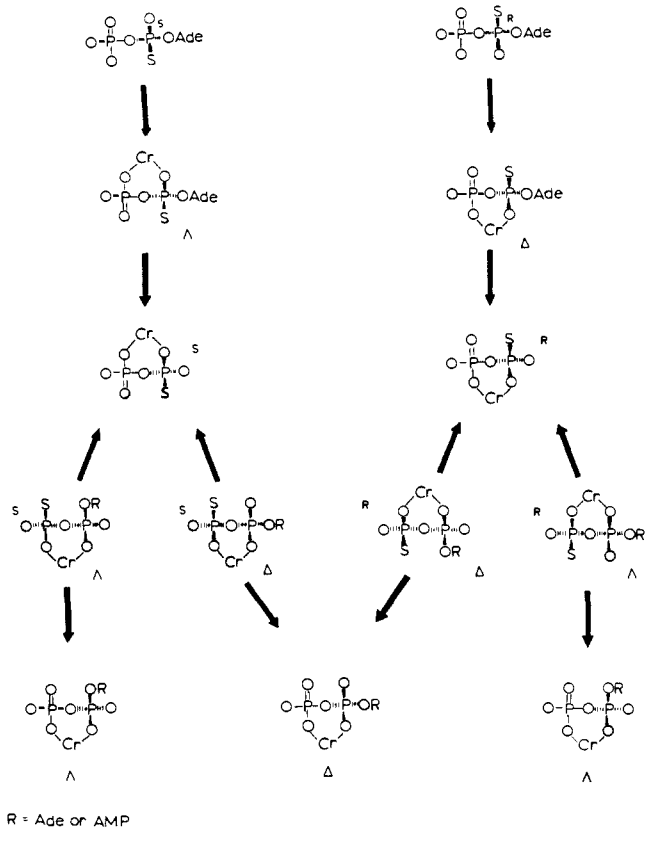
[a] Isomer number is based on order of elution from a reversed-phase HPLC column (no. 1 is first to elute). The old numbering system[7] was based on order of elution from a CHpA column (no. 1 is first to elute). For comparison, the CHpA-based numbers 1 and 2 for the $Cr(H_2O)_4ADP$ isomers correspond to HPLC-based numbers 2 and 1, respectively. CHpA-based numbers 1, 2, 3, and 4 for $Cr(H_2O)_4ATP$ correspond to HPLC-based numbers 2, 1, 4, and 3, respectively. [b] Molar ellipticity units are deg $cm^2/dmol$. [c] These values were taken from Dunaway-Mariano and Cleland.[7] [d] These values were taken from Lin et al.[9] [e] Pseudoaxial is denoted by (a), and pseudoequatorial is denoted by (e).

the $Cr(H_2O)_4ATP\beta S$ proceeds with retention of configuration at the β-P[11] and with retention of the chelate ring conformation.

β,γ-Bidentate $Cr(H_2O)_4ATP\gamma S$ and α,β-Bidentate $Cr(H_2O)_4ADP\beta S$. The strategy employed in assigning the configurations at the chiral phosphorus atoms of each purified isomer is depicted in Scheme I and the results obtained are summarized in Table I.

The R_P and S_P isomers of ADPαS[12] were used to prepare the Δ and Λ α,β-bidentate $Cr(H_2O)_4ADP\alpha S$ screw-sense isomers, respectively. Previous studies had shown that Cr(III) is coordinated to the α-P–O rather than to the α-P–S in these complexes.[9] Thus, the (R_P)-ADPαS isomer gives rise to the Δ-$Cr(H_2O)_4ADP\alpha S$ isomer and the (S_P)-ADPαS isomer gives rise to the Λ-$Cr(H_2O)_4ADP\alpha S$ isomer. The P^1,P^2-bidentate $Cr(H_2O)_4PPS$ unit was to be excised from the individual $Cr(H_2O)_4ADP\alpha S$ isomers by using $NaIO_4$ to convert the $C_{3'}$ to an aldehydic carbon (by oxidative cleavage of the $C_{2'}$–$C_{3'}$ bond) and aniline hydrochloride to affect the $C_{5'}$–OPα bond cleavage (by Schiff base formation at $C_{3'}$ and deprotonation at $C_{4'}$).[11] The $NaIO_4$/aniline hydrochloride oxidation–elimination procedure had previously been used to cleave β,γ-bidentate $Co(NH_3)_4PPP$ from α,β-bidentate $Co(NH_3)_4ATP$[13] and to cleave P^1,P^2-bidentate $Co(NH_3)_4PPS$ from α,β-bidentate $Co(NH_3)_4ADP\alpha S$.[1] Because the water ligands make the $Cr(H_2O)_4ADP\alpha S$ complex sensitive to base-catalyzed ligand exchange, the oxidation–elimination procedure, which was to be carried out at pH 6, was first tested with the diamagnetic aqua complex α,β-bidentate $Rh(H_2O)_4ADP$.[14] The structure and purity of the P^1,P^2-bidentate $Rh(H_2O)_4PP$ complex[14] generated by the $NaIO_4$/aniline hydrochloride treatment of $Rh(H_2O)_4ADP$ were confirmed by ^{31}P NMR (singlet, +8.0 ppm, pH 4). Following this successful demonstration, the oxidation–elimination procedure was performed on the $Cr(H_2O)_4ADP\alpha S$ isomers. The CD spectrum of the $Cr(H_2O)_4PPS$ enantiomer derived from the Λ-$Cr(H_2O)_4ADP\alpha S$ diastereoisomer displayed a positive Cotton effect at 610 nm while that derived by this procedure from the Δ-$Cr(H_2O)_4ADP\alpha S$ diastereomer showed a negative Cotton effect (Figure 3; Table I).

Scheme I. Outline of the Approach Taken in Determining the Configurations at the Chiral Phosphorus Atoms of the Four $Cr(H_2O)_4ATP\gamma S$ Isomers and the Four $Cr(H_2O)_4ADP\beta S$ Isomers



The second step in the analysis of the γ-P configuration of the $Cr(H_2O)_4ATP\gamma S$ isomers and the β-P configuration of the $Cr(H_2O)_4ADP\beta S$ isomers was the excision of the P^1,P^2-bidentate $Cr(H_2O)_4PPS$ unit from the parent complex. In the case of the $Cr(H_2O)_4ATP\gamma S$ complex this was accomplished with the enzyme

(11) The desulfurization of the uncomplexed thionucleotide occurs with inversion of configuration. Retention of configuration results from "adjacent" attack on the thiophosphoryl phosphorus center. Models of the Cr(III)–thionucleotide complexes indicate that regardless of whether the chelate ring assumes a chair or twist-boat conformation adjacent attack of the water is sterically more favorable than "in line" attack.

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

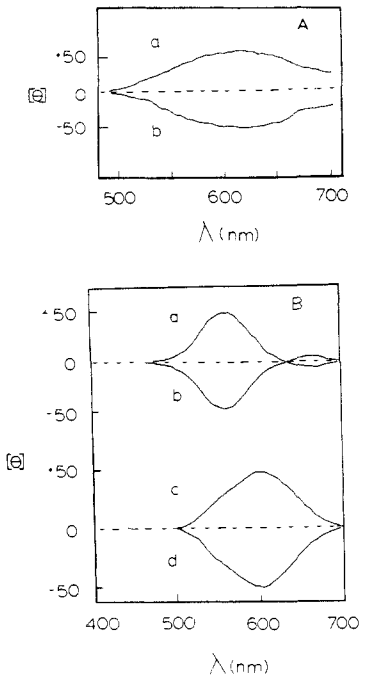


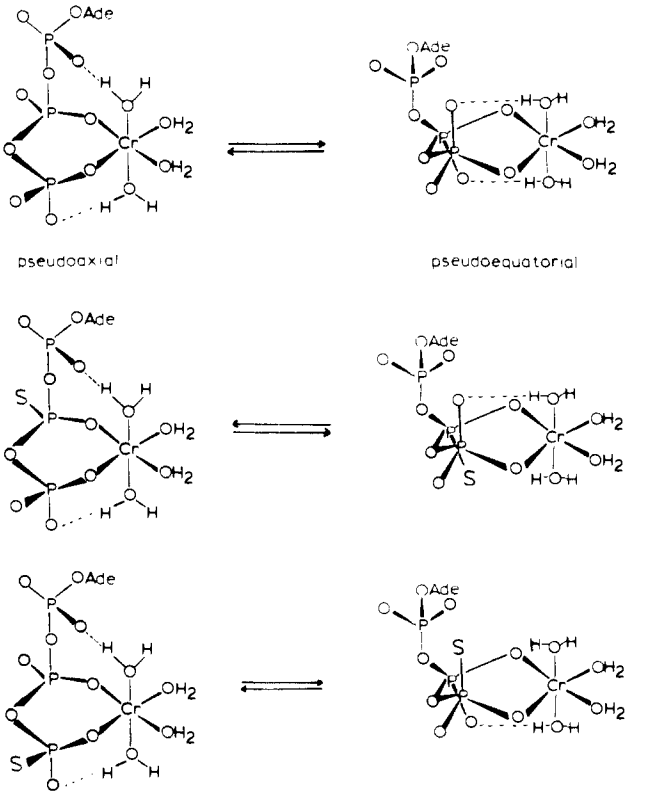
Figure 3. Circular dichroism spectra: A, $Cr(H_2O)_4PPS$ (pH 4) generated from (a) (S_P)-$Cr(H_2O)_4ADP\alpha S$ and (b) (R_P)-$Cr(H_2O)_4ADP\alpha S$ with $NaIO_4$/aniline hydrochloride;|B, $Cr(H_2O)_4PPS$ (pH 4) generated from $Cr(H_2O)_4ATP\gamma S$ isomers (a) 2, (b) 1, (c) 4, and (d) 3.

nucleotide pyrophosphatase.[15] The ability of this enzyme to catalyze the cleavage of metal–ATP complexes was first tested with the diamagnetic complex β,γ-bidentate $Rh(H_2O)_4ATP$.[14,16] The proton-decoupled ^{31}P NMR spectrum of a mixture generated from the reaction $Rh(H_2O)_4ATP$ with nucleotide pyrophosphatase at pH 5.9 was characterized by singlets at +8.0 ppm (P^1,P^2-bidentate $Rh(H_2O)_4PP$) and at +1.4 ppm (AMP). As expected, the ratio of the areas of the two singlets was 2:1. It was necessary to include the enzyme alkaline phosphatase in the nucleotide pyrophosphatase/$Cr(H_2O)_4ATP\gamma S$ reaction mixtures in order to convert the AMP formed to adenosine. This prevents product inhibition of the nucleotide pyrophosphatase by AMP and thus reduces the period required for the reaction to reach completion.

The visible CD spectra of the $Cr(H_2O)_4PPS$ enantiomers derived from the four $Cr(H_2O)_4ATP\gamma S$ isomers are shown in Figure 3. The CD spectra of the $Cr(H_2O)_4PPS$ enantiomers generated from $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 3 show negative Cotton effects, while the CD spectra of the $Cr(H_2O)_4PPS$ enantiomers derived from $Cr(H_2O)_4ATP\gamma S$ isomers 2 and 4 show positive Cotton effects. Comparison of these CD spectra with those of the $Cr(H_2O)_4ADP\alpha S$-derived $Cr(H_2O)_4PPS$ enantiomers allows assignment of the $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 3 the *R* configuration at the γ-P and isomers 2 and 4 the *S* configuration at the γ-P (Table I).

Attempts to excise the $Cr(H_2O)_4PPS$ unit from the $Cr(H_2O)_4ADP\beta S$ isomers failed. The oxidation–elimination reaction sequence that was used to cleave the $Cr(H_2O)_4PPS$ unit from the $Cr(H_2O)_4ADP\alpha S$ isomers was not successful in the case of the $Cr(H_2O)_4ADP\beta S$ isomers because of the sensitivity of the β-P sulfur atom to oxidizing agents. We were unable to displace the $Cr(H_2O)_4PPS$ unit from $C_{5'}$ using the nucleophiles thiourea and azide. Likewise, attempts to cleave the $C_{5'}$ O-P_α bond by the catalytic actions of alkaline phosphatase, 5′-nucleotidase, and apyrase were unsuccessful. Thus, the assignment of the β-P configurations of the $Cr(H_2O)_4ADP\beta S$ isomers can not be accomplished by the $Cr(H_2O)_4PPS$ relay method, and it must await the development of an alternate approach.

Scheme II. Proposed Structures of the Λ-$Cr(H_2O)_4ATP$,[7] the Λ-$Cr(H_2O)_4ATP\beta S$, and the (γ-S,β-Λ)-$Cr(H_2O)_4ATP\gamma S$ Isomers



The configuration of the α-P in the $Cr(H_2O)_4ADP\beta S$ isomers and the configuration of the β-P in the $Cr(H_2O)_4ATP\gamma S$ isomers were assigned by converting the isomers to the corresponding $Cr(H_2O)_4ADP$ or $Cr(H_2O)_4ATP$ isomers with bromine (see Table I). The conversions were found to be quantitative, with each thionucleotide isomer giving rise to a single $Cr(H_2O)_4ATP$ or $Cr(H_2O)_4ADP$ isomer. The CD spectra and HPLC retention times of the $Cr(H_2O)_4ADP$ and $Cr(H_2O)_4ATP$ isomers having known screw-sense configurations were compared with those of the $Cr(H_2O)_4ADP$ and $Cr(H_2O)_4ATP$ samples derived from the $Cr(H_2O)_4ADP\beta S$ and $Cr(H_2O)_4ATP\gamma S$ isomers (Figures 1 and 2).[7,9] In this manner, $Cr(H_2O)_4ADP\beta S$ isomers 1 and 3 were shown to have the Λ (or *R*) α-P configuration while isomers 2 and 4 have the Δ (or *S*) configuration.[17] In addition, $Cr(H_2O)_4ATP\gamma S$ isomers 2 and 3 were shown to have the Λ (or *R*) configuration at the β-P while isomers 1 and 4 have the Δ (or *S*) configuration at the β-P (Table 1).

Isomerism in β,γ-Bidentate Tetraaqua Chromium(III) Complexes of ATP, ATPβS, and ATPγS. β,γ-Bidentate $Cr(H_2O)_4ATP$ was previously shown to consist of four diastereoisomers.[7] Two of the stereoisomers possess the Λ (or *R*) configuration at the β-P, while the other two possess the Δ (or *S*) configuration. The isomers that have the same β-P configuration are comparable in energy (K_{eq} = 1–2) and are relatively stable at or below 25 °C in slightly acidic aqueous solutions (E_{act} = 17–20 kcal/mol, pH 6.0).[7,18] Kinases show a high degree of stereoselectivity toward the β-P epimers but display little or no discrimination between the isomers having the same β-P configuration.[19] Aside from differences in chromatographic properties (reversed-phase HPLC columns[8] and CHpA columns[7]), the isomers of the same screw sense also show differences in the magnitude of their

(15) We acknowledge and thank Peter Tipton and W. W. Cleland of the University of Wisconsin for bringing the β,γ-bidentate $Cr(H_2O)_4ATP$ P_α–OP_β bond-cleaving activity of nucleotide pyrophosphatase to our attention.

(16) Shorter, A. L.; Lin, I.; Dunaway-Mariano, D. *Biochemistry* **1984**, *23*, 3349.

(17) When stored in solution at pH 3.5, 4 °C isomers 1 and 3 slowly (over a period of days) equilibrated, as did isomers 2 and 4. Previous studies have shown that α-P epimerization in $Cr(H_2O)_4ADP$ and $Cr(H_2O)_4ADP\alpha S$ does not occur at a significant rate under these conditions.[9] Thus, β-P epimerization in $Cr(H_2O)_4ADP\beta S$ occurs much more readily than does α-P epimerization.

(18) Gruys, K. J.; Gregory, P. R.; Schuster, S. M. *J. Inorg. biochem.* **1986**, *28*, 67.

(19) Dunaway-Mariano, D.; Cleland, W. W. *Biochemistry* **1980**, *19*, 1506.

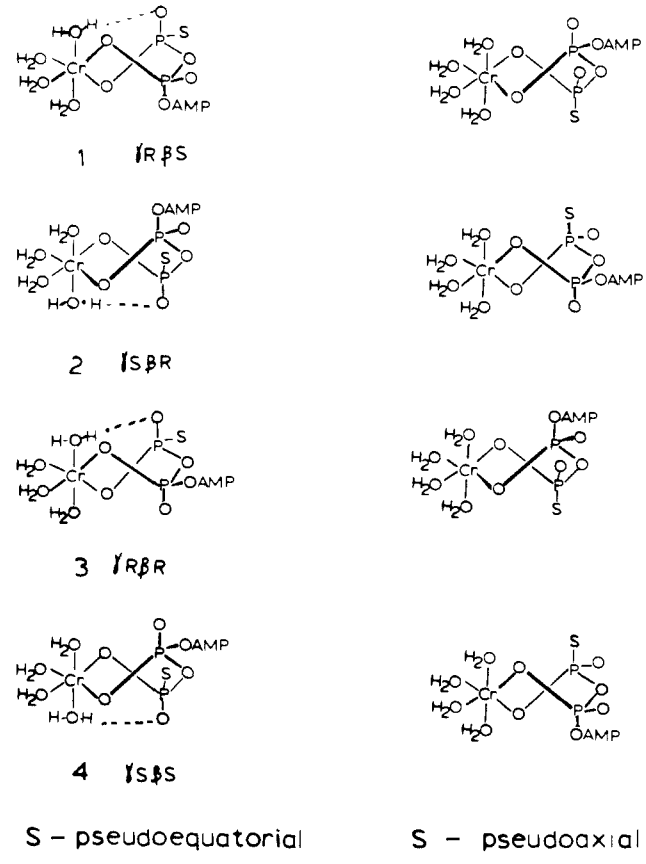
molar ellipticity at the 575-nm λ_{max}.[7] Specifically, isomers 1 and 2 have ca. twice the molar ellipticity at 575 nm as have isomers 4 and 3 (Figure 2). To account for the "extra set" of Cr-$(H_2O)_4$ATP isomers Dunaway-Mariano and Cleland[7] suggested that the chelate ring of the Cr$(H_2O)_4$ATP complex may possess some degree of rigidity. Two sets of twist-boat ring conformers were proposed, with one set having the AMP group in the pseudoequatorial position on the ring and the other set having AMP in the pseudoaxial position (see Scheme II). The twist-boat conformation of the ring was chosen since it provides for maximal staggering between the γ-P and β-P exocyclic oxygen atoms. Intramolecular hydrogen bonding between water ligands and phosphoryl oxygen atoms was thought to contribute to the stability of the conformers. As depicted in Scheme II, each of the conformers might be stabilized by hydrogen bonding between a water ligand and a γ-P oxygen atom. The AMP-pseudoaxial isomer could potentially be further stabilized by hydrogen bonding between a second water ligand and an α-P oxygen atom, while the AMP-pseudoequatorial isomer might be further stabilized by hydrogen bonding between a second water ligand and the exocyclic β-P oxygen atom.

Because of the paramagnetism of the chromium ion, NMR studies of the conformation of the chelate rings of the Cr-$(H_2O)_4$ATP isomers cannot be successfully carried out. Disappointingly, the diamagnetic Co$(NH_3)_4$ATP and Rh$(H_2O)_4$ATP complexes, which are subject to NMR-based conformational analysis have yet to be resolved into stable conformers.[20] The only available information on the chelate ring conformations of exchange-inert metal–ATP complexes derives from the crystal structures of complexes of pyrophosphate and tripolyphosphate.[21] With few exceptions the chelate ring conformations observed for these complexes are twist-boat or boat. Furthermore, hydrogen bonding between phosphorus oxygen atoms and metal water ligands is apparent. Although we do expect that the intramolecular hydrogen bonds formed with the acidic Cr(III) water ligands will be significantly stronger than intermolecular hydrogen bonds to solvent water molecules, we do not know to what extent the crystal-packing forces and the observed intermolecular hydrogen bonding (involving a nearest neighbor or a water molecule present in the crystal) have altered the "solution" conformations of these complexes.

If the chelate ring of Cr$(H_2O)_4$ATP does exist in two stable conformations, we expect that substitution of a chelate ring atom or group should perturb the conformer population and stability. However, on the basis of earlier work[7] we know that the tetraaquachromium complexes of the ATP analogues adenylylimido diphosphate, adenylmethylene diphosphonate, and α,β-methylene ATP each exist as four stable isomers. Although precise measurements of the stabilities of these isomers as compared with those of Cr$(H_2O)_4$ATP have not been made, it is apparent from the data that has been reported[7] that the structural differences between the chelate rings of the chromium–ATP analogue complexes and the chelate ring of Cr$(H_2O)_4$ATP are not significant enough to cause large differences in isomeric composition.

In the present study we have examined the effect of substituting a sulfur atom for the exocyclic γ-P oxygen and for the exocyclic β-P oxygen atom of Cr$(H_2O)_4$ATP. Both of these atoms are presumed to be engaged in intramolecular hydrogen bonding (Scheme II). If the proposed hydrogen-bonding interaction between a water ligand and an exocyclic γ-P oxygen atom contributes significantly to the stabilities of the chelate ring conformers, then we expect the Cr$(H_2O)_4$ATPγS conformers in which the sulfur atom occupies the pseudoequatorial position to predominate. Likewise, if hydrogen bonding between a water ligand and the β-P exocyclic oxygen makes a significant contribution to the stability of the AMP-pseudoequatorial conformer, then replacement of this oxygen atom by a sulfur atom may result in a shift in the conformer equilibrium in favor of the conformer in which the AMP moiety occupies the pseudoaxial (hydrogen-bonding) position.

Chart I. Representations of the Eight Possible Cr$(H_2O)_4$ATPγS Diastereoisomers. Isomers Labeled 1–4 Are Thought To Correspond to the Four Cr$(H_2O)_4$ATPγS Isomers Separated with HPLC



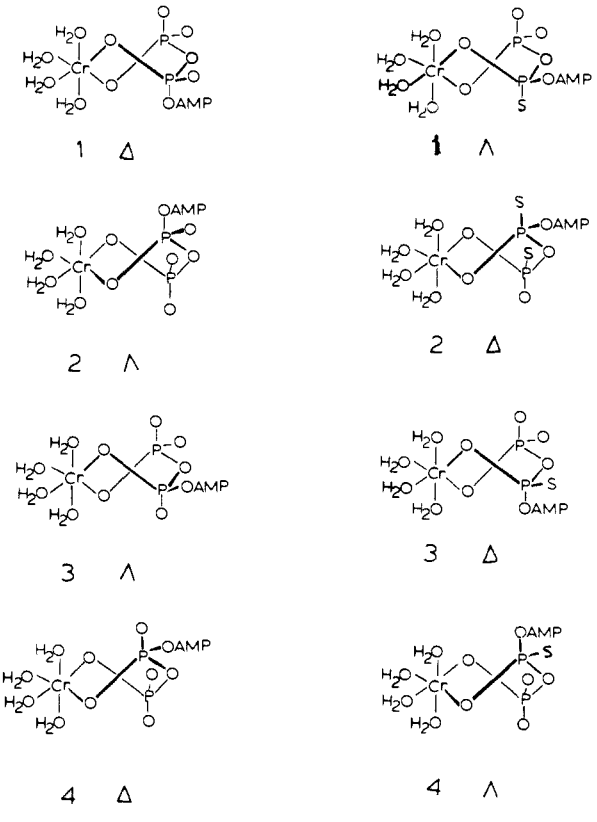
First, we will examine the effect of sulfur atom substitution of the exocyclic β-P oxygen atom of Cr$(H_2O)_4$ATP on the isomer composition. Pure (R_P)- and (S_P)-ATPβS diastereoisomers were used in preparing the Λ and Δ β,γ-bidentate Cr$(H_2O)_4$ATPβS isomers. As indicated in Figure 1, each screw-sense isomer exists as two isomers that are present in a ca. 1:1 ratio. The CD spectral properties of the two sets of isomers are very similar to those of the Cr$(H_2O)_4$ATP isomers (Figure 2). Specifically, the sign of the Cotton effect observed in the CD spectra of these isomers reflects the screw sense of the isomer, while the magnitude of the ellipticity at the CD λ_{max} reflects the chelate ring conformation of the isomer. As with the Cr$(H_2O)_4$ATP isomers, one set of Cr$(H_2O)_4$ATPβS conformers (differing in screw sense) has ca. twice the molar ellipticity than does the other set. Moreover, treatment of the individual Cr$(H_2O)_4$ATPβS isomers with bromine to replace the sulfur atom with an oxygen atom leads to selective formation of the corresponding Cr$(H_2O)_4$ATP isomer having the same screw-sense and chelate ring conformation. Thus, replacement of the exocyclic β-P oxygen atom of Cr$(H_2O)_4$ATP with a sulfur atom does not significantly alter isomer stability or population. Therefore, if hydrogen bonding between a water ligand and the β-P exocyclic oxygen atom does take place, it is of little consequence in determining conformational preference.

On the other hand, replacement of one of the exocyclic γ-P oxygen atoms of Cr$(H_2O)_4$ATP with a sulfur atom does lead to a change in the isomer population. Unlike the Cr$(H_2O)_4$ATP or Cr$(H_2O)_4$ATPβS complexes, the Cr$(H_2O)_4$ATPγS complex possess two chiral phosphorus atoms (γ-P, β-P). However, only half the possible eight isomers depicted in Chart I were observed. The four Cr$(H_2O)_4$ATPγS isomers were shown to be the γ-P and

(20) Because the NH_3 and H_2O ligands of the Co$(NH_3)_4$ATP and Rh-$(H_2O)_4$ATP complexes are not as acidic as the H_2O ligands of the Cr-$(H_2O)_4$ATP complex, intramolecular hydrogen-bonding interactions may not be strong enough to stabilize the Co$(NH_3)_4$ATP and Rh$(H_2O)_4$ATP chelate ring conformers in an aqueous environment.

(21) For a review on this subject see: Sundaralingam, M.; Haromy, T. P. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; (Frey, P.A., Ed.; Elsevier: New York, 1985; pp 249–266.

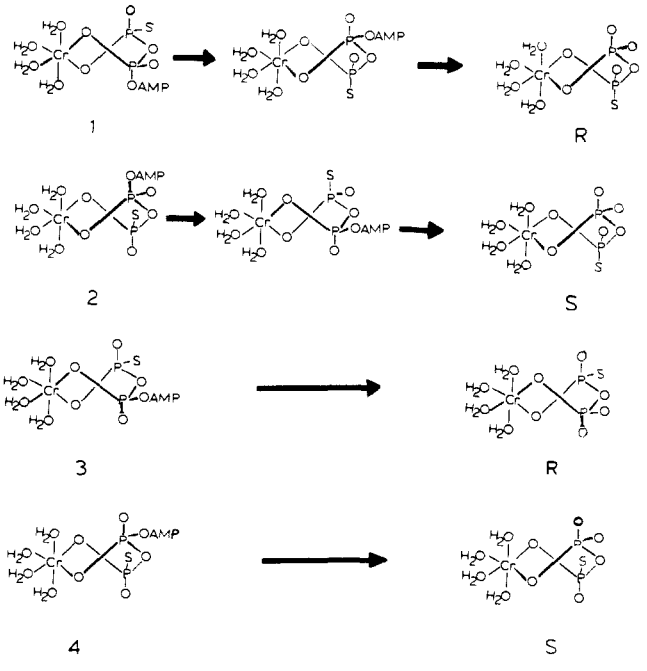
Chart II. Representations of the Structures of the $Cr(H_2O)_4ATP$ Isomers 1–4 and the $Cr(H_2O)_4ATP\beta S$ Isomers 1–4



β-P configurational isomers. Furthermore, the CD spectra of these four isomers closely resemble those of the four $Cr(H_2O)_4ATP$ isomers. Each isomer, when treated with bromine, converted to a single $Cr(H_2O)_4ATP$ isomer having matching HPLC elution order and CD properties (Figure 1 and 2) (viz. $Cr(H_2O)_4ATP\gamma S$ isomer 1 → $Cr(H_2O)_4ATP$ isomer 1, $Cr(H_2O)_4ATP\gamma S$ isomer 2 → $Cr(H_2O)_4ATP$ isomer 2, etc.). It thus appears that each $Cr(H_2O)_4ATP\gamma S$ configurational isomer can assume only one stable chelate ring conformation. Of the eight isomers shown in Chart I we predict that the four isomers (listed on the left-hand side of Chart I) having the γ-P oxygen in the pseudoaxial (hydrogen-bonding position) and the sulfur atom in the pseudoequatorial positions are the most stable. If this prediction is correct, then our results indicate (as shown in Chart II) that $Cr(H_2O)_4ATP$ isomers 1 and 2 have the AMP-pseudoaxial conformation and isomers 3 and 4 have the AMP-pseudoequatorial conformation, while the $Cr(H_2O)_4ATP\beta S$ isomers 1 and 2 have the pseudoequatorial conformation and isomers 3 and 4 have the pseudoaxial conformation. These assignments rest upon the critical assumption that these isomers are in fact the stable chelate ring conformers proposed by Dunaway-Mariano and Cleland.[7] However, since the P^1,P^2-bidentate $Cr(H_2O)_4PPS$ enantiomers appear to possess some degree of conformational rigidity (see below), we are probably on the right track. Whether the conformational isomers are twist-boats as previously suggested or chairs cannot be resolved on the basis of the present data.

Conformational Rigidity of the P^1,P^2-Bidentate $Cr(H_2O)_4PPS$ Chelate Ring. The CD spectra of the $Cr(H_2O)_4PPS$ enantiomers generated from the corresponding $Cr(H_2O)_4ATP\gamma S$ diastereoisomers by treatment with nucleotide pyrophosphatase are depicted in Figure 3. The set of enantiomers generated from $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 2 give rise to mirror-image CD spectra, as do the set of enantiomers generated from $Cr(H_2O)_4ATP\gamma S$ isomers 3 and 4. Interestingly, the two sets of CD spectra are clearly different. One set of spectra displays a λ_{max} of 550 nm while the second displays a λ_{max} of 600 nm. It appears that the two $Cr(H_2O)_4PPS$ enantiomers generated from $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 2 must in some way differ in conformation from the two $Cr(H_2O)_4PPS$ enantiomers generated from $Cr(H_2O)_4ATP\gamma S$ isomers 3 and 4 and from those generated from CrADPαS. At this point in time we can only speculate on the nature and the origin of these conformational differences. It is possible that nucleotide pyrophosphatase catalyzes the addition of a water molecule to the α-P of the $Cr(H_2O)_4ATP\gamma S$ complex only when the complex assumes a certain conformation in the active site. For example, the α-P of the $Cr(H_2O)_4ATP\gamma S$ might be sterically accessible for attack by the H_2O in the ternary nucleotide pyrophosphatase complex when, and only when, the AMP moiety is directed away from the metal center. This would be the case if it occupied the pseudoequatorial position on the chelate ring. Thus, only two of the $Cr(H_2O)_4ATP\gamma S$ isomers (viz. 3 and 4) would possess the correct conformation for reaction. Reaction of these two isomers would produce two $Cr(H_2O)_4PPS$ enantiomers in which the sulfur atom occupies the pseudoequatorial position (see Scheme III), as is presumably the case with the isomers derived from CrADPαS. Reaction of $Cr(H_2O)_4ATP\gamma S$ isomers 1 and 2 would require that the enzyme induce their pseudorotation to the S-pseudoaxial, AMP-pseudoequatorial conformation. Reaction from this conformation would produce two $Cr(H_2O)_4PPS$ enantiomers in which the sulfur atom occupies a pseudoaxial position on the ring. Although hydrogen bonding between the pseudoequatorial thiophosphoryl oxygen atom and a water ligand cannot take place, hydrogen bonding between the pseudoaxial oxygen atom of the phosphoryl group and a water ligand can occur. This hydrogen-bonding interaction and/or the unfavorable diaxial interactions that would occur during pseudorotation appear to present a sufficient energy barrier to ring flipping as to render this $Cr(H_2O)_4PPS$ conformer stable enough to observe at 25 °C.[22]

Scheme III. Representations of the Four $Cr(H_2O)_4ATP\gamma S$ Isomers and their Conversions to the $Cr(H_2O)_4PPS$ Enantiomers with Nucleotide Pyrophosphatase



In conclusion, we suggest that the interrelatedness that exists between the isomeric compositions of β,γ-bidentate $Cr(H_2O)_4ATP$, β,γ-bidentate $Cr(H_2O)_4ATP\beta S$, and β,γ-bidentate $Cr(H_2O)_4ATP\gamma S$ and the differences that exist between the CD spectra of the $Cr(H_2O)_4ATP\gamma S$/nucleotide pyrophosphatase generated $Cr(H_2O)_4PPS$ enantiomers support the proposal of

(22) Rapid racemization of the $Cr(H_2O)_4PPS$ enantiomers[23] makes the study of conformer interconversion difficult. In the case of the $Cr(H_2O)_4ATP$ complex, β-P epimerization was slow enough relative to conformer interconversion as to allow investigators to study conformer equilibration by CD.

(23) Lin, I.; Knight, W. B.; Hsueh, A.; Dunaway-Mariano, D. *Biochemistry* **1986**, *25*, 4688.

stable $Cr(H_2O)_4ATP$ chelate ring conformers. Interestingly, the comparable relative substrate activities of the $Cr(H_2O)_4ATP$ chelate ring conformers observed with hexokinase and glycerokinase[19] suggest that these enzymes efficiently alter the conformation of the isomers when they bind to the active site.

Experimental Section

General Procedures. ^{31}P NMR spectra were recorded at 25 °C by using either a Varian XL-100 (operating at 40.51 MHz) or an IBM WP 200 SY (operating at 81.02 MHz) NMR spectrometer. NMR samples contained 0.3 mM EDTA in 10% D_2O. Chemical shifts are reported (ppm) downfield (+) or upfield (−) from a 0.1 M D_3PO_4 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 Cr(III) complexes of the adenine nucleotides were determined by measuring solution absorption at 260 nm (ϵ 15400). The concentration of $Cr(H_2O)_4PPS$ was determined by measuring solution absorption at 595 nm (ϵ 24). High-pressure liquid chromatography was carried out by using an IBM LC19533 or Beckman 332 HPLC equipped with a Hitachi 100-10 variable wavelength detector, an Altex C-18 reversed-phase analytical column (4.6 mm × 25 cm, 1 mL/min flow rate) or a Du Pont C-18 reversed-phase preparative column (21.2 mm (i.d.) × 25 cm, 4 mL/min flow rate), and 0.01–0.10 M potassium methanesulfonate (with or without 5% MEOH) at pH 2.2 as an isocratic eluant. The cycloheptaamylose (CHpA) column (1.5 × 46 cm) was prepared as previously described.[24] All enzymes, nucleotides, buffers, and Dowex resins were obtained from Sigma Chemical Co. P^1,P^2-Bidentate $Cr(H_2O)_4PP$,[25] β,γ-bidentate $Cr(H_2O)_4ATP$,[7] α,β-bidentate $Cr(H_2O)_4ADP$,[7] α,β-bidentate $Cr(H_2O)_4ADP\alpha S$,[9] and β,γ-bidentate $Rh(H_2O)_4ATP$[14,16] were prepared according to published procedures. The screw-sense isomers of these complexes were separated by reversed-phase HPLC. Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) was prepared according to the method of Webb and Trentham.[27] The R_P and S_P isomers of adenosine 5′-*O*-(2-thiotriphosphate) were prepared according to the procedure of Eckstein and Goody.[12]

Preparation of $Cr(H_2O)_4ATP\gamma S$, $Cr(H_2O)_4ATP\beta S$, and $Cr(H_2O)_4ADP\beta S$. $CrCl_3\cdot 6H_2O$ (10 mL of 20 mM, pH 4.2) was added to 10 mL of 20 mM ATPγS or ADPβS (pH 5). The resulting solutions were adjusted to pH 5.5 with $KHCO_3$ solution and allowed to stand 10 min at room temperature. The $Cr(H_2O)_4ATP\beta S$ was prepared by heating a 10-mL solution, 10 mM in ATPβS and in $CrCl_3\cdot 6H_2O$ (pH 3), at 70 °C for 20 min. The $Cr(H_2O)_4ATP\gamma S$ and $Cr(H_2O)_4ATP\beta S$ reaction solutions were adjusted to pH 4.5 and directly absorbed onto a 2 × 10 cm Dowex-1 (Cl^-) column, while the $Cr(H_2O)_4ADP\beta S$ reaction solution (pH 4.5) was first passed through a 2 × 10 cm Dowex-50 (NH_4^+) column before it was loaded onto the Dowex-1 column. The $Cr(H_2O)_4ADP\beta S$ was washed through the Dowex-1 column with H_2O and then concentrated in vacuo. The $Cr(H_2O)_4ATP\beta S$ and $Cr(H_2O)_4ATP\gamma S$ complexes were focused off the Dowex-1 columns with 10 mM HCl after the columns were washed with 100 mL of H_2O. The eluants were adjusted to pH 4.5 prior to concentration in vacuo. Diastereoisomers were separated by using a Du Pont C-18 reversed-phase preparative HPLC column (4.5 mL/min) and 10 mM potassium methanesulfonate (pH 2.2) as eluant.

Desulfurization of $Cr(H_2O)_4ATP\gamma S$, $Cr(H_2O)_4ATP\beta S$, and $Cr(H_2O)_4ADP\beta S$. The use of bromine in the desulfurization process was adapted from the procedure of Lowe et al.[26] Solutions of the individual isomers at pH 3.5 (3 mM, 30 μL) were treated with 2 μL of bromine for 2 min, and then the excess bromine was removed in vacuo. The CD spectra and HPLC profiles of the reaction mixtures were compared with those measured for solutions of $Cr(H_2O)_4ATP$ or $Cr(H_2O)_4ADP$ diastereoisomers having known configuration.

Cleavage of β,γ-Bidentate $Rh(H_2O)_4ATP$ to P^1,P^2-Bidentate $Rh(H_2O)_4PP$ and AMP. The 2-mL reaction mixture contained 13 mM β,γ-bidentate $Rh(H_2O)_4ATP$, 150 units of nucleotide pyrophosphatase, and 50 mM K^+MES (pH 5.9). After a 2-h incubation period at 25 °C, the reaction mixture was made 1 mM in EDTA and then analyzed by ^{31}P NMR techniques.

Conversion of $Cr(H_2O)_4ATP\gamma S$ to $Cr(H_2O)_4PPS$. Reaction solutions (1.2 mL) contained 50 mM K^+MES (pH 5.5), 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 9.2 mM pure $Cr(H_2O)_4ATP\gamma S$ diastereomer, 140 units of nucleotide pyrophosphatase, and 35 units of alkaline phosphatase. An HPLC profile of the reaction measured after a 2-h reaction period showed that >90% of the $Cr(H_2O)_4ATP\gamma S$ had been consumed. The solution was adjusted to pH 5 before it was passed through a 1 × 15 cm Sephadex G-25 column 4 °C; 10 mM K^+MES (pH 5) as eluant). Column fractions that contained the $Cr(H_2O)_4PPS$ and unreacted $Cr(H_2O)_4ATP\gamma S$ were adjusted to pH 4 and concentrated in vacuo. A trace amount of $[^{32}P]Cr(H_2O)_4PP$ was added to the concentrate prior to chromatographing it on a 1.5 × 46 cm CHpA column with 10 mM 1,4-bis(2-hydroxyethyl)piperazine hydrochloride (pH 4). The $Cr(H_2O)_4PPS$ coeluted with the $[^{32}P]Cr(H_2O)_4PP$, thus allowing the column chromatography to be monitored by using scintillation counting techniques.

Preparation of $Cr(H_2O)_4PPS$ from $Cr(H_2O)_4ADP\alpha S$. The mixture of α,β-bidentate $Cr(H_2O)_4ADP\alpha S$ α-P epimers[9] (15 mL, 15 mM, pH 4.5) was separated by using preparative reversed-phase HPLC (95% 0.1 M potassium methanesulfonate plus 5% MeOH as eluant, pH 2.2). The individual $Cr(H_2O)_4ADP\alpha S$ isomers were passed through a G-10 Sephadex column (10 mM K^+MES as eluant, pH 5.5, 4 °C) in order to remove the methanesulfonate salt. Solutions containing the individual diastereoisomers, concentrated in vacuo to 5 mM (3 mL) and adjusted to pH 6, were treated with 1 equiv of $NaIO_4$ for 5 min and then with 8 equiv of mercaptoethanol for 5 min. Liberation of $Cr(H_2O)_4PPS$ by β-elimination was then accomplished by treatment of the solution with 0.3 M aniline hydrochloride (pH 5). The product was purified on a Dowex-50 (H^+) column by using water as an eluant. The $Cr(H_2O)_4PPS$ enantiomers were obtained in ca. 20% yields.

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(26) Lowe, G.; Tansley, G.; Cullis, P. M. *J. Chem. Soc., Chem. Commun.* **1982**, *595*.
(27) Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1980**, *255*, 1775.