Characterization of Isomers of Monoamminechromium–ATP and Their Use in Mapping Enzyme Active Sites†

Jill Rawlings,‡ David C. Speckhard,§ and W. W. Cleland*

Institute for Enzyme Research, University of Wisconsin—Madison, 1710 University Avenue, Madison, Wisconsin 53705

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ABSTRACT: Twelve isomers formed by the reaction of monoamminechromium(III) with ATP have been synthesized. Isomerism in this system results from chirality around the β-phosphorus of the ATP, the position of the ammonia ligand, the relative orientation of the ammonia and the AMP, and the presence of ring-puckering conformers. By using chromatography on cross-linked cycloheptaamylose, reverse-phase C-18 HPLC, and cation-exchange FPLC, these isomers have been separated and purified. Their structures have been identified by (1) cleavage by periodate, followed by elimination in the presence of diethylenetriamine and subsequent phosphate insertion to give Λ, Δ, or meso facial monoamminechromium tripolyphosphate with molar ellipticities of +240, −240, or 0 deg cm^2 dmol^{-1} at 550 nm, respectively, (2) cleavage by nucleotide pyrophosphatase to give meridional or facial monoamminechromium pyrophosphate, (3) spectral data, and (4) rates of interconversion of isomers. All possible isomers are seen except those with ammonia syn to AMP. Since the substitution of ammonia for water in the inner coordination sphere appears to diminish affinity for enzymes when the ammonia is in contact with the protein but not when it faces the solvent, these isomers are useful for mapping of enzyme active sites. Their use as probes of enzyme structure is illustrated by their behavior with yeast hexokinase.

The magnesium complex of ATP is a substrate for many enzymes. When this complex is β,γ-bidentate, the β-phosphorus is chiral, giving rise to Λ and Δ screw sense isomers,[1] and when the α-phosphate is coordinated, the α-phosphorus is chiral as well. The chirality of the MgATP substrate has been determined for a number of enzymes by the use of inert coordination isomers of tetraaquochromium–ATP or tetraamminecobalt(III)–ATP (Cleland, 1982) or by the use of chiral β- and/or α-sulfur-substituted ATP together with either Mg^{2+} or Cd^{2+}, since Mg^{2+} is preferentially coordinated to oxygen and Cd^{2+} to sulfur (Eckstein, 1985).

However, this information does not give a complete picture of how MgATP binds to an enzyme. In particular, it is not known which of the water molecules in the coordination sphere of the Mg^{2+} are in contact with the enzyme surface and may be important for binding and which are exposed to the solvent. In theory, this information should be available from X-ray studies on enzymes with bound nucleotide, but in few cases are such data available. Another approach is to use ammonia-substituted inert coordination complexes of ATP. Janson and Cleland (1974) and Danenberg and Cleland (1975) showed that binding of CrATP to yeast hexokinase and other kinases became weaker as the number of ammonias in the coordination sphere increased. These results suggest that by synthesizing complexes with one ammonia selectively placed in the different possible coordination positions and determining the dissociation constants from an enzyme, one could tell which coordination positions face the solvent (presumably no effect on binding) and which face the enzyme (diminished binding). This paper describes the synthesis of monoamminechromium complexes of ATP, the separation and characterization of the isomers, and their use in mapping the active site of yeast hexokinase.

MATERIALS AND METHODS

Yeast hexokinase, glucose-6-phosphate dehydrogenase, nucleotide pyrophosphatase, alkaline phosphatase, charcoal, and ATP were from Sigma. Chromium metal (99.99%) was from AESAR. Ion-exchange resins were from Bio-Rad. Cycloheptaamylose gel was prepared according to the method of Cornelius and Cleland (1978). CrATP was synthesized as in Dunaway-Mariano and Cleland (1980a). CD and UV–visible spectra were measured with a Jasco J41 spectropolarimeter and a Cary 118 spectrometer, respectively. Adenine was determined from A_{260} (ϵ = 15 400), chromium as in Postmus and King (1955), and ammonia according to the method of Speckhard et al. (1991).

Chromatography. Mono-S HR 16/10 or HR 5/5 Pharmacia cation-exchange columns were used at room temperature in a Pharmacia FPLC system with a UV–visible detector with sensitivity of 0.002 OD/cm. The eluant was 1 mM H_2SO_4. For reverse-phase C-18 HPLC chromatography with 10 mM methanesulfonic acid, pH 2.3, as the solvent, a preparative size Microsorb column from Rainin (25 × 2.14 cm) or an analytical column from PhaseSep (25 × 0.46 cm) was used at room temperature. Cycloheptaamylose columns (200 × 1 cm) were run at 4 °C with 10 mM Mes, pH 5.5, as the eluant.

Kinetics. Kinetic measurements with hexokinase used a glucose-6-phosphate dehydrogenase assay at 25 °C following the appearance of NADPH at 340 nm (Dunaway-Mariano & Cleland, 1980b). To prevent isomer interconversion, the

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‡ Permanent address: Department of Chemistry, Auburn University at Montgomery, Montgomery, AL 36117.

§ Permanent address: Department of Chemistry, Loras College, Dubuque, IA 52001.

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[1] In this notation, the reference axis is a line through the metal perpendicular to the chelate ring, and the bond from the chelate ring to the rest of the molecule is the skew line defining a left-hand (Λ) or right-hand (Δ) helix (Cornelius & Cleland, 1978).

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reactions were followed for only 5 min. Data were fitted to eq 1,

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

which describes competitive inhibition where V is maximum velocity, K is the Michaelis constant, K_i is the inhibition constant, A is MgATP concentration, and I is inhibitor concentration.

Monoamminechromium Sulfate. The synthesis is based on that of Ardon and Mayer (1962), but because of the difficulty of obtaining pure product, the details are presented here. Rigorously anaerobic conditions were necessary to prevent the presence of any hexaaquochromium species. Nitrogen gas was purified by bubbling through a chromous solution. Chromium metal (32 mmol, 99.99%) was dissolved in 64 mL of 1 M HCl; the chromium from AESAR dissolved in 12–48 h. When this solution was added to a stirred, degassed solution of 16 mmol of sodium azide in 160 mL of 1 M HCl, a large amount of nitrogen gas was produced. After 10 min, the solution was applied to a 22- × 1.2-cm column of AG50W-X8 ion-exchange resin (H^+ form, 200–400 mesh) protected from light. The column was washed with 0.5 M $HClO_4$ until the green $Cr(H_2O)_5Cl^{2+}$ was separated from the purple $CrNH_3(H_2O)_5^{3+}$. The purple band was then eluted with 2 M H_2SO_4. After addition of ethanol to the solution to 70% by volume and chilling of the solution on ice, purple microcrystals of $[CrNH_3(H_2O)_5]_2(SO_4)_3$ formed.

Monoamminechromium–ATP. A 100-mL solution, 10 mM each in Na_2ATP and monoamminechromium, pH 3, was heated for 8 min at 75 °C in the dark. After the solution was cooled on ice, the pH was adjusted to 4.5, and the solution was adsorbed on a 12- × 3-cm column of AG1-X2 (Cl^-, 200–400 mesh). The column was washed with 25 mM HCl, which eluted the blue monomeric monoammine complex, leaving a blue-green, more negatively charged material behind. This solution was used for further chromatography after adjustment to pH 5 and concentration on a 3- x 1-cm column of AG-1, using 0.1 M HCl for elution.

Periodate and Diethylenetriamine Degradation. A 10 mM excess of periodic acid was added to the nucleotide solution at pH 3.5. After 10 min, 10 μL of ethylene glycol per milliliter of sample was added. After another 10 min, diethylenetriamine chloride, pH 4.0, was added to a concentration of 0.35 M, and the pH was readjusted to 4 if necessary. Elimination rates were followed by changes in CD.

Cleavage by Nucleotide Pyrophosphatase. Alkaline phosphatase (110 units) was added to 0.3-1 mM monoamminechromium–ATP solutions containing 10 mM Mes, pH 5.5, at room temperature in 1-mL volume. Nucleotide pyrophosphatase (5 units) was added, and after 3 min the reaction was quenched with 10 μL of 1 M $HClO_4$ and cooled on ice. A slurry of 0.1 mL of acid-washed charcoal (50 mg/mL) was added, and the solution was filtered. The pyrophosphate complex formed was detected with a cation-exchange FPLC column with visible detection at 580 nm.

Isomer Interconversion Experiments. Interconversions of purified isomers were followed chromatographically using both the analytical reverse-phase HPLC column and the mono-S HR5/5 cation-exchange FPLC column. While isomers were equilibrating, they were kept in the dark.

Enzymatic Purification of Isomer 4Δ. Isomers 4Δ and 4Λ were difficult to separate, so neither sample was completely pure. The last portion of isomer 4Λ was removed from a sample of 4Δ by incubating a 6 mM solution of the monoamminechromium complex containing 20 mM Mes, pH 5.85, 40 mM glucose, and 1100 units of yeast hexokinase in 1 mL at room temperature in the dark for 8 h. The hexokinase was removed with a Centricon-3 microconcentrator, and purified isomer 4Δ was isolated by chromatography on the prep cation-exchange column. A similar enzymatic purification procedure employing pyruvate kinase and glycolate could be used to remove traces of isomer 4Δ from a solution of 4Λ (Dunaway-Mariano & Cleland, 1980b).

RESULTS

Possible Isomers. There are a number of possible isomers of β,γ-bidentate monoamminechromium–ATP. The β-phosphorus is asymmetric, and this leads to Λ and Δ screw sense isomers.[1] The ammonia and the two coordinated phosphates of the ATP can be facial or meridional, and the ammonia can be syn or anti to the AMP that is attached to the chelate ring. In addition, ring-puckering isomers can occur when a coordinated water is syn to AMP, since a strong hydrogen bond from this water to the α-phosphate helps stabilize an otherwise unfavorable axial arrangement of AMP. Thus Dunaway-Mariano and Cleland (1980a) found four rather than two isomers of tetraaquochromium–ATP (axial and equatorial ring-puckering isomers of Λ and Δ screw sense isomers). These ring-puckering isomers interconvert slowly enough to be separated, since a strong hydrogen bond also forms between a γ-phosphate oxygen and a coordinated water in both isomers. The axial ring-puckering isomer of monoamminochromium–ATP with ammonia facial and syn to AMP will not have the stabilization of a strong hydrogen bond to the α-phosphate, however, so only an equatorial configuration is predicted. These considerations lead to 14 predicted isomers, as diagrammed in Figure 1.

Separation of Isomers. The isomers have been numbered on the basis of their elution position on C-18 reverse-phase HPLC columns. Where two isomers coelute and one has a Λ CD spectrum and the other a Δ CD spectrum, these designators are added to the number (thus **2Λ** and **2Δ**). A variety of chromatographic methods were necessary to separate all observed isomers. Cycloheptaamylose chromatography fractionated the mixture into several groups: an early-eluting mixture of Λ isomers (**2Λ**, **4Λ**, and **5**), the early Δ isomers (**1**, **2Δ**, and **4Δ**), the late Δ isomers (**6**, **9**, and **10**), and some late-eluting Λ isomers (**3**, **7**, and **8**). Each crude fraction was then further separated on a C-18 reverse-phase HPLC column or a cation-exchange FPLC column.

It was also possible to use the reverse-phase HPLC column by itself to isolate several isomers in pure form (**1**, **7**, **8**, **9**, and **10**). The cation-exchange column was also useful for isolating isomers **3** and **6** without further or prior separation. Typical elution profiles from the reverse-phase and cation-exchange columns are shown in Figure 2. Table I shows the chromatographic methods needed to obtain each isomer, as well as elution times.

Isomers 4Λ and 4Δ were the hardest to purify, since only cycloheptaamylose chromatography separated them partially. Incubation of isomer 4Δ with yeast hexokinase and glucose was used to remove the residual 4Λ isomer. A similar incubation of isomer 4Λ with pyruvate kinase and glycolate should remove residual 4Δ (Dunaway-Mariano & Cleland, 1980b).

Isomer Properties. Each isomer contained chromium, adenine, and ammonia in a 1:1:1 ratio within experimental error. The CD wavelength maxima and specific ellipticities are in Table II. By comparison with the isomers of tetra-

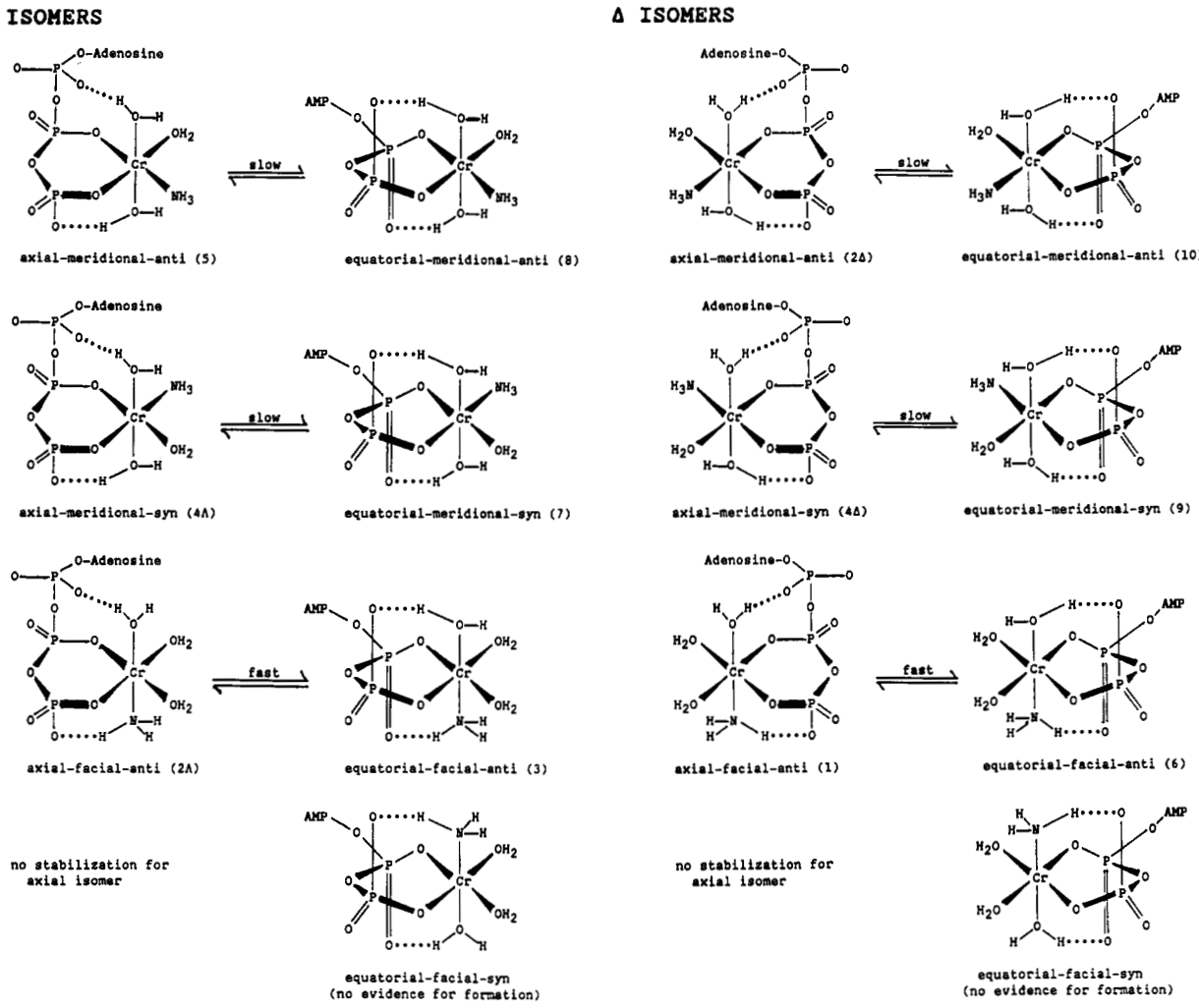

FIGURE 1: Predicted isomers formed from the reaction of monoamminechromium ion with ATP. (Left) Λ isomers. (Right) Δ isomers. The isomer numbers used in this article are also included for reference.

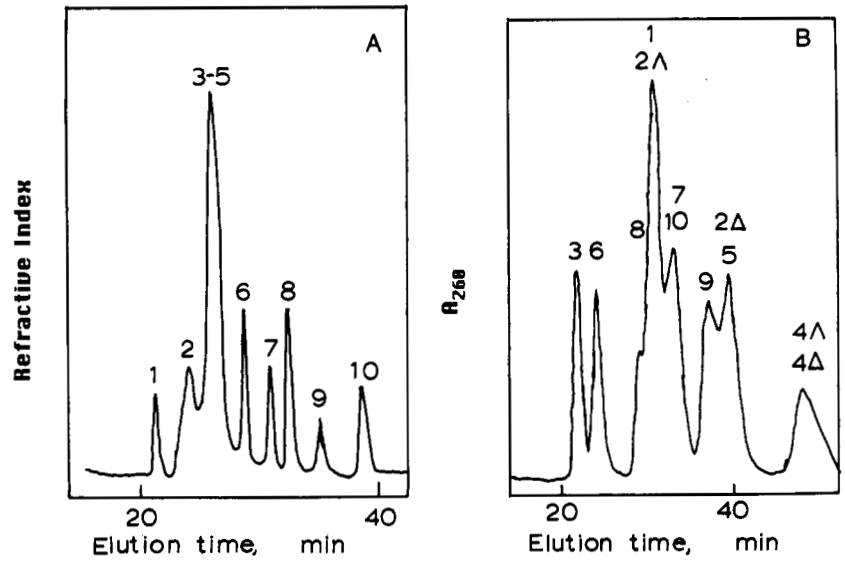

FIGURE 2: Typical elution patterns for monoamminechromium–ATP isomers. (A) Prep reverse-phase C-18 HPLC column. Flow rate 5 mL/min, elution with 10 mM methanesulfonate, pH 2.3. Detection by refractive index. (B) Prep cation-exchange FPLC column. Flow rate 3.67 mL/min, elution with 1 mM H_2SO_4. Detection by UV absorbance. The numbers identify similar isomers.

aquochromium–ATP (Dunaway-Mariano & Cleland, 1980a), the isomers with positive ellipticity at 610-640 nm and negative ellipticity at 540–570 nm are Λ isomers, while those showing the opposite pattern are Δ. This assignment is confirmed by the fact that the Λ isomers are substrates for yeast hexokinase. The maxima of the visible spectra are also given for each isomer in Table II, along with the full configuration determined as outlined below.

Isomer Cleavage by Periodate and Elimination with Diethylenetriamine. Monoamminechromium–ATP isomers were degraded to tripolyphosphate complexes by cleavage of the ribose ring with periodate and then elimination by

Table I: Purification of Monoamminechromium–ATP Isomers

isomer	purification method[a]	elution times (min) reverse phase	cation exchange
1	(middle CHpA), C-18	21	31
2Λ	early CHpA, cat ex, C-18	24	31
2Δ	middle CHpA, C-18, cat ex	25	40
3	(late CHpA), cat ex	27	22
4Λ	early CHpA, cat ex, enz	27	49
4Δ	middle CHpA, cat ex, enz	27	49
5	early CHpA, C-18, cat ex	27	40
6	(middle CHpA), cat ex	30	24
7	(late CHpA), C-18	31	33
8	(late CHpA), C-18	33	29
9	(middle CHpA), C-18	35.5	37
10	(middle CHpA), C-18	37.5	33

[a] Parentheses indicate that this step is optional but does give final fractions with higher concentrations. CHpA, 100- × 1-cm column of cross-linked cycloheptaamylose eluted with 10 mM Mes, pH 5.5. C-18, prep size C-18 reverse-phase HPLC column eluted with 10 mM methanesulfonic acid, pH 2.3, flow rate 5 mL/min; cat ex, Mono-S HR 16/10 cation-exchange FPLC column eluted with 1 mM H_2SO_4, flow rate 4 mL/min; enz, removal of residual opposite screw sense isomer by incubation with yeast hexokinase (for 4Δ) or pyruvate kinase (for 4Λ).

formation of a Schiff base with diethylenetriamine (Speckhard et al., 1991). While the newly formed tripolyphosphate complex should be bidentate, it undergoes rapid insertion of the newly freed phosphate group to give a facial tridentate complex. As shown in Figure 3, such a complex can be chiral or achiral. The achiral or meso isomer comes from an original ATP complex in which ammonia was meridional and trans or anti to the β-phosphate. Thus the isomers that form a tripolyphosphate complex showing no residual CD spectrum (**2Δ, 5, 8,** and **10**) can be unambiguously assigned as meridional and anti. These complexes have a several-nanometer shift to shorter wavelengths in their visible spectrum compared to the spectra of the other monoamminechromium–ATP complexes (see Table II).

The rate of the elimination reaction can also be used to determine whether the isomers have an axial or equatorial ring-puckering conformation. For tetraaquochromium–ATP, the axial ring isomers have a slower rate of elimination (60–120 min) than the equatorial isomers (7–8 min) if the reaction is carried out at pH 3.5 (the rates are faster and more similar at higher pH or when formate buffer is present to assist in the elimination). Facial triamminechromium–ATP complexes also show this behavior (Speckhard et al., 1991). Thus a slow rate of elimination is diagnostic for the axial isomers. The elimination rates, maximum ellipticities of the CD spectra of the resulting tripolyphosphate complexes, and assigned configurations are in Table II.

Degradation with Nucleotide Pyrophosphatase. This enzyme reacts with bidentate ATP complexes to form AMP and a pyrophosphate complex (Lin & Dunaway-Mariano, 1988). For monoamminechromium–ATP isomers, the resulting pyrophosphate complex will be meridional or facial, depending on the configuration of the original isomer.

Chromatography on a cation-exchange FPLC column separates facial and meridional monoamminechromium pyrophosphate complexes (Edens et al., 1993). ATP isomers **2Δ, 5, 8,** and **10**, which on the basis of the periodate/diethylenetriamine cleavage are meridional isomers, gave primarily the pyrophosphate complex with the longer retention time on the cation-exchange column. The faster eluting isomer is thus the facial one.

The analysis is somewhat complicated by the fact that the meridional monoamminechromium pyrophosphate isomer converts to the facial one within 15 min at pH 5.5 and room temperature. The reaction is much slower at lower pH or colder temperature. The conditions used in this work (3 min at 22 °C, pH 5.5) allowed enough product for detection but minimized the interconversion. The pure facial isomer was detected after enzymatic reaction with facial ATP isomers, but the pyrophosphate product formed by the reaction of meridional ATP complexes had some facial isomer in it, presumably resulting from the interconversion reaction. The pyrophosphate complex formed from each isomer was used in assigning the configurations in Table II.

Isomer Interconversion. Ammonia loss was not observed from monoamminechromium–ATP isomers for several weeks when they were kept in the dark, even at pH 5.5 and 22 °C. The isomers themselves were not stable indefinitely, interconverting in a variety of ways. For comparison, the axial-equatorial ring-puckering pairs of tetraaquochromium–ATP were stable in the dark for 4 days at pH 2.5, 22 °C, with only small amounts of the other ring isomer or opposite screw sense isomers formed. At pH 5.5, however, an equal amount of the other ring isomer formed within 6 h, along with smaller amounts of the opposite screw sense isomers.

This type of behavior was also observed for some pairs of monoamminechromium–ATP isomers. Thus isomers **5** and **8** and isomers **2Δ** and **10** were quite stable at pH 2.5 but clearly interconverted with each other at pH 5.5 within 6 h, with a very much slower conversion to other isomers or to ones with the opposite screw sense. However, isomers **2Λ** and **3** and isomers **1** and **6** were less stable, forming a substantial

Table II: Spectral Data of Monoamminechromium–ATP Isomers and Their Tripolyphosphate Degradation Products

isomer	CD spectrum[a] λ_{max} (nm)	θ	λ_{max} (nm)	θ	visible spectrum λ_{max} (nm)	degradation product[b] CD, θ	$t_{1/2}$ (min)	configuration[c]
1	620	−50	555	+1700	582	+240	14	Δ-ax-fac-anti
2Λ	630	+80	560	−1100	582	−240	12	Λ-ax-fac-anti
2Δ	635	−660	555	+1440	578	0	12	Δ-ax-mer-anti
3	610	+520	550	−820	582	−240	5	Λ-eq-fac-anti
4Λ	640	+40[d]	550	−460[d]	582	+30[d]	18	Λ-ax-mer-syn
4Δ	640	−60[d]	550	+270[d]	582	−50[d]	15	Δ-ax-mer-syn
5	620	+90	555	−750	580	0	10	Λ-ax-mer-anti
6	610	−290	550	+880	581	+240	4.5	Δ-eq-fac-anti
7	630	+360	540	−540	581	+240	5	Λ-eq-mer-syn
8	615	+710	545	−420	578	0	5	Λ-eq-mer-anti
9	640	−300	570	+330	581	−240	4.5	Δ-eq-mer-syn
10	620	−570	545	+590	578	0	4.5	Δ-eq-mer-anti

[a] Units are deg cm^2 dmol^{-1}. [b] CD spectrum[a] at 550 nm of monoamminechromium tripolyphosphate after treatment with periodate and diethylenetriamine. The half-time is for the CD change during the elimination. [c] Ax, axial; fac, facial; eq, equatorial; mer, meridional. For structures, see Figure 1. [d] These isomers are difficult to separate, and these samples were not pure. The true θ values are thus larger than these.

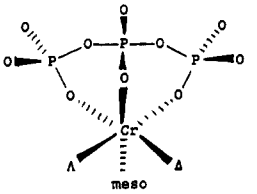

FIGURE 3: Monoamminechromium tripolyphosphate complexes resulting from degradation of monoamminechromium–ATP isomers with periodate and diethylenetriamine. Ammonia is in the position shown, with water in the other two positions. The screw sense is determined by a line connecting the terminal phosphorus atoms and a line between the coordinated waters. CD spectra: θ = +240 deg cm^2 dmol^{-1} (Λ); θ = −240 (Δ); θ = 0 (meso).

Table III: Inhibition Constants as Competitive Inhibitors of Yeast Hexokinase[a]

isomer	K_i (μM)
Tetraaquochromium–ATP	
Λ	0.04 ± 0.005
Δ	0.63 ± 0.05
Monoamminechromium–ATP	
Λ-eq-fac-anti (**3**)	0.90 ± 0.09
Λ-ax-fac-anti (**2Λ**)	0.45 ± 0.08
Λ-eq-mer-anti (**8**)	0.05 ± 0.006
Λ-ax-mer-anti (**5**)	0.05 ± 0.005
Λ-eq-mer-syn (**7**)	0.10 ± 0.01
Λ-ax-mer-syn (**4Λ**)	0.06 ± 0.007
Δ-eq-fac-anti (**6**)	1.65 ± 0.20
Δ-ax-fac-anti (**1**)	4.5 ± 0.4
Δ-eq-mer-anti (**10**)	2.0 ± 0.3
Δ-ax-mer-anti (**2Δ**)	1.8 ± 0.2
Δ-eq-mer-syn (**9**)	1.7 ± 0.1
Δ-ax-mer-syn (**4Δ**)	0.8 ± 0.07[b]

[a] pH 5.8, vs MgATP; 50 mM MES, 10 mM glucose. [b] Incubation with yeast hexokinase used to remove residual Λ isomer prior to use.

amount of the other isomer within 24 h at pH 2.5. Isomers **2Λ** and **3** also formed significant amounts of isomers **4Λ** and **7**, while isomers **1** and **6** also formed **4Δ** and **9**.

The other isomers did not form pairs that were as clear cut. Thus isomers **4Λ** and **7** and isomers **4Δ** and **9** did interconvert, but a significant amount of isomers **2Λ** and **3** formed when following **4Λ** or **7**, and **1** and **6** were noticeably present with **4Δ** or **9**. At longer times, formation of the other isomers was also observed.

Inhibition Studies. Monoamminechromium–ATP isomers were competitive inhibitors vs MgATP with yeast hexokinase at pH 5.8. The inhibition constants for the purified isomers are in Table III.

DISCUSSION

Number of Isomers. Although 14 isomers are predicted for monoamminechromium–ATP, only 12 have been isolated and characterized. Thus either the missing two isomers are cochromatographing and are not separated or they are not formed. We believe that the latter is the case. These are isomers with ammonia facial and syn to AMP and should not show ring-puckering isomerization. Such isomers cannot form strong hydrogen bonds to the α-phosphate, since coordinated ammonia forms much weaker hydrogen bonds than coordinated water. Thus tetraaminecobalt(III)–ATP shows only two screw sense isomers and no ring-puckering isomerization (Cornelius et al., 1977). The lack of an α-phosphate results in no ring-puckering isomerization in ADP complexes with chromium (Dunaway-Mariano & Cleland, 1980a).

The facial syn isomers of monoamminechromium–ATP not only lack the syn-coordinated water required for axial ring-puckering stabilization, but in the equatorial configuration of AMP on the chelate ring there will not be a strong hydrogen bond between a γ-phosphate oxygen and a coordinated water (the hydrogen bond will be to the coordinated ammonia, as shown in Figure 1). These isomers will thus be less thermodynamically stable than the others in Figure 1. The reason they are not formed at all, however, probably is that no kinetic path exists for their formation.

Table IV: Expected Isomers of Monoamminechromium–ATP if γ-Phosphate H-Bonds Steer β-Phosphate Insertion[a]

initial insertion relative to NH_3	H-bond from γ-phosphate oxygen	to coord water[b]	insertion of β-phosphate oxygen	gives isomer
cis	*pro-R*	trans	(*pro-R*)	Λ-ax-fac-syn (does not form)[c]
cis	*pro-R*	trans	*pro-S*	Δ-eq-fac-anti (**6**)
cis	*pro-R*	cis-R	*pro-R*	Λ-ax-mer-anti (**5**)
cis	*pro-R*	cis-R	*pro-S*	Δ-eq-mer-anti (**10**)
cis	*pro-R*	cis-L	(*pro-R*)	NR[d]
cis	*pro-R*	cis-L	(*pro-S*)	NR[d]
cis	*pro-S*	trans	*pro-R*	Λ-eq-fac-anti (**3**)
cis	*pro-S*	trans	(*pro-S*)	Δ-ax-fac-syn (does not form)[c]
cis	*pro-S*	cis-R	(*pro-R*)	NR[d]
cis	*pro-S*	cis-R	(*pro-S*)	NR[d]
cis	*pro-S*	cis-L	*pro-R*	Λ-eq-mer-anti (**8**)
cis	*pro-S*	cis-L	*pro-S*	Δ-ax-mer-anti (**2Δ**)
trans	*pro-R*	cis	*pro-R*	Λ-ax-mer-syn (**4Λ**)
trans	*pro-R*	cis	*pro-S*	Δ-eq-mer-syn (**9**)
trans	*pro-S*	cis	*pro-R*	Λ-eq-mer-syn (**7**)
trans	*pro-S*	cis	*pro-S*	Δ-ax-mer-syn (**4Δ**)

[a] Note that isomers **2Λ** (Λ-ax-fac-anti) and **1** (Δ-ax-fac-anti) are not formed initially but must result from isomerization from isomers **3** and **6**, respectively. [b] Position of coordinated water relative to ammonia. cis-R and cis-L distinguish the two cis waters when looking at chromium from the inserted γ-phosphate, with NH_3 at the top. [c] The ax-fac-syn isomers do not form because of the lack of proper α-phosphate H-bond steering. The eq-fac-syn isomers do not form because the γ-phosphate oxygens do not H-bond to the NH_3 but rather to one of the coordinated waters. [d] No reaction because NH_3 would have to be displaced.

After the initial insertion of the γ-phosphate in place of a water in the inner coordination sphere of the monoamminechromium ion (which can take place either cis or trans to the ammonia), a strong hydrogen bond should form between one of the two remaining oxygens on the γ-phosphate and a coordinated water cis to the insertion point. These possibilities are outlined in Table IV. This hydrogen bond between the γ-phosphate and a coordinated water then limits the insertion of the β-phosphate to a specific position. Insertion of one of the β-phosphate oxygens gives an axial ring-puckering isomer, and insertion of the other gives an equatorial one (an example is shown in Figure 4). Presumably, axial isomers form only when a strong hydrogen bond to the α-phosphate steers insertion of the β-phosphate. Coupled with the proviso that only water can be displaced from the coordination sphere, these principles, as illustrated in Table IV, explain the formation of all observed isomers except **1** and **2Λ**, which presumably form from isomerization of **6** and **3**, respectively, under the conditions of the experiment. In particular, this analysis shows that facial syn isomers cannot form.

Isomer Characterization. Three possible tridentate monoamminechromium tripolyphosphate complexes would be expected from the periodate cleavage and elimination with diethylenetriamine, as shown in Figure 3. Isomers with ammonia trans or anti to the β-phosphate are the only ones predicted to form a meso product with no residual CD spectrum. These isomers (**2Δ**, **5**, **8**, and **10**) thus have the meridional anti configuration, and, on the basis of their longer

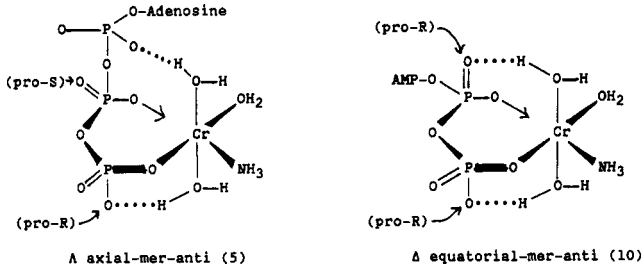

FIGURE 4: Steering of β-phosphate insertion. In the examples shown, the γ-phosphate has inserted cis to ammonia, and the *pro-R* oxygen of the γ-phosphate has formed a hydrogen bond to a cis water (cis-R in the notation of Table IV). (Left) The α-phosphate forms a hydrogen bond to the cis-L water, thus requiring insertion of the *pro-R* oxygen of the β-phosphate to give isomer **5**. (Right) If AMP assumes an equatorial configuration, the *pro-S* oxygen of the β-phosphate is inserted, giving isomer **10**.

half-times for elimination, the first two are axial and the other two are equatorial ring-puckering isomers.

The fact that isomers **2Δ**, **5**, **8**, and **10** have the meridional arrangement enabled us to identify the elution order of the facial and meridional isomers of monoamminechromium pyrophosphate from the cation-exchange FPLC column by degrading monoamminechromium–ATP isomers with nucleotide phyophosphatase, which removes AMP from such complexes. This degradation then allows the assignment of each isomer as facial or meridional. Those isomers that are meridional but give a chiral tripolyphosphate complex after treatment with periodate and diethylenetriamine are predicted to give the Λ isomer when the original screw sense was Λ and the Δ isomer when the original screw sense was Δ. The facial anti isomers are predicted to give the Λ tripolyphosphate isomer when their original screw sense was Δ and the Δ isomer when the original screw sense was Λ. The data in Table II follow this pattern and show that the molecular ellipticity at 550 nm of the Λ isomer of monoamminechromium tripolyphosphate is +240 deg cm^2 dmol^{-1} and that of the Δ isomer is −240. The only exceptions were the observed ellipticities from the degradation of isomers **4Λ** and **4Δ**, which are difficult to separate so that only partially purified samples were available.

Other information exists to confirm some of the isomer assignments. Lin and Dunaway-Mariano (1988) showed that axial isomers of tetraaquochromium–ATP elute before equatorial ones from cycloheptaamylose. In agreement with this pattern, we find that the early eluting Λ isomers (**2Λ**, **4Λ**, and **5**) and the early eluting Δ isomers (**1**, **2Δ**, and **4Δ**) are axial, while the late eluting Λ isomers (**3**, **7**, and **8**) and the late eluting Δ isomers (**6**, **9**, and **10**) are equatorial.

A number of isomers elute from the cation-exchange FPLC column in pairs that have the same ammonia position but opposite screw sense. However, not all isomers with ammonia in the same position elute together. For example, isomer **2Λ** elutes after isomer **3**. This column thus seems capable of separating the isomers on the basis of their ammonia positions and their ring conformations but not on screw sense. On this basis, the ring-puckering conformations should be the same for isomers **1** and **2Λ**, **3** and **6**, **4Λ** and **4Δ**, **7** and **9**, **8** and **10**, and **5** and **2Δ**, in agreement with our assignments.

Isomer Interconversion. Dunaway-Mariano and Cleland (1980a) discovered that there were two isomers of each screw sense of tetraaquochromium–ATP which interconverted rapidly at pH 5.5 but more slowly at pH 3 or below. These were assigned as a pair of ring-puckering isomers with axial and equatorial arrangements of AMP. The same behavior is seen with meridional monoamminechromium isomers, and in each case the interconversion rates are similar to those observed with tetraaquochromium–ATP isomers. The facial isomers of monoamminechromium–ATP, however, form pairs of more rapidly equilibrating isomers (**2Λ** and **3**, **1** and **6**); these isomers interconvert even at pH 2.5. The axial configuration in this case is stabilized only by the strong hydrogen bond to the α-phosphate, and the γ-phosphate can only hydrogen bond weakly to the coordinated ammonia. The equatorial configuration has only a strong hydrogen bond from the γ-phosphate to coordinated water, plus a weak one from the β-phosphoryl oxygen to ammonia. Thus these ring-puckering isomers are not as stable as those in which two hydrogen bonds form to coordinated waters, and they interconvert more rapidly.

The facial isomers of monoamminechromium–ATP show a significant conversion to the syn meridional isomers having ammonia trans to the γ-phosphate but very little conversion to the anti meridional isomers. Also, the syn meridional isomers show a significant amount of conversion to the other ring-puckering conformer or to the facial isomers but very little conversion to the anti meridional isomers. This behavior can be explained by looking at these interconversions as being either a dissociative process or an interchange (I_d) process, with only one water molecule in the complex that is particularly labile. As the bond to this water molecule is broken, a specific five-coordinate intermediate (or, for the interchange process, a transition state) would be formed, which would then rapidly add water.

Assuming that the chelate ring freezes the geometry of the coordinated phosphate oxygens, one must become equatorial and the other one axial in the trigonal-bipyramidal intermediate in the ammonia migration reaction. When the water that leaves to give the intermediate is trans to a coordinated phosphate oxygen, that oxygen is necessarily equatorial in the intermediate, and the other coordinated one is necessarily axial. Since water attack on the intermediate is in the equatorial plane, the group trans to the axial-coordinated phosphate oxygen *cannot migrate, but is frozen in place*, unless the intermediate has time to pseudorotate prior to reaction with water. This explains the failure of the meridional anti isomers (**5** and **8**; **2Δ** and **10**) to show ammonia migration, if the most labile water in the coordination sphere is the one trans to the coordinated γ-phosphate.

In the facial anti complexes, if the water that is trans to the γ-phosphate is preferentially lost, the proposed trigonal-bipyramidal intermediate would have ammonia, water, and the γ-phosphate in the equatorial plane. When water adds in this plane, the facial isomer would reform in two of the three possible points of attack, but water could also add so that the meridional syn complex having the ammonia trans to the γ-phosphate would form, as observed experimentally. There is no way that ammonia could end up trans to the β-phosphate, since the water in this position is axial in the intermediate and not free to migrate unless pseudorotation occurs.

In the meridional syn complexes with ammonia trans to the γ-phosphate, there is no water trans to the γ-phosphate, as in the above complexes. If the water that is trans to the β-phosphate is lost, the original complex is reformed, since the ammonia will be trapped in axial position in the resulting intermediate. However, if the water that is cis to both phosphates and anti to AMP is lost, a five-coordinate intermediate is formed that in two of the three modes of water attack will produce a facial anti isomer, as observed experimentally (the other mode reforms the starting isomer). If the water cis to both phosphates but syn to AMP is lost initially, the resulting intermediate could, in theory, form a facial syn

complex, but we have not observed this, possibly because of the lower thermodynamic stability of such complexes.

Inhibition Studies with Yeast Hexokinase. The inhibition constants for monoamminechromium–ATP isomers in Table III clearly show that the enzyme preferentially binds certain isomers. As Dunaway-Mariano and Cleland (1980b) showed, there is a clear preference for Λ isomers over Δ ones, with inhibition constants under 1 μM for the former and over 1 μM for the latter.

The inhibition constants for the Λ facial isomers are much higher than those for the meridional isomers, showing that water in the facial position is important for binding. The Λ isomers having ammonia trans to the β-phosphate are almost as effective as the Λ isomers of tetraaquochromium–ATP, suggesting that this coordination position faces the solvent, rather than the enzyme. The other meridional Λ isomers are also very good inhibitors, showing that this position has only minimal interaction with the protein. These data should be of value in interpreting the structure of hexokinase with a bound metal–nucleotide complex once such a crystal structure is obtained.

In each case, the ring-puckering isomers of the same complex have dissociation constants that are very similar, suggesting that during the binding process the hydrogen bonds stabilizing the axial or equatorial ring puckering are broken and perhaps replaced by ones to the enzyme. Similar results were found by Dunaway-Mariano and Cleland (1980b) for the isomers of tetraaquochromium–ATP.

These experiments show the value of monoamminechromium isomers for mapping the intimate interactions between bound metal–nucleotide complexes and enzyme active sites. Now that the absolute configurations of these isomers are known, they should prove very useful for this purpose.

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