Preparation and Properties of Chromium(III) Adenosine 5′-Triphosphate, Chromium(III) Adenosine 5′-Diphosphate, and Related Chromium(III) Complexes†

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ABSTRACT: A γ-monodentate complex of Cr(III) and ATP was formed in 10% yield from $CrCl_3$ by heating at 80 °C for 2 min and purified on Dowex-50-H^+. β,γ-Bidentate Cr(III) complexes of ATP and several ATP analogues were prepared in yields from 35 to 75% by reaction of the nucleotide with $CrCl_3$ at pH 5.7 and 4 °C. The complexes were separated into four stereoisomers on cycloheptaamylose columns. Two of the bidentate CrATP isomers gave CD spectra with a peak at 640 nm ([θ] = +430 or +580) and a trough at 575 nm ([θ] = −1000 or −550 deg cm^2/dmol) and were substrates for hexokinase, showing them both to have the Λ β-P configuration. The other two isomers were not substrates for hexokinase and had CD spectra which are almost mirror images of those described above; they must have the Δ β-P configuration. Epimerization and hydrolysis of the screw sense isomers were both base-catalyzed processes requiring the participation of several hydroxide ions in the rate-limiting reaction steps. The isomers were reasonably stable below pH 6, and at pH 5.6 epimerization occurs at room temperature with a half-life of 10 days and is faster than hydrolysis. The isomers with the same β-P configuration appear to be chelate ring conformers having twist boat geometry with the α-phosphate in either a pseudoaxial or pseudoequatorial position. The pseudoaxial conformer is probably stabilized by strong hydrogen bonds between coordinated water molecules and the γ-phosphate and α-phosphate oxygens, while the pseudoequatorial conformer is stabilized by hydrogen bonds to the γ-phosphate and β-phosphate oxygens. The activation energy for conformer interconversion at pH 5.6 is 17 kcal/mol, K_{eq} is approximately 1, and the half-life for conformational equilibration at 25 °C is 32 h. Bidentate Cr complexes of ATP analogues gave absorption and CD spectra quite similar to those of CrATP, but they varied significantly in their ability to inhibit hexokinase, with K_{is} values ranging from 10 nM for Cr(8-BrATP) to 410 μM for Cr(β,γ-methylene-ATP). Tridentate CrATP is formed from bidentate CrATP at low pH and is readily purified on cycloheptaamylose columns. CrPPP formed from tripolyphosphate by the procedure used for bidentate CrATP is tridentate, as are the products of degradation of either bidentate or tridentate CrATP by treatment with periodate followed by aniline. The α-phosphate epimers of Cr-$(H_2O)_4$ADP, Cr$(NH_3)_4$ADP, and Co$(NH_3)_4$ADP were separated on cycloheptaamylose columns, and, on the basis of the CD spectra, the relative ability of the isomers to inhibit creatine kinase, and the known stereochemistry of the creatine kinase reaction, it appears that the isomers with negative ellipticity are Δ.

ATP exists in the cell as a complex with Mg^{2+}, but because the various coordination isomers of MgATP equilibrate more rapidly than the NMR time scale, it is not possible to tell how many isomers exist in solution or which ones are the substrates for a given enzyme. The latter problem can be solved by using exchange-inert complexes of ATP with metals such as Cr(III) or Co(III). CrATP was first prepared by DePamphilis & Cleland (1973), who postulated that it was a tridentate chelate with one oxygen of each phosphate in the coordination sphere. These authors noted that stereoisomers should be possible because the α- and β-phosphates became chiral upon coordination, but isomer separation was not achieved. Danenberg & Cleland (1975) showed that 25% of CrATP as they prepared it bound very tightly to hexokinase in the presence of glucose and was a substrate, forming a CrADP–glucose-6-P complex which was released from the enzyme very slowly. Since Cr$(NH_3)_4$ATP was also a substrate, they correctly deduced that β,γ-bidentate CrATP was the active species and that their CrATP was 25% bidentate and 75% tridentate. The paramagnetism of Cr(III) makes it impossible to use NMR to characterize its complexes, but the closely related Co(III) complexes are readily studied by NMR, and Cornelius et al. (1977) used ^1H and ^{31}P NMR to establish the structures of bidentate and tridentate ATP complexes of tri- and tetraamminecobalt(III). They noted the existence of the four predicted diastereomers of tridentate Co$(NH_3)_3$ATP, as well as the two diastereomers of Co$(NH_3)_4$ATP, but did not separate any of these. Cornelius & Cleland (1978) then used hexokinase to convert one diastereomer of Co$(NH_3)_4$ATP to Co$(NH_3)_4$ADP–glucose-6-P, which was readily separated from the unused isomer and reconverted enzymatically into the active isomer of Co$(NH_3)_4$ATP. The two purified isomers of Co$(NH_3)_4$ATP had opposite CD spectra and, when degraded by removal of adenosine, gave crystalline enantiomeric Co-$(NH_3)_4$PPP complexes without loss of chirality. Merritt et al. (1978) determined by X-ray the structure of one of these and thus showed that hexokinase was specific for the Λ isomer (the Δ isomer has AMP attached to the other oxygen of the β-phosphate):[1]

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[1] The screw sense nomenclature is that of Cornelius & Cleland (1978). In this system, 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 either a left-hand (Λ) or a right-hand (Δ) helix.

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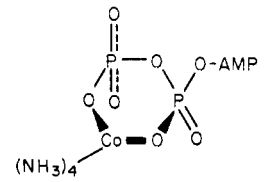


Since CrATP is a substrate for all of the kinases we have tested [see Dunaway-Mariano & Cleland (1980)], while Co$(NH_3)_4$ATP is not used by most of them, it is important to develop methods for separating CrATP isomers and identifying which is which, as well as to find conditions under which they are stable. Fortunately, the properties of Co(III) and Cr(III) nucleotide complexes are so similar that the information learned by NMR studies of the former is probably applicable to the latter. In the present study we report the preparation of monodentate and bidentate CrATP, the chromatographic separation of the latter into four stereoisomers, and the characterization of these isomers. We have also prepared and separated the bidentate isomers of the chromium complexes of a number of ATP analogues. The separation and characterization of bidentate Cr$(NH_3)_4$ATP, Cr$(NH_3)_4$ADP, and Cr$(H_2O)_4$ADP stereosiomers are reported, and we will describe the preparation and chromatographic properties of CrPPP and of tridentate CrATP, which has been only partially separated into isomers (Cleland & Mildvan, 1979).

Materials and Methods[2]

General. Yeast hexokinase, glucose-6-phosphate dehydrogenase, ATP, ADP, 2′-dATP, β,γ-NHATP, α,β-MeATP, β,γ-MeATP, ion-exchange resins, Sephadex G-25 resin, and Pipes were from Sigma Chemical Co. ATPγS was from Boehringer Mannheim and 2′,3′-didATP was from P-L Biochemicals. Cycloheptaamylose gel (CHpA) was prepared by the method of Cornelius & Cleland (1978). CD spectra and UV–visible absorption spectra were measured by using a Jasco J41 spectropolarimeter and Cary 118 spectrophotometer, respectively. Molar ellipticities are reported in deg cm^2/dmol. Hexokinase inhibition studies were carried out at 25 °C by using a Beckman DU monochrometer, a Gilford optical density converter, and a 10-mV recorder. Adenosine was quantitated by measuring absorbance at 260 nm, assuming $\epsilon = 15\,400$. Phosphate determinations were carried out by the method of Chen et al. (1956), and chromium(III) determinations were carried out by the method of Postmus & King (1955). The pK_a of adenosine was determined from the change in ϵ_{284} as a function of pH. Each measurement was made by adding an aliquot of CrATP solution at 4 °C to buffer and quickly measuring the absorbance at 284 nm. Inhibition studies were analyzed by fitting data to eq 1, and pK_a calculations were carried out by fitting data to eq 2, using FORTRAN programs of Cleland (1967) which assume equal variance for v or log A^{obsd}:

$$v = \frac{VS}{K(1 + I/K_{is}) + S} \quad (1)$$

$$\log A^{obsd} = \log \left(\frac{A^b + A^a K_a/[H^+]}{1 + K_a/[H^+]} \right) \quad (2)$$

In eq 1, v is initial velocity, V is the maximum velocity, S is substrate concentration, I is inhibitor concentration, K is the Michaelis constant, and K_{is} is the inhibition constant. In eq 2, A^{obsd} is experimental absorbance, A^b is the limiting value at low pH, A^a is the limiting value at high pH, and K_a is the acid dissociation constant.

Ion-Exchange Resin. Dowex-50-X2-H$^+$ (200–400 or 100–200 mesh) was used in this work. The 2% cross-linking is important, since higher percentage cross-linking does not allow proper penetration of the beads by CrATP. Before use, the resin was converted to the K$^+$ form with 1 N KOH and bleached with Br_2 dissolved in KOH (the dark brown color changes rapidly to very pale yellow). After being washed with water, the resin was then converted to the H$^+$ form with 6 N HCl and washed with water. Resin treated in this manner is nearly white, and colored compounds are readily observed on columns.

Preparation of γ-Monodentate and β,γ-Bidentate CrATP by Two-Minute Heating at 80 °C. To 250 mL of 20 mM Na_2ATP was added, with stirring, 250 mL of 20 mM $CrCl_3$. The resulting solution was rapidly brought to 80 °C in a boiling water bath. After 2 min at 80 °C (maintained by alternately immersing and removing the flask), the reaction was terminated by pouring the solution into a 2-L Erlenmeyer cooled in a salt–ice bath. Purification was accomplished by adsorbing the crude mixture onto a 22 × 1.5 cm column of Dowex-50-H$^+$ at 4 °C and washing the column with deionized water until three distinct bands became apparent. The dark blue top band containing hexaaquochromium was removed, and the resin containing the second blue-green band was transferred to a 1.2-cm diameter column containing 2 cm of fresh resin. Monodentate CrATP was then eluted from this column with 0.5 M $HClO_4$. The elutate was adjusted to pH 3 with saturated $KHCO_3$, filtered, and stored at 4 °C (10% yield). Bidentate CrATP was obtained from the resin of the third band of the first Dowex column in a similar manner, adjusted to pH 5, filtered, and stored at 4 °C (yield, 10%). Bidentate CrATP should not remain on Dowex-50-H$^+$ more than 1 h, because conversion to tridentate isomers is acid catalyzed (see below). Where isoionic material containing no traces of perchlorate was desired, bidentate CrATP was slowly focused off the resin by using 0.3 M aniline, the aniline was immediately removed from the eluate by extraction with 3 × 5 volumes of ether, and the residual ether was then removed in vacuo.

When only monodentate CrATP is desired, the following alternate method may be used. To 50 mL of 100 mM Na_2-ATP is added with stirring 50 mL of 100 mM $CrCl_3$. To this solution is added 400 mL of water at 85 °C (resulting temperature, 76 °C). After 2 min the reaction mixture is cooled and chromatographed as described above. The yield is 7% monodentate and 1.4% bidentate CrATP. The spectral properties of monodentate CrATP (pH 3) are as follows: UV max 257 nm; visible max 599 nm ($\epsilon = 17$) and 419 ($\epsilon = 18$); visible min 496 nm ($\epsilon = 4$), shoulder at 677 nm ($\epsilon = 5$); visible CD, peak at 605 nm ([θ] = +27).

Preparation of Bidentate CrATP. Method A. This compound was prepared in 60–75% yield from reaction of Na_2ATP and $CrCl_3$ at 80 °C (pH 3; 10 min), followed by Dowex-50-H$^+$ column chromatography at 4 °C as described above. *Method B.* A solution 10 mM each in Na_2ATP and freshly dissolved $CrCl_3$ was cooled to 4 °C and then adjusted slowly with good

[2] Abbreviations used: ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); 2′-dATP, 2′-deoxyadenosine 5′-triphosphate; 2′,3′-didATP, 2′,3′-dideoxyadenosine 5′-triphosphate; 8-BrATP, 8-bromoadenosine 5′-triphosphate; α,β-MeATP, α,β-methyleneadenosine 5′-triphosphate; β,γ-MeATP, β,γ-methyleneadenosine 5′-triphosphate; β,γ-NHATP, adenylyl imidodiphosphate; ADP, adenosine 5′-diphosphate; Pipes, K salt of piperazine-*N*,*N*′-bis(2-ethanesulfonate); Mes, K salt of 2-(*N*-morpholino)ethanesulfonate; CHpA, cycloheptaamylose cross-linked with epichlorohydrin; BHEP, Cl salt of *N*,*N*′-bis(hydroxyethyl)piperazine.

stirring to pH 5.7 with saturated $KHCO_3$. After standing at room temperature for 30 min, the solution was cooled, adjusted to pH 2, and then either chromatographed on a Dowex-50-H^+ column at 4 °C in the manner described above or passed through a Millipore filter and placed on a CHpA column as described below. *Method C.* A 3 mM solution of monodentate CrATP at pH 3, 4 °C, was adjusted to pH 5.5 with saturated $KHCO_3$ and allowed to stand at room temperature for 10 min. The resulting solution was determined by CHpA column chromatography to be 90% pure bidentate CrATP. Bidentate CrATP is most stable when stored at 4 °C as a dilute solution (1–10 mM) in the range of pH 4–5.7.

Separation of Bidentate Isomers on a CHpA Column. Bidentate CrATP was concentrated in a rotary flash evaporator using a vacuum pump with a large dry ice trap to supply the vacuum, a recovery flask cooled by an ice–water bath, and water at 10 °C running over the sample flask. Ten milliliters of a 30–40 mM solution of bidentate CrATP (pH 5.3–5.8) was loaded on a 245 × 1.3 cm CHpA column at 4 °C and the column eluted with 10 mM Pipes, pH 5.5, at a gravity-driven flow rate of 6 mL/h. The 1.6-mL fractions were examined by CD spectropolarimetry, and those enriched in the desired isomer were pooled. Each of the isomer-enriched solutions from two such chromatographies conducted simultaneously was combined, concentrated as described above to 5 mL, and immediately rechromatographed on 235 × 0.7 cm columns (flow rate 5 mL/h) at 4 °C. After a second rechromatography on CHpA, isomers of ≥99% purity are obtained in concentrations of 0.5–1 mM. Such solutions retain the chirality of the β-phosphate for several days if stored between pH 3.5 and pH 5 and at 4 °C and will remain greater than 90% pure in the chelate ring conformer for 1 day.

Inhibition Studies. The chromium complexes were tested as inhibitors of hexokinase by using the glucose-6-phosphate dehydrogenase assay in which the appearance of NADPH was monitored spectrophotometrically at 340 nm. The reaction solutions (3 mL; pH 5.8–5.9) were 1 mM in citrate, 2 mM in $Mg(OAc)_2$, 0.4 mM in NADP, 10 mM in glucose, 50 mM in Pipes, and 0.03–0.33 mM in ATP. The K_i values measured for the bidentate isomers of the chromium complexes of ATP and ATP analogues are reported in Table I.

Conversion of Bi- to Tridentate CrATP. Method A. Eight-milliliter aliquots of 1.5 mM bidentate CrATP isomers 1 and 4 were mixed with 2 g of dry Dowex-50X8-H^+ at 0 °C. After the specified time, the mixture was suction filtered (filtrate discarded) and the resin was quickly washed with 0.5 M $HClO_4$. The acid rinse was adjusted to pH 3.5 with saturated $KHCO_3$, filtered, and concentrated to 5 mL as described above. This solution was adjusted to pH 5.5 and chromatographed on a CHpA column (235 × 0.7 cm; 10 mM Pipes, pH 5.5; 4 °C), and the ratio of tridentate to bidentate CrATP present was determined from the elution profile. When full conversion was desired, 24-h reaction was used. *Method B.* A 0.5 mM solution of bidentate CrATP 0.5 M in $HClO_4$ was allowed to stand at 4 °C for 3 days. The solution was then adjusted to pH 5 with saturated $KHCO_3$, filtered, and concentrated to 40 mM. Five milliliters of this solution was then chromatographed on a 235 × 0.7 cm CHpA column at 4 °C with 10 mM Pipes (pH 5.6) as eluant, giving 4% polymer, 74% tridentate CrATP, and 22% bidentate CrATP.

Preparation of CrPPP. Method A. At 4 °C, 100 mL of 20 mM $CrCl_3$ was added to 100 mL of 20 mM tripolyphosphate (pH 3), and the solution was adjusted to pH 5.5 with saturated $KHCO_3$. After 30 min at room temperature, the reaction mixture was diluted twofold with water and adsorbed onto a 40 × 1.2 cm Dowex-1-Cl^- column equilibrated with 25 mM BHEP, pH 4. The column was washed with 200 mL of buffer, and the CrPPP was focused off by using the same buffer 0.1 M in KCl. One volume of acetone was added to the eluate at 0 °C and, after standing for 3 h at 0 °C, the solution was filtered and the green solid was dissolved in cold water (yield based on ϵ_{610} of 20, 65%). A portion of this solution was then made 0.5 M in $HClO_4$ and stored at 4 °C for 3 days. The reaction mixture was then adjusted to pH 4 with saturated $KHCO_3$, filtered, and chromatographed on a Dowex-1 column as described above (recovery of CrPPP, 90%). *Method B.* To a 30 mM solution of bi- or tridentate CrATP at pH 3 was added 2 equiv of sodium periodate. After 1 h in the dark at 25 °C, 10 μL of ethylene glycol per mL of reaction mixture was added. After 10 min, 3 volumes of 0.4 M aniline hydrochloride (pH 5) was added and the resulting solution was allowed to stand for 3 h. The mixture was then filtered, cooled to 0 °C, and suction filtered through 30 mL of Dowex-50-H^+ resin at 4 °C. The filtrate was adjusted to pH 4 and chromatographed on a Dowex-1 column as described above (yields based on ϵ_{610} of 20: 88% from bidentate CrATP; 61% from tridentate CrATP). Samples (0.5 mL) of the CrPPP preparation described above were chromatographed on Dowex-1-Cl^- columns (30 × 0.7 cm) by using a linear gradient (0 to 0.1 M) of KCl in 25 mM BHEP, pH 4, as the eluant. In each case, CrPPP eluted at 50 mM KCl.

Bidentate $Cr(NH_3)_4ATP$. Na_2ATP was reacted with $[Cr(NH_3)_4Cl(H_2O)]Cl_2$ and chromatographed on Dowex-50-H^+ as described by Danenberg & Cleland (1975). Final purification was carried out on a CHpA column (235 × 0.7 cm; 4 °C; 10 mM Pipes, pH 5.6). Fractions containing the bidentate $Cr(NH_3)_4ATP$ were pooled and used directly in the hexokinase reaction.

$Cr(NH_3)_4ADP$. A solution 10 mM each in NaADP and $[Cr(NH_3)_4Cl(H_2O)]Cl_2$ was heated at 80 °C for 10 min as described by DePamphilis & Cleland (1973). The solution was cooled, concentrated to 50 mM, and adjusted to pH 5.5, and the two isomers were separated by chromatography on a 235 × 0.7 cm CHpA column at 4 °C (10 mM Pipes, pH 5.6, as the eluant; the centers of the peaks were at 106 and 150 mL of eluate): yield, 80%.

$Cr(H_2O)_4ADP$. This compound was prepared in 50–65% yield either by heating a solution 10 mM each in NaADP and $CrCl_3$ to 80 °C for 10 min or by adjusting the solution to pH 5.6 at 4 °C with saturated $KHCO_3$ and allowing it to stand at room temperature for 20–30 min. The crude reaction mixture was either chromatographed directly on a CHpA column or first partially purified on a Dowex-50-H^+ column (water wash, followed by 0.5 M $HClO_4$ elution at 4 °C and back-titration to pH 5.5 with $KHCO_3$) and then chromatographed on the CHpA column.

Results

Preparation of γ-Monodentate and β,γ-Bidentate CrATP. Monodentate and bidentate CrATP were each isolated in 10% yield from a reaction mixture prepared by heating a solution 10 mM each in $CrCl_3$ and Na_2ATP for 2 min at 80 °C. Purification of the mixture was carried out at 4 °C on a Dowex-50-H^+ column using water as the eluant. During column loading the unreacted $Cr(H_2O)_6^{3+}$ adsorbed as a tight blue band at the top of the resin bed, while Na^+, monodentate CrATP, and bidentate CrATP adsorbed below this band. As the column was washed with water, the monodentate and bidentate CrATP traveled through the Na^+ band. While the monodentate CrATP focused on the fresh H^+ resin just under

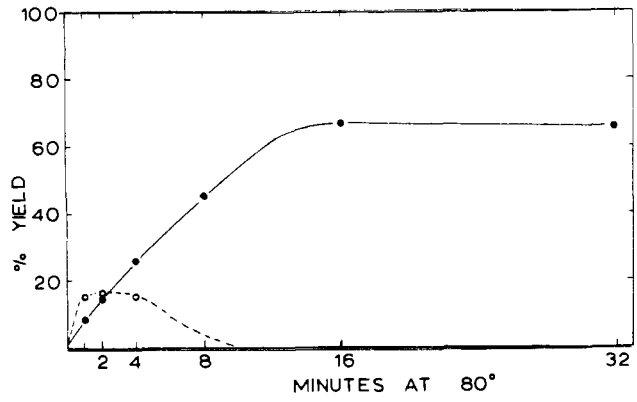


FIGURE 1: Time course for bidentate CrATP (●) and monodentate CrATP (○) formation from reaction of Na_2ATP with $Cr(ClO_4)_3$ (10 mM; pH 3) at 80 °C. By 8 min, little or no monodentate material remained.

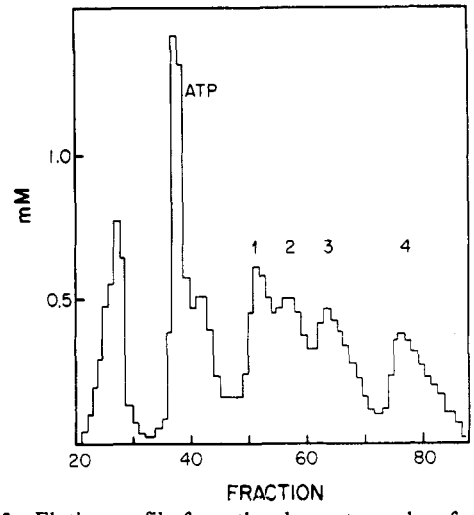


FIGURE 2: Elution profile from the chromatography of a CrATP reaction mixture on a 235 × 0.7 cm CHpA column at 4 °C using 10 mM Pipes, pH 5.6, as the eluant. The fraction volume was 1.6 mL and the flow rate was 5 mL/h. The bidentate isomers are numbered 1–4. The concentration plotted is that of adenine (from A_{260}). CrATP was prepared by titrating a solution 10 mM each in ATP and $CrCl_3$ to pH 5.7 at 4 °C and filtering it through a Millipore filter.

the Na^+ band, the bidentate CrATP continued to move slowly down the column. Once the monodentate (blue-green) and bidentate (apple-green) bands were visibly separated, the resin comprising the bands could be transferred to separate columns and the CrATP could be eluted. Monodentate CrATP could be stored for months at 4 °C and pH 3 without significant decomposition, but above pH 4 it is rapidly converted to bidentate isomers (see below). Bidentate CrATP may be stored at either pH 3 or pH 5.

In order to define conditions for optimum product yield, we maintained a reaction mixture 10 mM each in $Cr(ClO_4)_3$ and Na_2ATP at 80 °C. Samples were removed at various times, rapidly cooled in a salt–ice bath, and assayed for monodentate and bidentate CrATP by chromatographing them on small Dowex-50-H^+ columns in the manner described above. The results in Figure 1 suggest that monodentate CrATP is best prepared by heating the reaction mixture for only 1 min, while bidentate CrATP would be obtained in highest yield from mixtures heated for 10 min. From the time course in Figure 1, it appears that monodentate CrATP is the initial reaction product and serves as progenitor of bidentate CrATP. Thus, as one would predict, monodentate CrATP (5 mM; pH 3) is converted to bidentate CrATP in near quantitative yield simply by heating at 80 °C for 10 min.

Bidentate CrATP can also be prepared in 65–75% yield in a milder method by adjusting the pH of the Na_2ATP and $CrCl_3$ solution to pH 5.7 at 4 °C. This method is particularly useful for analogues which will decompose on heating to 80 °C and has become the method of choice for CrATP synthesis in this laboratory. Finally, almost pure bidentate CrATP preparations were obtained from monodentate CrATP simply by adjusting the pH from 3 to 5.7. This method has the advantage of giving bidentate CrATP whenever needed without subsequent chromatography.

Preparation of Tridentate CrATP and CrPPP. Heating a solution 10 mM each in $CrCl_3$ and Na_2ATP at 80 °C for up to 30 min at pH 1–4 produced only 5–10% tridentate CrATP, and little or no tridentate material resulted when the pH of $CrCl_3$ and ATP mixtures was raised to 6. However, bidentate CrATP was converted smoothly in 70–80% yield to tridentate CrATP in strong acid (0.5 M $HClO_4$ or Dowex-50-H^+ resin; 2–3 days 4 °C). Tridentate CrATP elutes from CHpA columns in the same location as free ATP does in Figure 2 and is thus easily separated from bidentate CrATP, although free ATP is removed only by chromatographing on Dowex-50-H^+. The partial separation of tridentate CrATP on Dowex-50-H^+ columns into two mixtures of isomers and the CD spectra of these isomers are described by Cleland & Mildvan (1979). Tridentate CrATP has an absorption spectrum with maxima at 433 and 616 nm (7–9 nm above those of bidentate isomers) and ϵ values of 21 for both peaks (somewhat less than for bidentate isomers). The CD spectrum at pH 5.5 of the mixture of tridentate isomers shows a positive peak at 595 nm ([θ] = +800) and smaller negative ones at 665 and 690 nm ([θ] = −63 and −162, respectively). The tridentate complex is more stable than bidentate CrATP. The original CrATP studied by DePamphilis & Cleland (1973) was about 75% tridentate because of the exposure to Dowex-50-H^+ during purification.

Formation of CrPPP and Degradation of CrATP to CrPPP. CrPPP was readily formed from sodium tripolyphosphate and $CrCl_3$ by adjusting the pH from 3 to 5.5 at 4 °C. A portion of the product was then exposed to 0.5 M $HClO_4$ at 4 °C for 3 days, but both this material and the original preparation appeared tridentate on the basis of their chromatographic behavior at pH 4 on Dowex-1-Cl^- columns. Bidentate and tridentate CrPPP should have at pH 4 a single and a double negative charge, respectively (the pK values of the terminal phosphates being around 2 when coordinated to chromium and around 6 when not coordinated), and since both preparations of CrPPP were eluted at chloride concentrations of 50 mM, they are clearly dianions. This conclusion is supported by titrations which showed two pK values between 1 and 2.5 and no other pK less than 8. The visible spectra of the CrPPP preparations were identical and similar to that of tridentate CrATP (λ_{max} at 432 and 612 nm). We conclude that, in contrast to the α-phosphate of bidentate CrATP, the terminal phosphate of bidentate CrPPP rapidly becomes coordinated at pH 5.5; possibly this group when doubly ionized catalyzes its own insertion in the coordination sphere by acting as a general base to remove a proton from a coordinated water.

When either bidentate or tridentate CrATP was converted to CrPPP by treatment with periodate at pH 3, followed by aniline at pH 5, the product was tridentate CrPPP. The ellipticity of pure bidentate isomer 2 of CrATP was not altered during the oxidation with periodate but disappeared during aniline treatment within a 2-h period. Bidentate CrPPP may be stable at pH 3, but so far it has not been isolated.

Separation of Bidentate CrATP Isomers. The bidentate

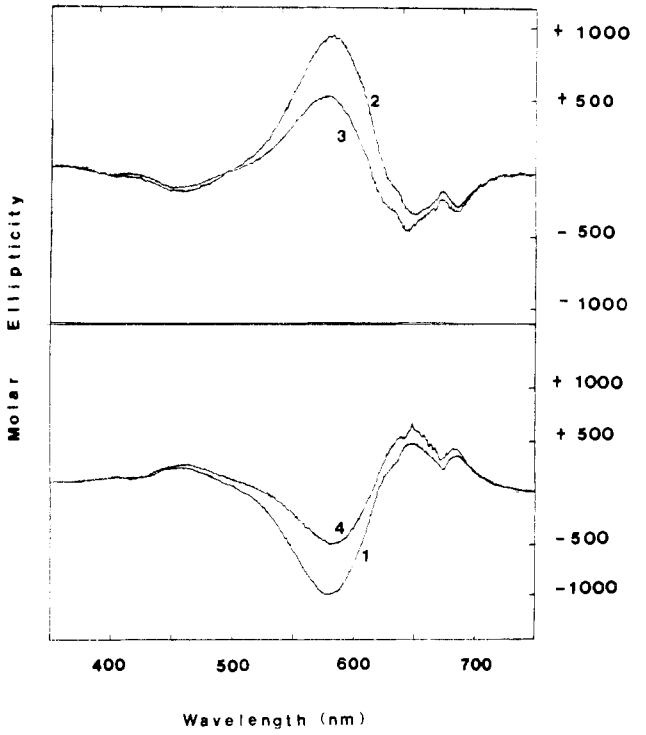


FIGURE 3: CD spectra of the four bidentate CrATP isomers in 10 mM Pipes, pH 5.6, at 25 °C. The spectra are numbered in the order in which the isomers are eluted from the CHpA column (see Figure 2).

isomers were separated on a CHpA column (Figure 2). The first peak eluting from the column is presumed to be polymeric material, the second peak is largely ATP (this is also where tridentate CrATP elutes, but none was present in this experiment), and the following four peaks are bidentate CrATP isomers. With heavier loading, the peaks are not as well resolved as those in Figure 2, but by determining the CD spectrum of each fraction, it is simple to decide which fractions to combine. At pH 5.6 and 4 °C the isomers were of sufficient stability to be rechromatographed to 99% purity. The homogeneity of the isomer preparations was judged by their CD spectra (described below) and by their substrate activity with various kinases [described in Dunaway-Mariano (1980)].

The chromium/phosphate/adenine ratio of each of the isomers was 1:3:1, and the UV spectra at both pH 2 and pH 5.6 were indistinguishable from those of ATP under the same conditions. The visible spectra for the individual isomers at pH 5.6 were quite similar (peaks at 426 and 607 nm, with ϵ values of 30 and 25) and did not differ significantly from those measured at pH 2. The pK_a for loss of a proton from N-1 of the adenine ring of each isomer was the same as that for ATP, 3.8. Similarly, the pK_a values for ionization of the γ-phosphate (about 2.2) of each isomer were judged to be very similar on the basis of the observed chromatographic behavior of the four isomers on Dowex-50-H^+ columns.

As shown in Figure 3, the CD spectra of isomers 1 and 4 at pH 5.6 are mirror images of those of isomers 2 and 3. The CD spectra of the isomer pairs (1 and 4; 2 and 3) which show the same sign of ellipticity at 575 nm differ only by the amplitude of this ellipticity. Reduction in pH appears to affect the elliptical properties of the isomer pair 1 and 4 the same way; thus, at pH 2 the ellipticity of both isomers at 575 nm is reduced by 50%, while that at 640 nm is reduced by 40%. The UV CD spectra of bidentate isomers 1 and 2 at pH 5.6 showed a negative peak at 257 nm ([θ] = −2500 and −2700, respectively). In comparison, the molar ellipticity of isomers 3 and 4 at 257 nm was less (−1800), while the value for ATP at 257 nm was −2950. At pH 2 the molar ellipticity of the isomers at 257 nm was smaller than that of ATP (−1900 to −2400 vs. −3500), and a positive peak at 280 nm ([θ] = +500 to +625) was present in the spectra of the CrATP isomers but not in that of ATP.

Interconversions of Bidentate CrATP Isomers. Bidentate CrATP isomers 1 and 4 were observed to interconvert below pH 6 much more rapidly than they were converted to isomers 2 and 3. Similarly, isomers 2 and 3 could be brought to equilibrium with little isomer 1 and 4 formation. At pH 7, however, epimerization becomes so fast that all four isomers are equilibrated. The interconversions of the isomers were monitored by measuring the rate of change in molar ellipticity at 575 nm. All studies were carried out with isomers 1 and 4, since they were most easily obtained pure in large quantities, and their interconversion was most easily observed by chromatography on CHpA columns.

The relative stabilities of isomers 1 and 4 at pH 5.6 were determined at 61 °C by heating the pure isomer solutions (0.5 mM) until no change in $[\theta]^{575}$ was observable and then immediately chromatographing the equilibrated solutions on a CHpA column at 4 °C. The elution profiles indicated that the equilibrated solutions contained isomers 1 and 4 in a 1:1 ratio and in addition contained about 10% of isomers 2 and 3. The same 1:1 ratio of isomers 1 and 4 was observed for a solution of isomer 4 that was allowed to reach equilibrium at 40 °C. Thus, over the temperature range of 40–61 °C, the K_{eq} for interconversion of Λ bidentate conformers is ~ 1.

The activation energy for the interconversion of isomers 1 and 4 was determined from the change in $[\theta]^{575}$ by measuring the temperature dependence of the first-order rate constant for conversion of isomer 1 to 4 or isomer 4 to 1 at pH 5.6. The data were analyzed assuming that the approach to equilibrium from both directions is first order and thus that k_{obsd} from the slopes of the first-order plots is twice the rate constant for conversion of isomer 1 to 4, or vice versa. These values of k_{obsd} were used to construct an Arrhenius plot, and the activation energy for the conformational isomerization was calculated from the slope of this plot to be 17 kcal/mol.

The rate of epimerization of isomers 1 and 4, present in a 1:1 ratio, into isomers 2 and 3 was examined as a function of pH at 25 °C. Three-milliliter solutions 1 mM in the isomer 1 and 4 mixture were adjusted to the desired pH with saturated $KHCO_3$, and the rate of change in ellipticity was monitored in a 5-cm cell at 575 nm. The $[\theta]^{575}$ decreased from −775 to 0 as the reaction was completed. First-order plots of the data were biphasic, indicating that two or more competing base-catalyzed reactions, both of which involve destruction of chirality at the β-phosphate, were taking place. The first-order rate contants for these two processes were determined at several pH values and the results are shown in Figure 4. The faster of the two processes is epimerization and appears to involve three to four hydroxide ions, while the second process appears to be a hydrolysis reaction which involves two hydroxides. The nature of the hydrolysis reaction was further examined by adjusting a 3 mM solution of bidentate CrATP to pH 7, allowing it to stand for 30 min, adjusting the pH to 5.6, and then chromatographing it on a 235 × 0.7 cm CHpA column at 4 °C (10 mM Pipes, pH 5.6). The reaction products, present in 40% yield, eluted significantly in front of bidentate CrATP as two partially resolved peaks. The material possessed no ellipticity in the visible region, adsorbed tightly to Dowex-50-H^+ resin, and upon standing slowly liberated CrADP. On the basis of its elution volume from the CHpA

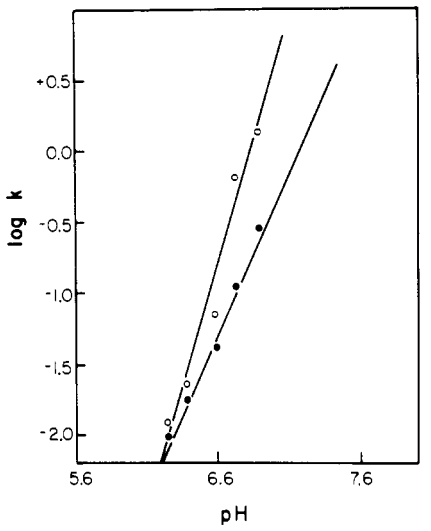


FIGURE 4: pH profile of the epimerization (○) and hydrolysis (●) of bidentate CrATP isomers 1 and 4, as a 1:1 mixture at 25 °C. The units of k are min^{-1}.

column, its strong adsorption on Dowex-50-H^+, its visible spectrum which shows λ_{max} at 605 and 426 nm, and its slow conversion to CrADP, the primary product is tentatively identified as $Cr(H_2O)_4(ADP)(P_i)$ resulting from hydrolysis of ATP while coordinated to chromium.

The relative rates of conversion of bidentate isomers 1 and 4 to tridentate CrATP were determined at 0 °C in the presence of Dowex-50-H^+ resin. Reaction rates were reasonably constant up to 30% conversion. The initial conversion rate of isomer 1 to tridentate CrATP was approximately twice as fast as that of isomer 4. Unfortunately, interconversion of isomers 1 and 4 is also acid catalyzed, so that under the reaction conditions conversion of one bidentate isomer to the other occurred at a rate comparable to the rate of conversion to tridentate CrATP. Thus, the difference in the rate constants for the conversion of isomers 1 and 4 to tridentate CrATP may be significantly larger than the twofold difference actually seen.

Bidentate Chromium(III) Complexes of ATP Analogues. Bidentate Cr(8-BrATP), Cr(2′-dATP), Cr(2′,3′-didATP), Cr(α,β-MeATP), Cr(β,γ-MeATP), Cr(β,γ-NHATP), and Cr(ATPγS) were prepared by reaction of the nucleotide and chromium chloride (10 mM each) at pH 5.7, 4 °C, in 73, 65, 57, 63, 53, 46, and 35% yields, respectively. The visible spectra of the purified complexes were the same as that of bidentate CrATP. The bidentate isomers of each complex were separated on CHpA columns, and the column fractions were combined in the manner indicated in Figures 5 and 6. The elution profile for Cr(β,γ-MeATP) was similar to that of Cr(β,γ-NHATP), with isomers 1–3 eluting closely together in fractions 50–65 and isomer 4 eluting as a separate peak in fractions 78–85. The K_{is} values determined for the partially purified isomers as competitive inhibitors vs. MgATP of hexokinase and the CD data for these isomers are in Table I.

The substrate activities of the four Cr(ATPγS) isomers with hexokinase were tested as described in Dunaway-Mariano & Cleland (1980) by using 75 μM isomer, 20 μM hexokinase, 1 mM glucose, and 50 mM Mes, pH 5.5, at 25 °C for 30 min. Isomer 1 gave 30% of a single turnover, and isomer 2 gave 50% of a turnover (isomers 3 and 4 gave less than 10% of a single turnover), but these activities correspond to only 6–8% of the chromium complex present and probably result from contamination with isomer 4 of CrATP, which is eluted in the same location.

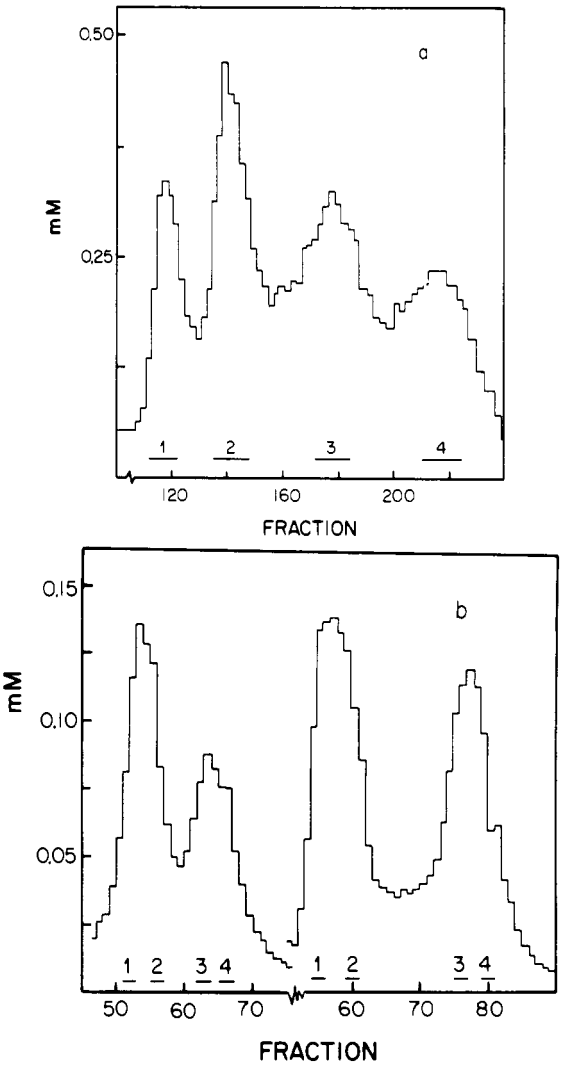


FIGURE 5: Elution profiles from the chromatography of reaction mixtures containing (a) Cr(8-BrATP), (b) (left) Cr(2′,3′-didATP), and (b) (right) Cr(2′-dATP) on a 235 × 0.7 cm CHpA column at 4 °C using 10 mM Pipes, pH 5.6, as the eluant. Only the portion of the elution pattern including the bidentate isomers is shown (the first part of the elution profile in each case looks like that in Figure 2).

$Cr(NH_3)_4ATP$. Unlike $Cr(H_2O)_4ATP$ [but similar to Co-$(NH_3)_4ATP$], $Cr(NH_3)_4ATP$ showed no separation of bidentate isomers on CHpA columns. The elliptical properties of the right-hand (Δ) isomer were determined by incubating a 5.5-mL solution 2.2 mM in $Cr(NH_3)_4ATP$, 90 mM in glucose, 28 μM in hexokinase, and 55 mM in Pipes, pH 5.9, at room temperature for 7 h and then recording the CD spectrum in a 10-cm cell. During this period the color of the reaction mixture changed from rose to lavender, and the molar ellipticity increased from 0 to +45 at 590 nm, to −100 at 520 nm, and to +35 at 410 nm. Although the λ_{max} positions were shifted relative to those of Δ $Cr(H_2O)_4ATP$, the basic CD spectrum was the same.

$Cr(NH_3)_4ADP$ and $Cr(H_2O)_4ADP$. When $Cr(NH_3)_4ADP$ was chromatographed on a CHpA column, it was separated into two peaks, as was observed for $Co(NH_3)_4ADP$ by Cornelius & Cleland (1978). Both isomers show a visible spectrum with peaks at 388 (ϵ = 28) and 524 nm (ϵ = 38). The CD spectrum of the first isomer eluted has negative ellipticity at 510 nm ([θ] = −170) and positive ellipticity at 580 and 390 nm ([θ] = +23 and +38). The CD spectrum of the second isomer was nearly a mirror image of that of the first isomer

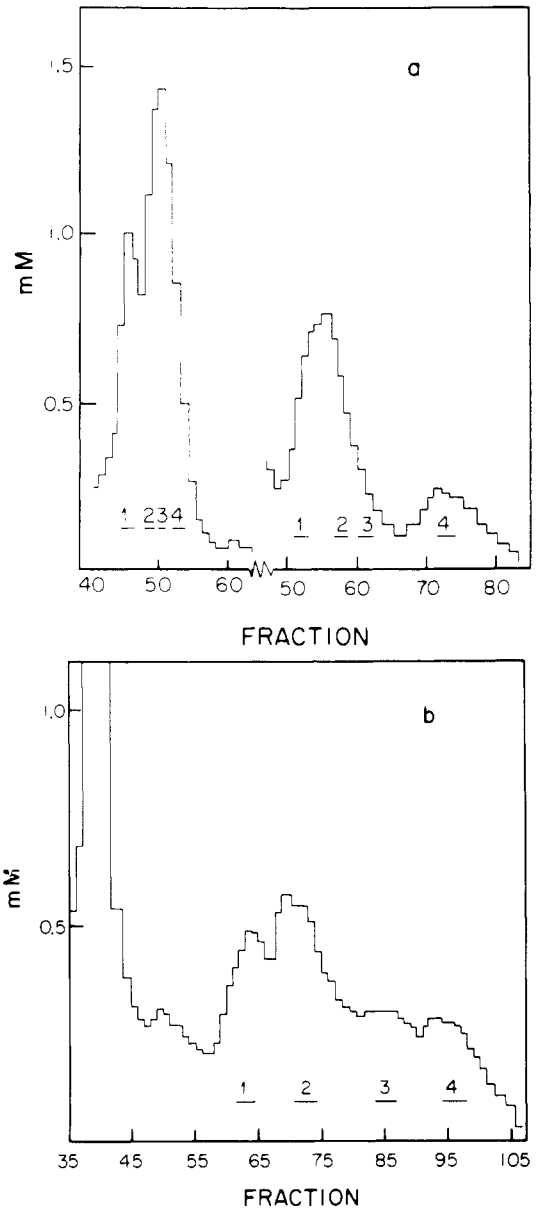


FIGURE 6: Elution profiles from chromatography of reaction mixtures containing (a) (left) Cr(α,β-MeATP), (a) (right) Cr(β,γ-NHATP), or (b) Cr(ATPγS) on a 235 × 0.7 cm CHpA column at 4 °C with 10 mM Pipes, pH 5.6, as the eluant. Only the portion of the elution pattern containing the bidentate isomers is shown.

([θ] = +210 at 510 nm, −37 at 400 nm, and −6 at 580 nm).

When concentrated to above 10 mM at pH 5.6, Cr(H_2O)$_4$ADP rapidly produced a blue-green substance having limited water solubility. This reaction occurs readily with Cr(H_2O)$_4$ADP, which has no net charge at pH 5.6, but does not occur with Cr(H_2O)$_4$ATP (at least at these concentrations), which has a net negative charge at this pH. We conclude that some form of polymerization is occurring. The elution profile from chromatography of a bidentate CrADP mixture concentrated sufficiently to contain this material is shown in Figure 7. It had a Cr/adenine ratio of 2:3, while the two CrADP isomers labeled I and II had a ratio of 1:1. The visible CD spectrum of the first polymer peak has maximum ellipticities (based on adenine) of +1000 at 450 nm and −1000 at 550 nm, while the second polymer peak shows a mirror image CD spectrum. The visible spectra of CrADP isomers I and II are the same (peaks at 426 nm, ϵ = 23, and 600 nm, ϵ = 21), and their UV CD spectra show peaks at 257 nm with [θ] = −4000 and −3100 for isomers I and II. The

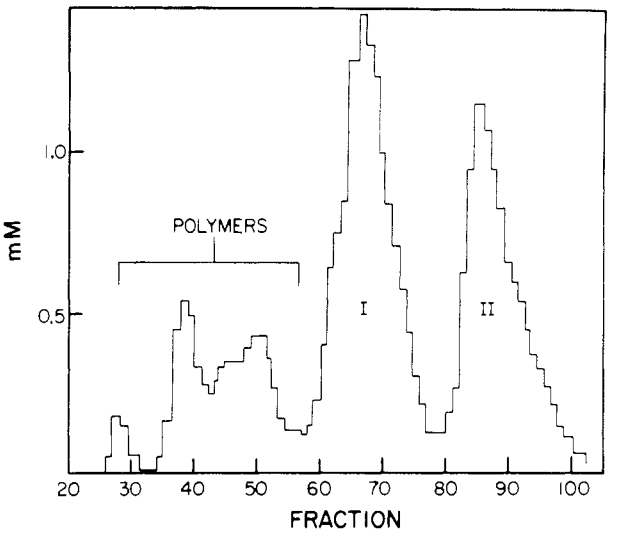


FIGURE 7: Elution profile from chromatography of a reaction mixture containing Cr(H_2O)$_4$ADP on a 235 × 0.7 cm CHpA column at 4 °C with 10 mM Pipes, pH 5.6, as the eluant.

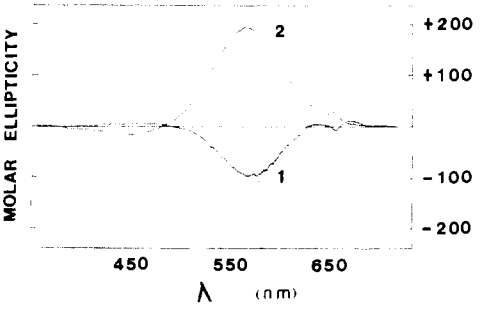


FIGURE 8: CD spectra of bidentate CrADP isomers in 10 mM Pipes, pH 5.6, at 25 °C. The spectra are numbered in the order in which the isomers are eluted from the CHpA column (see Figure 7).

visible CD spectra are shown in Figure 8 and give unequal peaks at 580 nm (−100 and +195 for isomers I and II). Like the Cr(NH_3)$_4$ADP isomers, and similar to the Co(NH_3)$_4$ADP isomers separated by Cornelius & Cleland (1978), the first Cr(H_2O)$_4$ADP isomer eluted from the column gave a CD spectrum dominated by negative ellipticity, and the second isomer gave a spectrum with largely positive ellipticity. However, unlike the two Cr(NH_3)$_4$ADP isomers, the two Cr(H_2O)$_4$ADP column peaks were not homogeneous. The CD spectrum of the fractions comprising the front edge of the isomer I peak indicated contamination with the polymeric substance described above. The CD spectra of the front and back halves of the isomer II peak were similar except that the spectrum of the front half showed maximum ellipticity at 570 nm, while that of the back half had a maximum at 580 nm. As described in Dunaway-Mariano & Cleland (1980), the K_{is} values for bidentate CrADP isomers I and II and for the polymeric substance indicated in Figure 7 as competitive inhibitors vs. MgADP of creatine kinase were determined. These values ranged from 117 to 262 μM for the polymeric substance, compared to 7 and 150 μm for bidentate isomers I and II.

Discussion

Preparation of Chromium(III) Complexes of ATP and ATP Analogues. Bidentate CrATP can be selectively prepared in good yield by heating a solution 10 mM each in Na_2ATP and $CrCl_3$ at 80 °C for 10 min, but up to 6% hydrolysis to ADP may occur (DePamphilis & Cleland, 1973). In the present study a milder procedure involving titration of the same reaction solution to pH 5.7 at 4 °C was developed for the

Table I: Properties of Bidentate Tetraaquochromium(III) Complexes of ATP and ATP Analogues

complex	isomer[a]	K_{is} vs. MgATP of hexokinase (μM)	[Θ] (deg cm²/dmol) at visible CD maxima 575 nm	640 nm
CrATP	1	0.029 ± 0.003	−1000	+430
	2	0.18 ± 0.01	+1000	−310
	3	0.30 ± 0.03	+550	−450
	4	0.029 ± 0.002	−550	+580
Cr(8-BrATP)	1	0.010 ± 0.001	−950	+560
	2	0.048 ± 0.005	+650	−330
	3	0.049 ± 0.004	+410	−353
	4	0.012 ± 0.001	−650	+490
Cr(2′-dATP)	1[d]	3.4 ± 0.2	−575	+260
	2[d]	6.0 ± 0.5	+480	−220
	3[d]	4.9 ± 0.3	+400	−250
	4[d]	6.0 ± 0.6	−375	+300
Cr(2′,3′-didATP)	1[d]	5.9 ± 0.3	+370	−180
	2[d]	10.3 ± 0.9	−260	+175
	3[d]	17 ± 4	−235	+220
	4[d]	15 ± 2	+260	−200
Cr(α,β-MeATP)	1		−1600	+320
	2[d]	66 ± 4[b]	+1000	−180
	3[d]		+1000	−125
	4[d]		−850	+290
Cr(β,γ-MeATP)	1[d]	400 ± 100	−540	+426
	2[d]	360 ± 20	+743	−571
	3[d]	260 ± 10	+1354	−800
	4	210 ± 20	−400	+460
Cr(β,γ-NHATP)	1[d]	2.9 ± 0.1	−820	+460
	2[d]	13.8 ± 0.5	+950	−720
	3[d]	10.0 ± 0.3	+1050	−770
	4	4.8 ± 0.2	−900	+750
Cr(ATPγS)	1	0.63 ± 0.02[c]	−330	+175
	2	0.216 ± 0.007[c]	+390	−99
	3	5.01 ± 0.09	+340	−117
	4	3.45 ± 0.09	−310	+155

[a] Numbered in order of elution from CHpA columns. [b] As a mixture of all four isomers. [c] See footnote 3 in the text. [d] Because complete separation of these isomers was not achieved (see Figures 5 and 6), they are probably not pure, and the K_{is} values and ellipticities of the pure isomers may differ somewhat from the values given here.

preparation of chromium(III) complexes of heat-labile nucleotides; the yield for this reaction averages 60% and no hydrolysis to ADP occurs. Thus, Cr(2′-dATP) is readily prepared by this method, while Janson & Cleland (1974) obtained only the chromium complex of deoxyribose 5′-tripolyphosphate by heating dATP and $Cr(ClO_4)_3$ at 80 °C. The reaction is base catalyzed and presumably involves deprotonation of coordinated water to induce ligand exchange. Chloride leaves the coordination sphere and is replaced by γ- and β-phosphate oxygens of ATP. The resulting bidentate CrATP complex is stable to ionization at this pH, and thus no further conversion to tridentate CrATP takes place. In a similar reaction, monodentate CrATP is converted into bidentate CrATP is 90% yield by raising the pH of the solution to 5.5–6.

By reaction of the nucleotide with chromium chloride at pH 5.6–5.9, bidentate Cr(ATP), Cr(ATPγS), Cr(β,γ-NHATP), Cr(β,γ-MeATP), Cr(α,β-MeATP), Cr(8-BrATP), Cr(2′-dATP), and Cr(2′,3′-didATP) complexes were prepared in yields ranging from 35 to 73%. Purification of the reaction mixtures was carried out on CHpA columns at 4 °C as shown in Figures 2, 5, and 6, and the column fractions were analyzed by spectrophotometric and/or spectropolarimetric and enzymatic techniques. In each case, the first material eluted is thought to be polymer, the second, free nucleotide, and the remaining material, the bidentate chromium nucleotide. It is clear from the elution profiles of CrATP (Figure 2), Cr(8-BrATP) (Figure 5a), and Cr(ATPγS) (Figure 6b) that each complex exists as four bidentate isomers. While the bidentate isomers of Cr(2′-dATP), Cr(2′,3′-didATP), Cr(α,β-MeATP), Cr(β,γ-MeATP), and Cr(β,γ-NHATP) are not as well separated by the CHpA column, the CD spectra of the column fractions (Table I) do demonstrate that each of these bidentate complexes is composed of four separable isomers.

Tridentate CrATP is formed readily from bidentate CrATP only by acid catalysis, while formation of monodentate CrATP or conversion of mono- to bidentate CrATP is not acid catalyzed. Presumably, protonation of a coordinated water leads to its expulsion, and insertion of the α-phosphate can then take place. In $Cr(H_2O)^{3+}$ ion, or in monodentate CrATP, the three or two positive charges on the complex may be sufficient to prevent a similar acid-catalyzed mechanism. One might predict that tridentate CrATP could be formed by base-catalyzed expulsion of water after ionization of a coordinated water in analogy to the mechanism for formation of mono- and bidentate CrATP, but since the pK of coordinated water in bidentate CrATP lies above 7 and hydrolysis of ATP to ADP and P_i and decomposition of the complex to free ATP and a polymeric hydroxide are competing reactions above pH 7, this is not a practical method of synthesis. Tridentate CrATP is readily purified by CHpA chromatography, but a clear picture of its isomeric content is not yet available (Cleland & Mildvan, 1979).

Stability of Bidentate CrATP Isomers. The stability of the bidentate CrATP isomers was examined in order to define conditions under which biochemical studies could be carried out. Below pH 6 the isomers are reasonably stable. For example, at pH 5.6 and 25 °C the half-life for conformational equilibration is 32 h while the half-life for screw sense equilibration is 10 days. Above pH 6 epimerization and hydrolysis of the bidentate isomers become quite rapid (see Figure 4). Mechanistically, the epimerization process appears quite complex, and from the pH profile in Figure 4 it appears that three to four hydroxide ions participate in the isomerization.

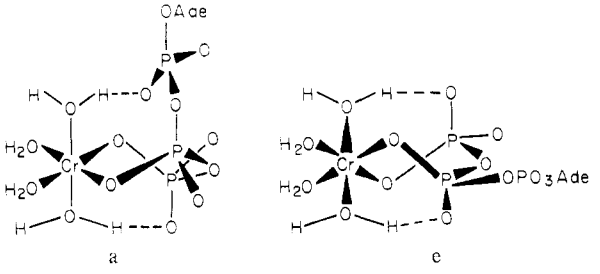
Properties of Chromium Complexes of ATP Analogues. The bidentate isomers of Cr(ATP analogue) complexes gave visible absorption spectra which were indistinguishable from those of the bidentate CrATP isomers and visible CD spectra which were quite similar to those of the CrATP isomers. On the other hand, their effectiveness as inhibitors of hexokinase is significantly less than that of the corresponding CrATP isomers, except for Cr(8-BrATP) (see Table I). Thus, substitution of the γ-phosphate oxygen, the β,γ-phosphate bridge oxygen, or the α,β-phosphate bridge oxygen with either a CH_2, NH, or S does apparently affect the electronic nature and/or geometry of the chelate ring but not so dramatically as to produce large changes in the spectral properties of the complex. Alteration of the ribose ring structure lowers affinity for hexokinase, but the 8-bromo substitution of the adenine ring enhances affinity, as previously noted by Danenberg & Cleland (1975).

Nature of the Four Bidentate Isomers. The interesting feature of the bidentate tetraaquochromium(III) ATP complexes is that they are each composed of four isomers, while β,γ-bidentate $Co(NH_3)_4ATP$ exists as two diastereomers, the left- (Λ) and right-hand (Δ) screw sense isomers differing in

configuration at the β-phosphorus (Cornelius & Cleland, 1978). Isomers 1 and 4 of bidentate CrATP gave CD spectra with negative ellipticity at 575 nm, while isomers 2 and 3 showed positive ellipticity at 575 nm. The CD spectra of isomers 1 and 2 were close to mirror images, as were those of isomers 3 and 4. On the basis of previous CD studies with $Co(NH_3)_4ATP$ isomers, it was suspected that isomers 1 and 4 were of a single screw sense, and isomers 2 and 3 had the opposite screw sense. This was confirmed by studies with yeast hexokinase, which uses the left-hand screw sense isomer of $Co(NH_3)_4ATP$ (Cornelius & Cleland, 1978). Since isomers 1 and 4 have substrate activity [see Dunaway-Mariano & Cleland (1980)], they are Λ isomers, and isomers 2 and 3 are Δ.

What then is the reason for there being two Λ and two Δ isomers? Since the ratio of isomers is not concentration dependent under conditions which allow isomer interconversion, we discard the possibility that dimers or other polymeric species are involved. Since all four isomers have the same pK for the adenine ring as ATP and Cr(8-BrATP), where N-7 is sterically blocked, shows four isomers, we can rule out coordination of the adenine ring as a cause for isomerization. Likewise, the presence of four isomers of Cr(2′-dATP) and Cr(2′,3′-didATP) rules out coordination of the ribose hydroxyls as the cause. Coordination of the second β-phosphate oxygen would give a β,β,γ-tridentate chelate that is symmetric and would not give the observed CD spectra. Coordination of the α-phosphate would give α,β,γ-tridentate CrATP which has a different CD spectrum, as described by Cleland & Mildvan (1979). Lastly, coordination of a second γ-phosphate oxygen would prevent the γ-phosphate from becoming protonated at low pH, and the compound would not be adsorbed on Dowex-50-H^+.

We thus feel we can rule out structural isomerization as the cause for four β,γ-bidentate CrATP isomers and are forced to conclude that isomers 1 and 2 are chelate ring conformers of isomers 4 and 3. The most probable ring geometry is the twist boat in which the phosphates are maximally staggered, and this conformation was observed in the crystal structure of $Co(NH_3)_4PPP$ (Merritt et al., 1978). There are two twist boat ring conformations which may be assumed by the two bidentate CrATP screw sense isomers. As shown below for the Λ isomers, one conformation places the AMP in the pseudoaxial position (a) and the other places it in the pseudoequatorial position (e). $Co(NH_3)_4PPP$ in the crystalline state is in the pseudoaxial conformation, stabilized by intramolecular hydrogen bonding between ammonia ligands and the pseudoaxial γ-phosphate oxygen and possibly the β,γ-phosphate bridge oxygen. Previous studies (Cornelius et al., 1977) suggest that the rate of conformational isomerization in $Co(NH_3)_4ATP$ is fast relative to the NMR time scale. In comparison to the NH_3 ligands of the cobalt complex, the H_2O ligands of the $Cr(H_2O)_4ATP$ complex are significantly more acidic and therefore capable of forming much stronger hydrogen bonds. The observed stability of the $Cr(H_2O)_4ATP$ conformers thus derives from strong intramolecular hydrogen bonds between one water ligand and the pseudoaxial oxygen on the γ-phosphate (as is shown for both a and e) and possibly another water ligand and either the β-phosphate oxygen (for the pseudoequatorial isomer e) or an α-phosphate oxygen (for the pseudoaxial isomer a).



a e

The first line of evidence for intramolecular hydrogen bonding to water ligands of the bidentate CrATP complex comes from studies in which significant differences in the paramagnetic effects on water proton relaxation were observed between the bidentate and tridentate isomers (Dunaway-Mariano et al., 1979). While for the tridentate isomers the relaxation rate is predominantly in the fast exchange region, the relaxation rate of the bidentate isomers is partially exchange limited. The proton escape rate from the coordination sphere of the tridentate CrATP isomers is approximately 1 order of magnitude greater than that of the bidentate isomers, while the number of exchangeable inner sphere water protons on chromium should be eight for the bidentate and six for the tridentate isomers. The lower escape rate from the bidentate isomers suggests that a portion of the water ligands of the bidentate complex are in fact restricted in their interaction with the water solvent. Intramolecular hydrogen bonding between the polyphosphate chain and these coordinated waters thus can account for the observations and supports the assignment of the bidentate isomers as chelate ring conformers.

If one oxygen is replaced by sulfur on the γ-phosphate of ATP and if the γ-phosphate coordinates to chromium only through the oxygen, as suggested by the spectral properties of Cr(ATPγS) and in analogy with the structure of Mg(ATPγS) (Jaffe & Cohn, 1978), the γ-phosphate as well as the β one becomes chiral, and four structural diastereomers should exist for Cr(ATPγS). If each of these formed two ring conformers, there would be 8 isolatable isomers. However, since the stability of the ring conformers probably derives largely from the hydrogen bond to the γ-phosphate oxygen in pseudoaxial position, replacement of oxygen on the γ-phosphate with sulfur, which is a very poor hydrogen-bond acceptor, should lead to only one stable ring conformer for each structural isomer with oxygen in the pseudoaxial and sulfur in the pseudoequatorial position on the γ-phosphate. Thus, we see only four isomers of Cr(ATPγS) (Figure 6b), and each of these should be a single ring conformer.

Interestingly, Cr(ATPγS) has little or no substrate activity with hexokinase,[3] although Mg(ATPγS) is a slow substrate. It is tempting to postulate that substrate activity requires hydrogen bonding of the noncoordinated oxygens on the γ-phosphate to positively charged groups on the protein and thus that the active isomer of Mg(ATPγS) is the one where the sulfur is chelated to Mg. The low proportion of this isomer present in solution would explain the low activity seen with Mg(ATPγS), as well as the absence of activity with Cr(ATPγS) where the inert coordination bonds would not allow formation of more such isomer after any small amount present did react.

While similar experiments to those with ATPγS have not yet been carried out with ATPβS or ATPαS, the isomers of Cr(α,β-MeATP) have been examined. The X-ray structure of bidentate $Co(NH_3)_4PPP$ (Merritt et al., 1978) indicates that in the crystalline state, hydrogen bonding occurs between the α,β-phosphate bridge oxygen and an ammonia ligand. Models

[3] Cr(ATPγS) isomers 1 and 2 are contaminated by CrATP isomer 4. The substrate activity of the Cr(ATPγS) isomer 1 and 2 preparations corresponds to 6–8% contamination by CrATP. This amount of CrATP isomer 4 would account for a reduction in the observed K_{is} from 2 to 0.3 μM.

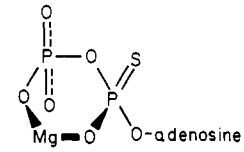
show, however, that the more electron-rich, free α-phosphate oxygens can also assume proper contact distance for hydrogen bonding. Thus, it was of interest to determine whether the α,β-phosphate bridge oxygen might participate in the hydrogen bonding in CrATP. The elution profile of bidentate Cr(α,β-MeATP) (Figure 6a) and the CD spectra and hexokinase inhibition data (Table I) measured for the column fractions indicate that bidentate Cr(α,β-MeATP), like bidentate CrATP, exists as four diastereomers having approximately equal stability. These data suggest that the α,β-bridge oxygen does not hydrogen bond to coordinated water in the pseudoaxial CrATP isomers.

A different approach taken to examine the geometries of the proposed bidentate CrATP conformational isomers was to measure the relative rates at which the conformers convert to tridentate CrATP. The α-phosphate of the pseudoaxial conformer is directed over the chromium center, while the α-phosphate of the pseudoequatorial one is directed away from chromium. Thus, prior to α-phosphate insertion the pseudoequatorial conformer should first undergo isomerizaton to a more reactive conformation. If this isomerization is much slower than conversion of the pseudoaxial isomer to tridentate CrATP, a large difference between the reaction rates of the two conformers having the same β-phosphorus configuration should be observed. Unfortunately, the strongly acidic conditions necessary for conversion of the bidentate isomers to the tridentate ones catalyze interconversion between the bidentate conformers, so that the difference in the measured reaction rates of the two Λ isomers was small, and CrATP isomer 1 formed tridentate CrATP only twice as fast as isomer 4. While these data suggest that isomer 1 is the pseudoaxial one, no firm assignment can be made, and work is continuing toward identification of the conformers.

Screw Sense of ADP Complexes with Cr(III) and Co(III). When one considers the CD spectra of various bidentate ATP complexes, it is clear that the maximum ellipticities of the chromium complexes are opposite in sign to that of the corresponding cobalt complex. Thus, Cornelius & Cleland (1978) showed that Λ $Co(NH_3)_4ATP$ has a positive CD peak at 560 nm, while the Δ isomer has a negative one. In the present study, Λ $Cr(H_2O)_4ATP$ showed a negative peak at 575 nm, and Λ $Cr(NH_3)_4ATP$ had a negative peak at 520 nm, while the Δ isomers of both compounds had positive peaks at their respective wavelengths.

A similar attempt was made to relate the α-phosphorus epimers of $Co(NH_3)_4ADP$, $Cr(NH_3)_4ADP$, and $Cr(H_2O)_4ADP$. All three complexes cleanly separated on CHpA columns into two diastereomers, labeled I and II in order of elution. While in the case of $Co(NH_3)_4ADP$ and $Cr(NH_3)_4ADP$ there was no indication of stable chelate ring conformers, the heterogeneity of the isomer II peak from the chromatography of $Cr(H_2O)_4ADP$ suggests that this complex might exist as four rather than two stable isomers. Interestingly, isomer I of each of three ADP complexes has negative maximum ellipticity, while isomer II has a positive peak. Thus, either the elution order of the α-phosphorus epimers of $Co(NH_3)_4ADP$ from the CHpA column is reversed from that of the chromium complexes or changing the metal does not change the sign of the CD peak of the ADP complexes as it does with the ATP complexes. In Dunaway-Mariano & Cleland (1980), we report that the $Cr(H_2O)_4ADP$ isomers are tight inhibitors of creatine kinase with the K_{is} of isomer I 21-fold smaller than that of isomer II. Similarly, the K_{is} values of isomer I of $Co(NH_3)_4ADP$ and $Cr(NH_3)_4ADP$ were at least three- and fivefold smaller than the K_{is} values for isomer II. Assuming that the observed binding selectivity is based on screw sense, then isomer I of each of the three ADP complexes has the same configuration at the α-phosphate, and the order of elution from CHpA columns is determined by screw sense and does not depend on the nature of the metal or the degree of ammonia substitution.

P. M. J. Burgers and F. Eckstein (personal communication) have recently discovered that creatine kinase uses ATPαS isomer B 5 times faster than isomer A when Mg^{2+} is the activator but uses isomer A 5000 times better than isomer B when Cd^{2+} was used. This Mg–Cd reversal of specificity, first used by Jaffe & Cohn (1978), proves that the metal is coordinated to the phosphate during the reaction, since Mg^{2+} preferentially coordinates to oxygen and Cd^{2+} preferentially coordinates to sulfur. Since ATPαS isomer B is *R* (Burgers & Eckstein, 1978), it is the Δ isomer of MgADPαS which is the reactive species:



Since isomer I of CrADP is more tightly bound than isomer II, we tentatively conclude that chromium or cobalt ADP complexes with negative ellipticity are Δ and those with positive ellipticity are Λ. $Co(NH_3)_4ATP$ and $Co(NH_3)_4PPP$ also show this pattern, and chromium ATP complexes are thus the only apparent exception to the rule. However, if, instead of the peak at 575 nm in CrATP, one considers the sign of the longest wavelength peak in the CD spectrum [640 nm in CrATP; 590 nm in $Cr(NH_3)_4ATP$], which is not present in the spectra of cobalt(III) complexes or in complexes of either metal with ADP, the rule holds for chromium ATP complexes as well.

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