Structural Preferences for the Binding of Chromium Nucleotides by Beef Heart Mitochondrial ATPase*

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The mono- and bidentate forms of adenosine 5'-diphosphate, chromium (III) salt (CrADP) were separated using Sephadex G-10 column chromatography. The isomeric purity of the two forms was monitored using high voltage electrophoresis and column chromatography. The same techniques were employed to assess the purity of the mono-, bi-, and tridentate forms of adenosine 5'-triphosphate, chromium (III) salt (CrATP).

Distinct differences in the interaction of beef heart mitochondrial ATPase with the various isomers of chromium nucleotides were seen in kinetic studies. Monodentate CrADP was a competitive inhibitor of the ATP hydrolysis activity of both purified ATPase and submitochondrial particles. However, when ATPase activity was examined, noncompetitive inhibition was observed. The bidentate isomer of CrADP did not affect ATPase activity. Enzymatic synthesis of the transition state analog of ATP synthesis and hydrolysis, P:\CrADP occurred exclusively with the monodentate isomer of CrADP. It was also found that only the mono- and tridentate forms of CrATP were potent inhibitors of ATP hydrolysis by beef heart mitochondrial ATPase. These results are discussed in terms of possible ATP synthesis and hydrolysis mechanisms.

The naturally occurring substrate utilized by beef heart mitochondrial ATPase is ATP in the presence of magnesium ion. Formation of a MgATP complex is required prior to catalytic activity (1). Several possible substrate geometries exist since one, two, or three of the phosphate groups could potentially coordinate to the metal. The particular form of the MgATP complex used by F1, is, however, unknown. Magnesium exchanges oxygen ligands so rapidly that it is difficult to study the role of the coordinated metal ion in the catalytic mechanism of ATP synthesis or hydrolysis. This experimental difficulty, however, is not shared by Cr(III)-nucleotide complexes. Cr(III) has an exchange rate with oxygen ligands so much slower than magnesium that it forms an essentially stable analog of the metal-nucleotide complex (2). Therefore, the Cr(III) nucleotides can conceivably be used in the study of the metal-complexed nucleotide as it interacts with the enzyme active center.

The effectiveness and suitability of chromium nucleotides as probes to examine enzyme kinetic mechanisms is well established (2-5). Cleland's laboratory has tested chromium nucleotides and chromium phosphates with several kinases. The enzymes displayed similar specificity for the base, but showed different specificities towards the various geometrical and optical isomers of the chromium nucleotides (6, 7). By characterizing those isomers of the chromium nucleotides that were the most effective enzyme inhibitors, Cornelius and Cleland (6) and Dunaway-Mariano and Cleland (7, 8) were able to deduce preferred substrate and product conformations for several kinases. Although it has been established that the Mg-nucleotide complex is the active substrate form for F1, activity, nothing is known concerning the conformation of metal-nucleotide complex bound to the enzyme.

The preparation of chromium nucleotides involves heating a chromium salt with the desired ligand followed by purification and concentration on an ion-exchange resin. According to the published procedure, the length of the heating period determines which isomer predominates. Unfortunately, this synthesis produces a significant amount of contamination by the alternate isomer which is not completely separated during the published purification step utilizing ion exchange chromatography (2, 3, 8).

Currently, a procedure is available to separate the various isomers of CrADP and CrATP using a cyclohexylamyllose gel (6, 8). However, the preparation of this gel is costly and tedious. Because of this problem, we have devised an alternate isolation procedure for these nucleotides that utilized commercially available Sephadex. This method of separating and monitoring the geometrical isomers of CrADP or CrATP presented is comparatively easy, rapid, and inexpensive. However, it does not resolve optical isomers.

Kinetic evidence is presented which shows monodentate CrADP is a more potent inhibitor of the hydrolysis reaction of both membrane-bound ATPase and soluble F1, from beef heart mitochondria. This specificity was unchanged whether ATP or IPT was used as substrate. In addition, synthesis of P:\CrADP by F1, occurs only with monodentate CrADP. A binding preference for the mono- and tridentate forms of CrATP is also demonstrated.

MATERIALS AND METHODS

Beef heart mitochondrial ATPase (F1) was isolated by the method of Sitts, Jeng, and Bar. Submitochondrial particles were prepared according to Kfelch and Pardee (19). Protein concentrations were measured by the Lowry procedure (20).

* Portions of this paper (including "Materials and Methods," Figs. 1 to 3, and Tables I-III) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-1710, cite authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
and is therefore designated monodentate CrADP. The relative process (bidentate CrADP) did not. Although each process product which effectively inhibited yeast hexokinase (monodentate form (which might be expected to be less tightly adsorbed and thus have a larger Stokes radius) is reasonable. The adsorption phenomena can be used to resolve geometrical isomers of Cr-nucleotides was achieved by using a long column containing Sephadex G-10 as the starting material. The original isomeric assignment of the mono- and bidentate isomers of Cr-nucleotides was performed by high voltage electrophoresis (8). The trailing of the peak was attributed to nonspecific adsorption of CrATP to the Sephadex since separation using BioGel resulted in a symmetrical peak (9).

The bidentate form of CrADP eluted before the monodentate form of CrADP produced by a 1-min heating of reactants yielded nonodentate CrADP reached 95% faster than the monodentate form. The bidentate form of CrADP occurred. Conversion to the bidentate form reached 95% conversion to bidentate CrADP occurred in a week.

The bidentate form of CrADP appeared to be thermodynamically more stable under conditions of pH 7.0 and low ionic strength (0.1 M Na-acetate). The monodentate form of CrADP is much less stable under these conditions. Nearly 100% conversion to the bidentate form had occurred (3-4 weeks) and remained stable for weeks.

Syntheses of CrADP isomers.

All three chelate isomers of CrATP were prepared by the procedures of Danenberg and Cleland (10). Either party was mentioned using a column (100 X 2.5 cm) of Sephadex G-10 and high voltage electrophoresis as indicated in Figure 1.

RESULTS

The original isomeric assignment of the mono- and bidentate forms of CrADP were from Cleland’s observation that CrADP produced by a 1-min heating of reactants yielded product which effectively inhibited yeast hexokinase (monodentate CrADP), whereas the product of a 10-min heating process (bidentate CrADP) did not. Although each process produced a mixture of the two forms, the synthesis and purification procedures were adequate for some experimental purposes.

When CrATP was first synthesized, attempts to separate unreacted ATP from CrATP were done using Sephadex G-10. As reported earlier (2), the CrATP peak was asymmetrical. This trailing of the peak was attributed to nonspecific adsorption of CrATP to the Sephadex since separation using BioGel resulted in a symmetrical peak (2). We have found that the adsorption phenomena can be used to resolve geometrical isomers of CrADP and CrATP. The resolution and separation of mono- and bidentate isomers of Cr-nucleotides was achieved by using a long column containing Sephadex G-10 as described under “Materials and Methods.”

Fig. 1 illustrates the adsorption elution profile obtained from a mixture of the two chelate isomers of CrADP. Heating a solution of CrCl3 and ADP for 1 min at 80 °C yielded predominantly a substance eluting from the column corresponding to the second peak in the elution profile. This compound inhibited yeast hexokinase (Fig. 2) at pH 5.8 (7) and is therefore designated monodentate CrADP. The relative area of the first peak in Fig. 1 increased as the heating time lengthened. The compound contained in this first peak did not inhibit hexokinase (Fig. 2) under the prescribed assay conditions and is therefore designated bidentate CrADP. The fact that CrAMP elutes from the column with one symmetrical peak (data not shown) supports the notion that these two peaks represent chelation isomers of CrADP, and not polymerization products.

That the bidentate form of CrADP eluted before the monodentate form (which might be expected to be less tightly coiled and thus have a larger Stokes radius) is reasonable. Due to the large degree of cross-linkage of Sephadex G-10, the
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Effect of separation based on the Stokes radius is outweighed by adsorption and charge effects when the ionic strength is less than 0.02 (10). Using 50 mM acetic acid as a solvent permits the small number of carboxyl groups on the Sephadex beads to interact effectively with the positively charged chromium nucleotides. Thus, the more positively charged monodentate CrADP elutes after the bidentate form. The designation of the isomeric assignment corresponds to previously published assignments (3, 7) and has been verified using high-voltage electrophoresis at pH 2.5. As would be expected under these conditions, the monodentate form of CrADP moved further towards the cathode (Table I).

Sephadex chromatography was also used for batch separation of concentrated mixtures of the CrADP isomers. Fractions containing individual isomers were recombined and concentrated on a small column containing Dowex 50. Fractions containing appreciable amounts of both isomers were combined, concentrated, and chromatographed using Sephadex G-10. A major advantage of the Sephadex G-10 chromatography procedure was the ability to monitor the isomeric purity of the CrADP solutions obtained by different synthetic or storage procedures.

Monodentate CrADP, prepared by a 1-min heating period, synthesized, and purified on Dowex 50 in 2 h, could be obtained in 95% or better (according to relative areas under the absorbance elution profiles) purity (see Fig. 1). After 3 days at pH 5.2, however, a significant portion had converted to bidentate CrADP (Fig. 1). Monodentate CrADP used in kinetic studies was either freshly synthesized or freshly separated by Sephadex chromatography. The conversion of monodentate CrADP could be retarded by storage in 0.1 M sodium acetate, pH 2.5-3.6.

Bidentate CrADP was obtained in high isomeric purity by conversion of the monodentate isomer. The bidentate isomer was thermodynamically favored under mildly acidic conditions at low ionic strength. Fig. 3 illustrated this conversion. Monodentate CrADP was synthesized and eluted from a column containing Dowex 50 with 0.1 M MES/KOH, pH 5.6. This peak was collected and a small aliquot was chromatographed using Sephadex G-10 yielding the absorbance elution profile seen in Fig. 3A. This shows primarily the monodentate form of CrADP. Fig. 3B is the absorbance profile 1 day, C is 6 days, and D is 14 days after synthesis and elution from Dowex 50. After 3 to 4 weeks, total conversion to bidentate CrADP was achieved.

Isolated beef heart mitochondrial ATPase catalyzes the hydrolysis of ATP to ADP and inorganic phosphate. The hydrolysis of ATP exhibits negative cooperativity in the absence of activating anions (11). The presence of the inhibitor CrADP amplified the cooperativity effect illustrated in Fig. 4A. The curvature of the lines made it difficult, if not impossible, to obtain a value for $K_i$. Nearly equal concentrations of mono- and bidentate forms of CrADP were used as inhibitors. The slight inhibition by bidentate CrADP may be partly due to a small amount of contaminating monodenate isomer. Monodentate CrADP was clearly a more potent inhibitor (Fig. 4A). In the presence of an activating anion (20 mM bicarbonate), the cooperativity effect was abolished (Fig. 4B), but again, the monodentate isomer effected greater competitive inhibition. The small amount of inhibition by bidentate CrADP relative to the control indicated this isomer does not bind to F₃ very well. As is shown in Fig. 5, submitochondrial particle ATPase was inhibited by monodentate CrADP and displayed negative cooperative inhibition with ATP as a substrate. Bidentate CrADP was not an effective inhibitor in submitochondrial particles (data not shown).

F₁-ATPase also hydrolyzed ITP but displayed no cooperativity effect. Again, bidentate CrADP (Fig. 6) was much less effective as an inhibitor than the monodentate isomer when ITP was the substrate. The bidentate isomer was such a poor inhibitor (Fig. 6) no attempt was made to determine a $K_i$. 

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**Figure 2:** Conversion of mono- to bidentate CrADP. CrADP stored in 0.1 M MES pH 5.6 showed no appreciable conversion. This preparation was then proportionately split into the monodentate form (Panel A) and the bidentate form (Panel B). The relative absorbance at 265 nm decreased to elution with 0.1 M MES/KOH pH 5.6 and continued to do so. The complete conversion to the bidentate form occurs after 3-4 weeks.
value. In Fig. 7A, micromolar concentrations of monodentate CrADP produced classical noncompetitive inhibition. A replot of slope and intercept versus inhibitor concentration (Fig. 7B) both showed a $K_I$ of 220 $\mu M$, indicating monodentate CrADP reacted equally with both the free enzyme and the enzyme-substrate complex.

$F_1$, produces a transition state analog of ATP synthesis, $P_i$-CrADP, from CrADP and inorganic phosphate. This same compound is also produced by hydrolysis of bidentate CrATP (9). As is illustrated in Fig. 8 by incorporation of free radioactive $P_i$, $^{32}P_i$-CrADP was produced only from monodentate CrADP. Although not shown, substitution of bovine serum albumin for $F_1$, omission of $F_1$, and substitution of MgADP for CrADP in the incubation mixture all gave essentially the same results as bidentate CrADP which failed to incorporate free $^{32}P_i$.

The three geometrical forms (mono-, bi-, and tridentate) of CrATP were resolved using a longer column containing Sephadex G-10 (2 meters) than that needed for the diphosphate compounds. Again, elution profiles were correlated to synthesis procedures and high voltage electrophoresis. The high voltage conditions employed clearly distinguish between the mono- and bidentate forms of CrATP (Table I). Although the high voltage conditions reported did not clearly resolve the bi- and tridentate forms of CrATP, these were readily observed by monitoring the absorbance elution profile from Sephadex G-10 chromatography. This column, however, takes $\sim 18$ h for complete sample elution and was used only to check isomer purity. The tridentate form eluted first, the bidentate form second, and monodentate CrATP eluted last (data not shown). These assignments agree with hexokinase inhibition behavior (3). It was observed that each preparation of monodentate CrATP and tridentate CrATP contained significant quantities of bidentate CrATP. Bidentate CrATP was prepared with essentially no contamination by the other isomers. Kinetic studies were done using all three forms, and linear competitive inhibition resulted. Inhibition due to contaminating bidentate CrATP in the mono- and tridentate CrATP preparations was accounted for using the equation for two mutually exclusive competitive inhibitors (12), as described in Table II. The $K_I$ values for the three forms of CrATP as ATP hydrolysis inhibitors are shown in Table II.

![Figure 5: Monodentate CrADP inhibition of submitochondrial ATPase activity.](image)

![Figure 6: Inhibition of F1-ATPase activity by CrADP.](image)

![Figure 7A: F1-ATPase activity inhibition by mono-CrADP.](image)

![Figure 7B: Monodentate CrADP is a noncompetitive inhibitor of ATP hydrolysis.](image)

![Figure 8: $P_i$-CrADP formation only by mono-CrADP.](image)
The 2-m Sephadex chromatography, however, permits quantitative analysis of the relative percentages of mono- and bidentate CrADP. It was analyzed by the relative UV absorbance intensities of the spots in many as 10 samples can be simultaneously and quantitatively purpose. Using the high voltage electrophoresis system, as the absorbance profile of the eluant from the Sephadex C-10 chromatography did not distinguish ADP from bidentate-MgADP decomposition could also be concurrently detected. The absorbance profile of the eluant from the Sephadex C-10 chromatography did not distinguish ADP from bidentate-MgADP were noted that fractions of mono-CrADP eluted from Dowex solutions or be able to correct for contaminants. Secondly, the method for routine analysis of these compounds.

**DISCUSSION**

Differences in the kinetic behavior of enzymes toward the various substrate geometrical isomers lead to two important consequences. First, it becomes imperative to work with pure solutions or be able to correct for contaminants. Secondly, the geometrical specificity can provide important clues to the natural substrate geometry. However, due to multiple synthesis products and isomer interconversion, it was essential for further research to be able to check rapidly the purity of the mono-, bi-, and tridentate forms of chromium nucleotides. Two independent techniques have been employed for this purpose. Using the high voltage electrophoresis system, as many as 10 samples can be simultaneously and quantitatively analyzed by the relative UV absorbance intensities of the spots in ~2 h. At pH 2.5, monodentate CrADP has one more positive charge than the bidentate form, allowing the two to be clearly distinguishable. Any free ADP resulting from CrADP decomposition could also be concurrently detected. The absorbance profile of the eluant from the Sephadex G-10 chromatography did not distinguish ADP from bidentate-CrADP. However, this second method provided a more quantitative analysis of the relative percentages of the two geometrical forms of CrADP. The chromatography was also useful for batch separation of the two CrADP forms from concentrated solutions containing a mixture of isomers.

It was observed that the synthesis method of heating CrCl₃ and ADP for 1 min (3) did not always produce the same relative percentages of mono- and bidentate CrADP. It was also noted that fractions of mono-CrADP eluted from Dowex 50 converted to the bidentate form at different rates, depending upon the pH of the resultant solution. Further work is needed to determine the optimum conditions for stabilization of mono-CrADP.

Similar variations occurred in per cent yield of desired isomers obtained from CrATP synthesis attempts (8). Although we were able to purify bidentate CrATP, the monoaand tridentate preparations contained varying amounts of contaminating bidentate CrATP after elution from Dowex. The 2-m Sephadex chromatography, however, permits quantification of the two components so that the necessary concentration corrections can be made.

The marked preference of both isolated F₁ and submitochondrial particle-bound F₁ for monodentate CrATP binding is significant. The tight binding of monodentate CrADP and synthesis of Cr₃-CrATP from only the monodentate isomer indicated that the natural substrate is probably monodentate MgADP. The ratio of monodentate MgADP to bidentate MgADP in solution has been estimated to be 1:27 (7). Thus, it is possible that when ATP synthesis occurs, the least abundant form of ADP is utilized and the remaining bidentate ADP will not cause substrate inhibition. It is also interesting to note that both mono- and tridentate CrATP bind tightly to F₁, but the bidentate form (again the most abundant) does not again, extrapolating to the native system where the primary function of ATPase is to synthesize ATP, the probable product released is bidentate MgATP. As the relatively high Kᵢ of bi-CrATP indicates, the bidentate MgATP released would not be likely to rebind to F₁, preventing possible product inhibition.

It has been previously proposed that mitochondrial ATPase contains two types of nucleotide binding sites (4, 11). A regulatory site is thought to bind specifically adenine nucleotides. The catalytic site, however, is nonspecific and hydrolyzes other nucleoside triphosphates as well. Both mono-CrADP and all three forms of CrATP were competitive inhibitors of ATP hydrolysis (Figs. 4 and 5) (Table II). In Figs. 6 and 7, however, noncompetitive inhibition of ITP hydrolysis by mono-CrADP was observed. This is consistent with the kinetic studies presented earlier (4, 11). It would appear that ITP cannot compete with CrATP for binding to the F₁ regulatory site, resulting in the observed noncompetitive inhibition. These data are difficult to reconcile with the proposed alternating site models proposed by Boyer and his colleagues (13–15).

It must be reemphasized that the methods of nucleotide separation and identification discussed here do not resolve optical isomers. Optical resolution can be achieved (6, 8), but is not required for all types of mechanistic studies. Currently, other groups are using chromium nucleotides prepared by the heating procedures and comparing the products by inhibition of yeast hexokinase (16, 17). We feel we have found a simpler method for routine analysis of these compounds.

If the binding of chromium nucleotides to F₁ is a reasonable model system to extrapolate to the naturally occurring Mg-nucleotide system, an interesting correlation results. Monodentate MgATP might be the ATP hydrolysis substrate and bidentate MgATP could be the ATP synthesis product. Since the equilibrium favors bidentate MgATP formation free in solution, the process of ATP synthesis would not be inhibited by the MgATP produced in the mitochondria. This could explain how ATP synthesis can occur in the presence of a substantial mitochondrial phosphate potential.

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**REFERENCES**