Proton and Phosphorus-31 NMR Study of the Dependence of Diadenosine Tetraphosphate Conformation on Metal Ions*

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Nancy H. Kolodny and Laura J. Collins

From the Department of Chemistry, Wellesley College, Wellesley, Massachusetts 02181 and the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Adenosine 5′-tetraphospho-5′-adenosine (ApA) plays a role in cellular metabolism in a wide variety of organisms. Because the divalent cations Mg2+ and Zn2+ are involved in the synthesis and function of ApA, the effect of divalent cations on the dinucleotide’s conformation is of interest. 1H and 31P chemical shift experiments were carried out as a function of Mg2+ concentration and pH. We propose that Mg2+ stabilizes the unusual ring-stacked conformation of ApA at pH > 2 by interacting with the β-phosphates. To further probe conformational effects, stable complexes of ApA with Co3+ were studied using 31P NMR. Co3+ forms two different bidentate complexes with ApA, independent of whether the other four octahedral coordination sites are occupied by ammonia or trimethylene-diamine. NMR results suggest that in one complex the Co3+ is coordinated to two β-phosphates and ring stacking is stabilized. In the other complex, Co3+ is coordinated to an α-phosphate and its neighboring β-phosphate and ring stacking is destabilized. These results further support the hypothesis that Mg2+ stabilizes the ring-stacked conformation by interacting symmetrically with the two β-phosphate groups.

To use NMR to probe the site(s) of metal ion interaction with the dinucleotide and the effects of this interaction on overall conformation, we have synthesized stable complexes of ApA with Co3+. Mg2+-nucleotide complexes are not amenable to such studies since ligand exchange is rapid. Octahedral complexes of Co3+ or Zn2+ with nucleotides, on the other hand, have dissociation times on the order of 10-6 s (4). The assumption that Co3+ can be used to study the behavior of Mg2+ is based on studies of Cr3+-ATP and Co3+-ATP complexes, in which the latter complexes were shown to specifically inhibit a number of enzymes which have Mg2+-ATP or other nucleotides as substrates (5). Furthermore, since the ionic radii of both Mg2+ and Co3+ are approximately 0.065 nm (6), the distance from the central metal ion to the ligand are very similar.

We have used 31P NMR to demonstrate coordination of the Co3+ to the dinucleotide and to identify which phosphate groups are bonding to the metal ion. When trivalent cobalt forms complexes with phosphate groups, it produces a decrease in the electron density around the phosphorus atom, thereby causing deshielding effects which shift the coordinated phosphate resonances downfield. Cornélis et al. (7) have reported downfield shifts of between 10 and 14 ppm for coordinated phosphate groups. 1H NMR chemical shifts of the adenine H-2 and H-8 resonances provided information concerning the conformation of the complexed dinucleotides.

**EXPERIMENTAL PROCEDURES**

ApA was purchased as the trilithium salt from Boehringer Mannheim, and 5′-AMP as the disodium salt from Sigma. Both were used without further purification. Solutions containing metal ions were prepared by mixing weighed quantities of the mono- or dinucleotide with solutions of MgCl2 or Co(NO3)2 of known concentration. Concentrations of initial metal chloride solutions were determined by titration with EDTA. Concentrations of mono- or dinucleotide solutions were determined by UV absorption spectroscopy (8). In order to minimize intermolecular nucleotide interactions, all solutions used for 1H and 31P NMR spectra were prepared so that the concentration of 5′-AMP or ApA was 0.008 M. Dilution beyond 0.008 M produced no changes in chemical shifts, suggesting that this concentration was low enough to avoid intermolecular interactions. An internal reference compound, tetramethylammonium chloride (5 = 3.180 ppm downfield from tetramethylsilane), was used at a concentration of 5 µM for all 1H spectra. 31P spectra were run with a reference solution of 8.5% D3PO4 in D2O in a concentric outer tube. Values are reported for pH although all solutions for NMR spectroscopy were prepared in D2O. Values were calculated by adding 0.4 to measured pH values.

Two ApA complexes were synthesized, one containing tetramethylammonium cobalt(III) and the other bis(trimethylammonium) cobalt(III). Starting materials, carbonatotetraammine cobalt(III) nitrate and carbonatobis(trimethylammonium) cobalt(III) chloride, were prepared as described elsewhere (8). The latter material was converted to the perchlorate salt prior to reaction with ApA.

Syntheses of the complexes were done using 25 or 50 mg of ApA.
The approach of Cornelius et al. (7) Co(NH3)2 ATP was followed, except that the dinucleotide reaction was run in D2O (99.8% D) instead of water. This was done so that the reaction mixture could be studied immediately by 1H NMR. The synthesis of trimethylenediamine Co(II) complexes with ATP has been described by Hediger and Milburn (9). We followed their approach with Ap,A instead of ATP, but again working at a 25-50 mg level.

1H NMR spectra were obtained at Wellesley College on a Varian CFT-20 spectrometer modified for protons at 80 MHz. Ninety-degree pulses were applied with no delay time. 31P NMR spectra were obtained at the NMR Resource at the National Magnet Laboratory at Massachusetts Institute of Technology on a Bruker HFX-270 spectrometer operating at 109.3 MHz. Ninety-degree pulses were applied with a delay time of 1 s and broad-band proton decoupling. Temperature was maintained at 24 °C for 1H and 31P spectra.

RESULTS AND DISCUSSION

1H NMR studies (2) of the dependence of the chemical shifts of the Ap,A adenine ring protons on pH suggested that this molecule undergoes a conformational transition with increasing pH. Whereas the rings appear to be unstacked below pH 4, they pass through an intermediate stage that has been called "folded" (2) and finally become stacked above pH 6. 1H NMR results of chemical shift studies of the interaction of Ap,A with Mg2+ are plotted in Figs. 1 and 2 as Δδ, difference in chemical shift between 5'-AMP and Ap,A in the presence of the same [Mg2+]. The comparison with 5'-AMP is made to correct for the chemical shift changes caused by protonation of the adenine ring (pK 4-5) and possible binding of the Mg2+ with the adenine ring (3). The upfield shifts of adenine H-8 (and to a smaller extent, H-2) at pH values up to 7 in the presence of Mg2+ suggest that ring stacking is stabilized by this cation. Maximum upfield shifts are reached when about one Mg2+ ion per Ap,A is present in solution, leading us to postulate the existence of a 1:1 association, in agreement with Holler et al. (3). At pH 7, the H-8 and H-2 resonances remain relatively constant with increasing [Mg2+]. There is a possibility that the differences between Ap,A and 5'-AMP H-8 chemical shifts in the presence of Mg2+ could be due to this proton's proximity to different numbers of negatively charged phosphate moieties in the two species rather than to conformational changes. In order to probe this, we monitored the pH dependence of 5'-ADP and 5'-ATP as well as that of 5'-AMP and found no significant difference in H-8 chemical shifts.3 How the magnesium cation interacts with Ap,A is demonstrated by 31P NMR spectra. Fig. 3 is a graph of chemical shift (with respect to 8.5% D3PO4) for the α- and β-phosphate groups of Ap,A alone in solution and in the presence of several concentrations of MgCl2. First, Ap,A must be considered in the absence of Mg2+. As pH increases in the absence of Mg2+, the β-phosphate resonance undergoes significant upfield shifts, whereas the α-phosphate resonance undergoes negligible upfield shifts. The shift of the β-phosphate resonance must be caused by an effect other than deprotonation since deprotonation is known to cause downfield, rather than up-

3 C. Redfield, private communication.
field, shifts. Gorenstein (10) has shown that changes in O-P-O bond angles lead to upfield shifts on either side of a minimum at 107°. Thus, we conclude that the conformational change which accompanies ring stacking must be occurring (at least in part) about the P-O-P bond between the β-phosphates.

Additional evidence for this conclusion comes from an analysis of Fig. 4. This well-resolved 31P NMR spectrum of ApA at pH 7.0 was analyzed as an AA'XX' system, with the α-phosphates A and A' and the β-phosphates X and X'. Coupling constants are: J(AA') = 5.73 Hz, J(AX) = 15.36 Hz, J(AX') = 3.02 Hz, and J(XX') = 10.77 Hz. The J(XX') coupling is considerably lower than that reported for the middle phosphates in the tetrapolyphosphate ion (15.0 Hz) by Glonke et al. (11), whereas our J(AX) has a similar value to theirs. In ApA then, as compared with the tetrapolyphosphate ion, the β-phosphates experience a distortion of their O-P-O bond angle and thus a different spin-spin coupling. The cause of this distortion we ascribe to the stacking of the adenine rings.

Whereas it would be desirable to obtain J(XX') values for ApA when it is associated with Mg2+, our 31P NMR spectra of this system was not sufficiently well-resolved to allow calculation of coupling constants. The hypothesis derived from 1H NMR data is, however, well-supported by 31P NMR chemical shift results. In the presence of Mg2+ at concentration ratios ranging from 0.38 to 3.8, [Mg2+]/[ApA], the change in chemical shift of the β-phosphates is opposite to that in the absence of Mg2+. For all ratios, the chemical shift is downfield from that of the free dinucleotide at pH 5, 6, and 7 and upfield at lower pH values. Two factors could account for this. It could be postulated that Mg2+ prevents ApA from assuming its ring-stacked conformation at pH values above 5, but this is obviated by 1H NMR results described above, which clearly demonstrate that ring stacking is occurring. The other factor is the association of Mg2+ with the β-phosphates. Association of metal ions with phosphate groups causes significant downfield shifts (=10 ppm, see below). The comparatively small downfield shifts in the presence of Mg2+ suggest the formation of a rapidly dissociating complex between the cation and the β-phosphates of ApA.

At pH values between 2 and 5 in the presence of Mg2+, however, the β-phosphates experience relatively constant, small upfield shifts. This is consistent with 1H NMR results which suggest that Mg2+ enhances the population of a conformation in which there is ring stacking at all pH values above 2. Thus, the β-phosphate chemical shifts in this pH range represent a balance between the upfield shift that accompanies ring stacking and the downfield shift that accompanies binding to Mg2+.

The chemical shift for the α-phosphates is relatively insensitive to pH and Mg2+. The α-phosphates thus appear neither to bind to Mg2+ nor to experience changes in their O-P-O bond angles due to ring stacking.

To confirm our hypothesis that Mg2+ binds to ApA in a 1:1 ratio at the β-phosphates, we synthesized coordination complexes of ApA (and, for comparison, ATP) with Co2+ as described above. ATP formed complexes with both tetraammine cobalt(III) and trimethylenediamine cobalt(III). 31P NMR spectra (not shown) suggest that coordination took place at either the α- and β-phosphates or the β- and γ-phosphates for the trimethylenediamine cobalt(III) complex and at the β- and γ-phosphates only for the tetraammine cobalt(III) complex (8).

The 31P NMR spectra (Fig. 5) for Co(NH3)4.ApA and Cotn2.ApA indicate that in each case the cobalt ion has formed more than one type of complex with ApA. Six distinct resonances can be observed for the Cotn2 .ApA complexes (Fig. 5B). It is somewhat more difficult to identify six separate signals in the tetraammine complex spectrum (Fig. 5A) due to poorer resolution. However, none of the peaks appear at positions of uncomplexed ApA. In light of complexes observed between ATP and Co2+ (7), we have assigned two of the signals to a β-β complex, whereas the remaining four signals are assigned to an α-β complex, in which one of the α-phosphate groups and its neighboring β-phosphate groups are not complexed. Table 1 presents the 31P chemical shifts for ApA, Co(NH3)4.ApA, and Cotn2.ApA. In Table II, differences in chemical shift for phosphates in uncomplexed ApA and in the cobalt complexes are shown. For each type of cobalt complex, shifts are reported for two differently coordinated ApAs, one in which an α-phosphate and its neighboring β-phosphate are associated with the central metal and one in which both β-phosphates are associated. Shifts for the complexed (c) and uncomplexed (u) phosphates are assigned, as well as for phosphates which are not complexed themselves but are adjacent to complexed phosphates (n).

The assignments of the six resonances in the 31P NMR spectra of Co(NH3)4.ApA and Cotn2.ApA can be best understood by referring to the following diagram (in which only those phosphate oxygens necessary to show binding sites are
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We assume that coordination of a phosphate group with Co$^{3+}$ affects the coordinated group resonance by shifting it down-field. At the same time, adjacent phosphate group resonances experience upfield shifts (7). Thus, in the $\alpha$-$\beta$ complex, both $\beta$-phosphate resonances should show downfield shifts, and both $\alpha$-phosphate resonances should show upfield shifts. If the complex is symmetrical, one resonance should be observed for the $\alpha$-phosphate and one for the $\beta$-phosphate. The $\alpha$-$\beta$ complex should exhibit four resonances, an $\alpha$-phosphate and a $\beta$-phosphate with large downfield shifts, a $\beta$-phosphate with an upfield shift, and an $\alpha$-phosphate with no shift due to complex formation.

Peaks a, c, d, and f in both spectra are assigned to the $\alpha$-$\beta$ complex. Peak a is the 8-ppm downfield shifted resonance of the coordinated $\alpha$-phosphate, peak c is the relatively unchanged resonance of the uncomplexed $\alpha$-phosphate, peak d is the 7-8-ppm downfield shifted resonance of the complexed $\beta$-phosphate, and peak f is the 1-2-ppm upfield shifted peak of its adjacent, uncomplexed $\beta$-phosphate. Peaks b and e are assigned to the $\beta$-$\alpha$ complex. Peak b is the 11-ppm downfield shifted resonance of the two coordinated $\beta$-phosphates. Peak d is the 3-ppm upfield shifted resonance of the two adjacent, uncoordinated $\alpha$-phosphates. No residual Ap$_4$A peaks are seen, suggesting that the complex formation reaction went to completion, yielding two products.

The identification of two products is confirmed by the $^1$H NMR spectrum which appears in Fig. 6. Changes in chemical shift of adenosine protons $H-8$ and $H-2$ between uncomplexed and complexed Ap$_4$A are in Table III. Two sets of $H-8$ and $H-2$ resonances are clearly visible. The downfield pair are

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### Table I

| $^3$P chemical shifts of Ap$_4$A, Co(NH$_3$)$_2$.Ap$_4$A, and Cotn$_2$.Ap$_4$A (ppm ±0.10) upfield from 8.5% D$_2$PO$_4$ at 24 °C | pH |
|---|---|
| Ap$_4$A | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 6.8 | 7.0 |
| $\alpha$ | 11.03 | 10.96 | 10.95 | 11.06 | 11.21 | 11.20 |
| $\beta$ | 21.91 | 21.65 | 21.63 | 22.26 | 22.97 | 22.98 |
| Co(NH$_3$)$_2$.Ap$_4$A $\alpha$-$\beta$ complex | 11.42 | 11.55 |
| $\alpha$ | 22.53 | 22.78 |
| $\beta$ | 1.55 | 1.52 |
| $\beta$ | 13.02 | 13.02 |
| Co(NH$_3$)$_2$.Ap$_4$A $\beta$-$\beta$ complex | 13.26 | 13.14 |
| $\beta$ | 11.13 | 11.17 |
| Cotn$_2$.Ap$_4$A $\alpha$-$\beta$ complex | 11.56 | 11.49 |
| $\alpha$ | 23.37 | 23.31 |
| $\beta$ | 2.72 | 2.81 |
| $\beta$ | 14.00 | 14.00 |
| Cotn$_2$.Ap$_4$A $\beta$-$\beta$ complex | 14.57 | 14.63 |
| $\beta$ | 11.08 | 11.10 |

* $u$, uncomplexed phosphate; c, complexed phosphate; n, uncomplexed phosphate adjacent to complexed phosphate.

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### Table II

| $^3$P chemical shifts (ppm, $\Delta\delta$(free Ap$_4$A − cobalt complex)) (±0.20 ppm) | pH |
|---|---|
| 4.0 | 5.0 | 6.8 |
| Co(NH$_3$)$_2$.Ap$_4$A $\alpha$-$\beta$ complex | +0.36 | +0.29 |
| $\alpha$ | +0.27 | -0.20 |
| $\beta$ | -9.51 | -9.68 |
| $\beta$ | -9.24 | -9.96 |
| Co(NH$_3$)$_2$.Ap$_4$A $\beta$-$\beta$ complex | +2.20 | +1.94 |
| $\alpha$ | -11.09 | -11.81 |
| Cotn$_2$.Ap$_4$A $\alpha$-$\beta$ complex | 0.03 | 0.14 |
| $\alpha$ | +0.63 | +0.55 |
| $\beta$ | +1.74 | 1.14 |
| $\beta$ | -8.21 | -7.85 |
| Cotn$_2$.Ap$_4$A $\beta$-$\beta$ complex | 0.03 | 0.15 |
| $\alpha$ | +3.64 | 3.86 |
| $\beta$ | -10.55 | -10.81 |

* $u$, uncomplexed phosphate; c, complexed phosphate; n, uncomplexed phosphate adjacent to complexed phosphate.

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### Table III

| $^1$H chemical shifts (ppm, $\Delta\delta$(free Ap$_4$A − cobalt complex)) (±0.01 ppm) | pH |
|---|---|
| 3.8 | 5.0 | 6.2 | 6.6 |
| Co(NH$_3$)$_2$.Ap$_4$A $\alpha$-$\beta$ complex | H-8 | -0.11 |
| H-2 | -0.12 |
| Co(NH$_3$)$_2$.Ap$_4$A $\beta$-$\beta$ complex | H-8 | +0.03 |
| H-2 | +0.03 |
| Cotn$_2$.Ap$_4$A $\alpha$-$\beta$ complex | H-8 | +0.08 | +0.09 | -0.10 |
| H-2 | +0.02 | +0.01 | -0.17 |
| Cotn$_2$.Ap$_4$A $\beta$-$\beta$ complex | H-8 | +0.32 | +0.19 |
| H-2 | +0.10 | +0.15 |
assigned to the $\alpha\cdot\beta$ complex and the upfield pair to the $\beta\cdot\beta$ complex. At pH 6.6, for example, one Co(NH$_3$)$_2$·Ap$_4$A complex has its peaks approximately 0.10 ppm downfield from the comparable peaks at that pH in Ap$_4$A. Since free Ap$_4$A has been shown to be in a ring-stacked conformation at pH 6.6, formation of this complex must destabilize the stacking of the adenine rings. The other pair of H-8 and H-2 peaks are slightly upfield of those of free Ap$_4$A at this pH, indicating a slight increase in ring stacking. This complex is likely to be the $\beta\cdot\beta$ complex, whereas the destacked, asymmetric complex is the $\alpha\cdot\beta$.

The pH behavior of the Cotn$_2$·Ap$_4$A complexes further confirms the existence of two different species. Free Ap$_4$A assumes an unusual folded conformation in the pH range 3–5 (2). This was shown by downfield shifts of H-8 and H-2 as compared with 5'-AMP. In both cobalt complexes, these downfield shifts are absent. In one case, which we assign to the $\beta\cdot\beta$ complex, considerable upfield shifts are seen. This is consistent with our observations for the effect of Mg$^{2+}$ on Ap$_4$A, where ring stacking and its resulting upfield shifts are promoted at pH values above 2. The other complex, $\alpha\cdot\beta$, seems to leave the adenosine groups in a position in which they do not experience any intramolecular interactions and thus behave like 5'-AMP.

Final confirmation of the assignment of the NMR results to the two different isomers of Co(NH$_3$)$_2$·Ap$_4$A and Cotn$_2$·Ap$_4$A awaits successful separation and perhaps x-ray crystallographic analysis. Attempts were made to accomplish separation using high pressure liquid chromatography, but the procedure led to complete degradation of the complexes. Nevertheless, it is clear from the results presented above that two products are formed and that the assignments made are consistent with the hypotheses that Mg$^{2+}$ forms $\beta\cdot\beta$ complexes. The biological significance of Mg$^{2+}$ stabilization of the unusual ring-stacked conformation of Ap$_4$A despite changes in pH may be that the cation enables the nucleotide to maintain this conformation in situations in which its local cellular environment experiences pH fluctuations. Free Ap$_4$A does not maintain such conformational stability.

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