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Investigation of the Structure, Protonation, and Reactivity of Tetraammine(imidodiphosphato)cobalt(III), a Substrate for Potato Apyrase†

Tuli P. Haromy, Wilson B. Knight, Debra Dunaway-Mariano,* and M. Sundaralingam*

ABSTRACT: The present study was undertaken to evaluate the suitability of polyphosphoroamidates as probes of proton transfer steps in enzyme-catalyzed phosphoryl transfer reactions. An inert coordination complex, P^1,P^2-bidentate $Co(NH_3)_4PNP$, was prepared and crystallized in both its neutral and fully protonated (hydrochloride) forms for X-ray analysis. Crystals of the neutral form $[Co(NH_3)_4HP_2O_6NH{\cdot}3H_2O]$ are monoclinic with space group $P2_1/c$ ($Z = 4$) and cell dimensions $a = 8.582$ (6) Å, $b = 16.128$ (6) Å, $c = 10.730$ (4) Å, and $\beta = 126.23$ (5)°. The hydrochloride $[Co(NH_3)_4H_3P_2O_6NH^{2+}{\cdot}2Cl^-]$ crystallizes in the orthorhombic space group $P2_12_12_1$ ($Z = 4$) with cell dimensions $a = 7.088$ (2) Å, $b = 10.345$ (2) Å, and $c = 17.589$ (3) Å. Both structures were solved by the heavy atom technique and refined to R indexes of 0.055 and 0.057 for the neutral and hydrochloride forms, respectively. The six-membered chelate ring in the neutral form assumes the chair conformation while the hydrochloride is found in a conformation intermediate between the boat and twist-boat conformations. Addition of HCl to the neutral complex fails to protonate the imido nitrogen atom to the quaternary charged state, rather the phosphate oxygen atoms of the acidified complex accept the two protons donated by the chloride ions. This accounts for the observed stability of $Co(NH_3)_4PNP$ in 6 M HCl. The potato apyrase reaction was examined with $Co(NH_3)_4PNP$, $Co(NH_3)_4PP$, imidodiphosphate (PNP), and pyrophosphate (PP) as substrates. Both PP and PNP were hydrolyzed to orthophosphate, the $Co(NH_3)_4PP$ complex was hydrolyzed to $Co(NH_3)_4(P)_2$, and $Co(NH_3)_4PNP$ was converted to $Co(NH_3)_4PP$ via a $Co(NH_3)_4(PO_4)(PO_3NH_3)$ intermediate. The observations suggest that apyrase transfers a proton to the nitrogen atom of both PNP and $Co(NH_3)_4PNP$ during the course of catalysis.

Structural analogues of ATP that are resistant to enzyme action can be of great utility in the study of catalytic mechanisms for ATP-dependent enzymes. In one such analogue, adenyl-5′-yl imidodiphosphate (AMP-PNP),[1] the β,γ-bridge oxygen atom of ATP is substituted with a nitrogen atom. AMP-PNP is principally used to study ATP binding to enzymes which are unable to cleave the P–N linkage (Yount, 1975). Solution studies of imidodiphosphate (PNP) itself have shown that hydrolytic cleavage of the P–N bond does not occur readily unless the nitrogen atom is fully protonated (Quimby et al., 1960). Thus, it appears that only those enzymes which can transfer a proton to the imido nitrogen atom of AMP-PNP during catalysis should be capable of catalyzing γ-phosphoryl transfer from AMP-PNP while those enzymes which either do not normally protonate the β,γ-bridge oxygen atom of ATP or are unable to protonate the bridge nitrogen atom of

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706 (T.P.H. and M.S.), and the Department of Chemistry, University of Maryland, College Park, Maryland 20742 (W.B.K. and D.D.-M.). *Received April 18, 1983.* This work was supported in part by National Institutes of Health Grant GM-17378 to M.S. and by National Institutes of Health Grant GM-28688 to D.D.-M. D.D.-M. acknowledges a research career development award from the National Institutes of Health (ES-00111). Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this work.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; AMP-PNP, adenyl-5′-yl imidodiphosphate; PNP, imidodiphosphate; PN, phosphoroamidate; PP, pyrophosphate; P, orthophosphate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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AMP-PNP should fail to react with AMP-PNP. Two enzymes known to catalyze the hydrolysis of AMP-PNP are *Escherichia coli* alkaline phosphatase (Yount et al., 1971a,b) and sarcoplasmic reticulum ATPase (Taylor, 1981).

AMP-PNP, as well as other P–N containing substrate analogues, might be useful as probes of proton transfer steps in enzymic phosphoryl transfer reactions as long as the P–N analogue is isosteric with the normal substrate. X-ray studies of tetrasodium imidodiphosphate (Larsen & Willett, 1974) and tetrasodium pyrophosphate (MacArthur & Beevers, 1957) have shown that the P–N–P and P–O–P bond lengths and angles are almost identical. Since most enzymes do not recognize the free polyphosphate substrates but instead require the metal complexed substrates, the initial phases of this work were devoted to evaluating the structural differences which might exist between the Mg^{2+} complexes of polyphosphates and polyphosphoroamidates.

The structures of kinetically labile metal–polyphosphate complexes (such as those formed by Mg^{2+}) have in recent years been examined by the study of exchange inert complexes prepared from Co(III) or Cr(III) which have a similar coordination geometry as Mg^{2+} (Cleland & Mildvan, 1979). All known $M^{III}ATP$ and $M^{III}PP$ complexes which act as enzyme substrates have six-membered bidentate chelate rings such as found in β,γ-bidentate MATP or in P^1,P^2-bidentate MPP. The X-ray structure of P^1,P^2-bidentate $Co(NH_3)_4PP$ (CoPP) has been previously reported (Merritt & Sundaralingam, 1980), and X-ray structures of the corresponding $Co(NH_3)_4PNP$ complex (CoPNP) are now reported both in the neutral and fully protonated forms. The substrate activities of these two complexes with potato apyrase are also reported. The structural and enzymatic data presented in this paper suggest that the P^1,P^2-bidentate MgPNP chelate ring can meet the stereoelectronic requirements for proton transfer to the nitrogen atom in the apyrase active site.

Experimental Procedures

Materials

Potato apyrase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, phosphorylase *a*, PNP, and all nucleotides and buffers used in this study were purchased from Sigma Chemical Co. The potato apyrase obtained from Sigma Chemical Co. was judged to be approximately 85% pure by SDS gel electrophoresis (7.5% acrylamide). $[^{32}P]Na_4PP$ was purchased from New England Nuclear. $Co(NH_3)_4PP$ was prepared according to Cornelius et al. (1977).

Methods

General. Liquid scintillation counting was carried out by using Beckman Ready-Solv MP scintillation fluid and an Intertechnique scintillation counter. UV/visible absorption spectra were recorded with a Perkin-Elmer 552 spectrophotometer while kinetic studies were carried out with a Gilford 250 spectrophotometer. NMR spectra (40.5 MHz) were recorded with a Varian XL-100 or IBM-200 spectrometer (25 °C; 10% D_2O solvent and 0.1 M D_3PO_4 external standard). ^{31}P chemical shifts downfield from D_3PO_4 are negative in sign and those upfield are positive in sign.

Preparation of P^1,P^2-Bidentate $Co(NH_3)_4PNP$. $[Co(NH_3)_4CO_3]NO_3$ (Schlessinger, 1960) was dissolved in 1 M HCl, diluted to a final concentration of 20 mM, and added to an equal volume of 20 mM imidodiphosphate. The resulting solution was adjusted to pH 6 with KOH and heated at 80 °C for 10 min. After cooling, the reaction mixture was adjusted to pH 2 with HCl and loaded on a Dowex 50-X2 (H^+) column. The column was washed with deionized water at 4 °C until two rose-colored bands separated. The resin containing the upper band was removed, and the $Co(NH_3)_4PNP$ was eluted from the lower band with 0.3 M aniline. The eluate was immediately washed with diethyl ether (yield of 15%).

The isoionic $Co(NH_3)_4PNP$ crystallizes when stored at a concentration of 20 mM at 4 °C. The visible spectrum of this complex is characterized by maxima at 525 and 375 nm. The ^{31}P NMR spectrum of $Co(NH_3)_4PNP$ at isoionic pH (4.1) is characterized by a singlet at −10.4 ppm. At pH 6.6 the resonance is observed at −11.5 ppm while in 6 M HCl the resonance occurs at −9.3 ppm. Crystals of the hydrochloride were prepared by dissolving the neutral CoPNP crystals in 0.2 M HCl and allowing the solution to slowly evaporate to dryness.

Crystal Structure Analyses. Data for both the neutral and acid forms were collected on an Enraf-Nonius CAD4 diffractometer using Cu Kα radiation (λ = 1.5418 Å). For the neutral form, 1229 unique intensities were measured up to a 2θ limit of 100°, of which 937 intensities with $I/\sigma(I) > 1.5$ were used for the structure analysis. Of a total of 1582 unique intensities collected for the hydrochloride, 1423 intensities with $I/\sigma(I) > 2$ were used for the structure analysis. The data were corrected for crystal decay, Lorentz, polarization, and absorption effects.

Both structures were solved by the heavy-atom technique and refined by the full matrix least-squares method by using anisotropic temperature factors and a counting statistics weighting scheme with the weights proportional to $1/[\sigma^2F + (0.01F_o)^2]$ (for additional information, see paragraph at end of paper regarding supplementary material).

In the neutral form, 19 hydrogen atoms were located by difference Fourier syntheses and refined with a fixed temperature factor of 4.0 Å^2. The only missing hydrogen atom H2(W3) was geometrically fixed. In the acid form, all 16 hydrogen atoms were located in difference Fourier syntheses. All protons except H(O2P2) were refined with fixed temperature factors of 4.0 Å^2. H(O2P2), which was found in a region of diffuse density, was fixed midway between O2(P2) and O3(P1). The final R index ($\sum||F_o| - |F_c||/\sum|F_o|$) was 0.055 and 0.057 for the neutral and acid forms, respectively.

Since the space group of the acid form is noncentrosymmetric, the correct enantiomeric conformation in the crystal was determined at this point by refining the enantiomeric structure with all coordinates inverted. (Although interconversion of the enantiomorphs could occur in solution, only one isomer is present in any given crystal.) The correct conformation displayed a significantly (0.02) better R index due to the effect of anomalous scattering confirming that the original coordinates were correct.

Apyrase Reactions. Apyrase concentrations were determined by assuming $A_{280}^{0.1\%} = 1.0$ (Valenzuela et al., 1972). Control reactions, not containing enzyme, were run along with all of the following reactions (25 °C).

(A) PP and PNP. The time course for the apyrase-catalyzed hydrolysis of PP and PNP was examined by using ^{31}P NMR spectroscopy. Reaction solutions (4 mL) contained 100 mM K^+Pipes (pH 6.7), 10% D_2O, 0.3 mM EDTA, 1.7 mM $CaCl_2$, 0.4 μM apyrase, and either 10 mM PP or 10 mM PNP. Both the PP and PNP reactions yielded orthophosphate as product. Repeating the PNP reaction with 11 μM apyrase also yielded only orthophosphate as product.

(B) $Co(NH_3)_4PP$. The time course for apyrase-catalyzed hydrolysis of $Co(NH_3)_4PP$ was examined over an 18-h period by using ^{31}P NMR spectroscopy. The 4-mL reaction solution contained 100 mM K^+Pipes (pH 6.7), 4.5 mM $CaCl_2$, 5.8 mM $Co(NH_3)_4PP$, and 67 μM apyrase. The ^{31}P NMR spectrum

measured after the reaction had been incubated for 2 h was characterized by a singlet at −4.4 [85%; $Co(NH_3)_4PP$] and at −11.1 ppm [15%; $Co(NH_3)_4(P)_2$]. At 5 h a new resonance at −0.9 ppm (P, 6%) was apparent in addition to those of $Co(NH_3)_4PP$ (67%) and $Co(NH_3)_4(P)_2$ (27%). At 12 h the P resonance accounted for 12% of the total material present, $Co(NH_3)_4PP$, 54%, and $Co(NH_3)_4(P)_2$, 31%. In addition, two new resonances were apparent, one at −13 ppm [$Co(NH_3)_4(H_2O)(HPO_4)$, 2%] and one at −24.3 ppm [$Co(NH_3)_4(PO_4)$, 2%]. At 18 h the amounts of P, $Co(NH_3)_4PP$, $Co(NH_3)_4(P)_2$, $Co(NH_3)_4(H_2O)(HPO_4)$, and $Co(NH_3)_4(PO_3)$ present in the mixture were 20%, 44%, 24%, 5%, and 7%, respectively.

(*C*) *$Co(NH_3)_4(P)_2$*. A 2-mL solution of 0.52 mM $Co(NH_3)_4[^{32}P]PP$ (SA = 7.5×10^9 cpm/mmol), 3.1 mM $CaCl_2$, 50 mM K^+Pipes (pH 6.6), and 0.28 mM apyrase was incubated for 64 min and then dialyzed against water for 105 min. The solutions on either side of the dialysis membrane were sampled over a 48-h period. Each aliquot was loaded onto columns (made from disposable pipets) which contained 2 mL of Dowex 50-X2 (50–100 mesh, H^+) resin. Each column was washed with 10 mL of H_2O and then with 10 mL of 2 M HCl. The radioactivity (>95% recovery) in each wash was measured by using liquid scintillation counting techniques. The percent uncomplexed phosphate present in each sample was then evaluated from the ratio of the water wash cpm to the total cpm. The percent uncomplexed P was plotted as a function of incubation time. The plots were biphasic; in each case the release of the first P from $Co(NH_3)_4(P)_2$ was faster than that of the second P. The rates of P release were evaluated from the slopes of these plots.

(*D*) *$Co(NH_3)_4PNP$*. A 6-mL solution containing 50 mM K^+Pipes (pH 6.6), 3.0 mM $CaCl_2$, 0.3 mM EDTA, 4.5 mM $Co(NH_3)_4PNP$, and 0.14 mM apyrase was examined over a 13-h reaction period by using ^{31}P NMR techniques. The chemical shift of $Co(NH_3)_4PNP$ (−11.5 ppm) could not be fully resolved from that of $Co(NH_3)_4(P)_2$ (−11.2 ppm). At 1 h the percent composition was judged to be 77% $Co(NH_3)_4PNP$ [some of which may actually be $Co(NH_3)_4(P)_2$] and 23% $Co(NH_3)_4PP$ (−4.4 ppm). At 6.4 h the percent composition had changed to 35% and 65%. At 13 h the sum of $Co(NH_3)_4PNP$ and $Co(NH_3)_4(P)_2$ represented 30%, $Co(NH_3)_4PP$, 31%, P (−1.0 ppm), 31%, bidentate $Co(NH_3)_4(PO_4)$, 5%, and monodentate $Co(NH_3)_4(H_2O)(HPO_4)$, 5%. After a 13-h reaction period the solution was dialyzed for 3.5 h against H_2O at 4 °C. Three equivalents [to Co(III) and Ca^{2+}] of EDTA were added and the solution heated for 15 min at 80 °C. ^{31}P NMR analysis revealed that 13% of the mixture was $Co(NH_3)_4PP$ (−4.4 ppm), 72% P (−1.0 ppm), and 15% PP (+7.6 ppm). An additional equivalent of EDTA was added to the solution before heating it for 15 min at 80 °C. ^{31}P NMR analysis revealed that 7% of this mixture was $Co(NH_3)_4PP$, 73% P, and 20% PP. The assignment of the PP resonance was ensured by adding authentic PP to the sample and measuring the ^{31}P NMR spectrum a second time. In order to compare the relative rates of conversion of $Co(NH_3)_4PP$ to $Co(NH_3)_4(P)_2$ and $Co(NH_3)_4PNP$ to $Co(NH_3)_4PP$, the two reactions were carried out under identical conditions and allowed to proceed to 10% conversion. The turnover numbers of $Co(NH_3)PNP$ and $Co(NH_3)_4PP$ were calculated as 3.2 and 3.9 h^{-1}, respectively.

Results

Crystal Structures. Atomic coordinates for the structures are given in Table I and ORTEP drawings are presented in Figure 1. Bond lengths, bond angles, and ring torsion angles are given in Figure 2.

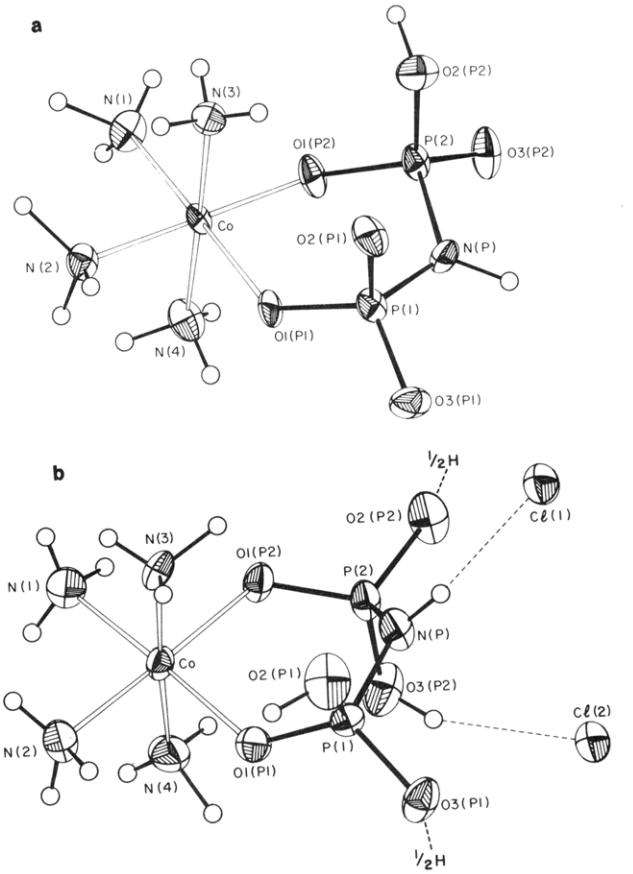


FIGURE 1: ORTEP (Johnson, 1976) drawings of neutral CoPNP (a) and of CoPNP dihydrochloride (b). Non-hydrogen atoms are represented by 50% probability ellipsoids, and hydrogen atoms are represented by spheres of arbitrary size. In the hydrochloride structure, O2(P2) and O3(P1) of neighboring molecules share one proton which is located midway between the oxygen atoms.

Chelate Ring Pucker. The chelate ring puckering can be desribed by three parameters: Q, θ, and ϕ (Cremer & Pople, 1975) where Q measures the amplitude of ring puckering, θ measures the degree of chair (θ = 0° or 180°) vs. boat (θ = 90°) character, and ϕ is the angle of pseudorotation between the various boat and twist–boat (TB) conformations (0° = boat, 30° = TB, 60° = boat, 90° = TB, ...). These parameters for the Co–O1(P1)–P(1)–(N or O)–P(2)–O1(P2) ring are as follows:

	Q (Å)	θ (deg)	ϕ (deg)	
CoPNP neutral	0.41	175	17	chair
CoPNP·2HCl	0.61	84	227	B/TB
CoPP	0.62	87	108	B/TB

The six-membered chelate ring of neutral CoPNP assumes a distorted chair conformation while a boat/twist–boat (B/TB) conformation is observed for the hydrochloride. All ring atoms except P(2) of the two structures can be nearly superimposed (maximum deviation of 0.13 Å) with a distance between P(2) atoms of 1.10 Å (Figure 3). An overlay of the neutral (chair) imidodiphosphate complex and the corresponding tetraammine(pyrophosphato)cobalt(III) complex, which assumes a B/TB conformation (Merritt & Sundaralingam, 1980), shows that five of the six ring atoms nearly overlap (maximum deviation of 0.07 Å) with the P(1) atoms displaced from each other by 1.01 Å (Figure 4). In the overlay, the equatorial O3(P1) atom of the imidophosphate complex is only 0.15 Å away from the equatorial O2(P1) atom of the pyrophosphate complex.

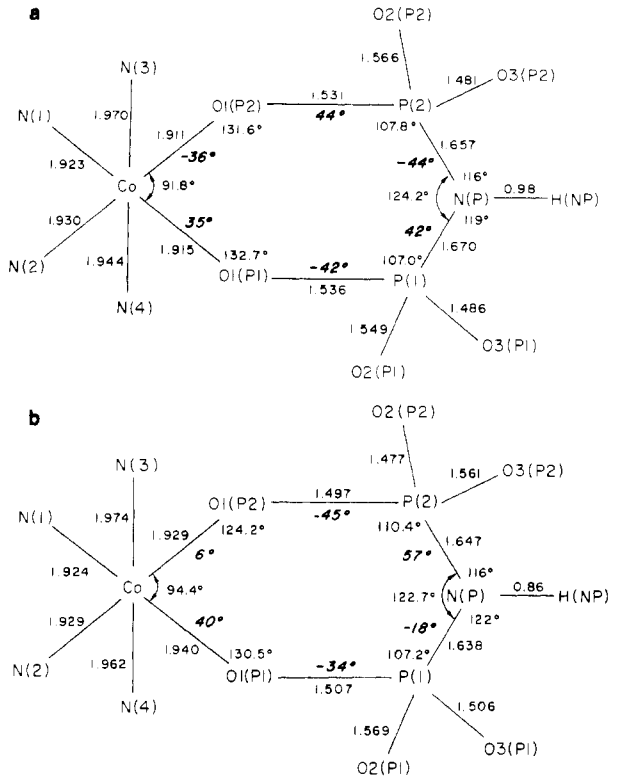


FIGURE 2: Bond lengths, ring bond angles, and torsion angles for neutral CoPNP (a) and the acid form of CoPNP (b). Torsion angles are shown in italics. For the neutral structure, estimated standard deviations are 0.007 Å for bond lengths, 0.5° for bond angles, and 0.7° for torsion angles. For the acid form the estimated standard deviations are 0.006 Å, 0.4°, and 0.6° for bond lengths, bond angles, and torsion angles, respectively.

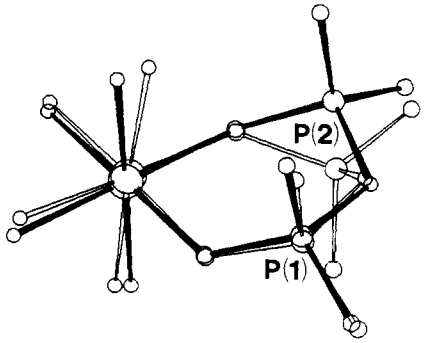


FIGURE 3: Overlay of the neutral imidodiphosphate complex (solid bonds) superimposed with the acid form (open bonds) showing near equivalence at all ring atoms except P(2).

Bond Lengths and Angles. The phosphate bond lengths differ significantly between the neutral and acid structures due to the degree of protonation. In the neutral structure the P–O bond lengths are within the expected ranges (Figure 2a) whereas most of the bond lengths in the hydrochloride structure display unusual characteristics (Figure 2b). In the acid form, the P–O bonds involved in metal ligation (1.507 and 1.497 Å) display a striking shortening of 0.03 Å compared to the neutral complex (1.536 and 1.531 Å). This shortening is compensated by a 0.02 Å lengthening of the Co–O coordinate bonds (1.940 and 1.929 Å) compared to the neutral form (1.915 and 1.911 Å). The P–N bonds (1.638 and 1.647 Å) also show a striking shortening of 0.02 Å compared to the neutral structure (1.670 and 1.657 Å). On the other hand, the P–O "double bonds" (1.506 and 1.477 Å) are in the normal range even though they share a proton in a very short hydrogen bond.

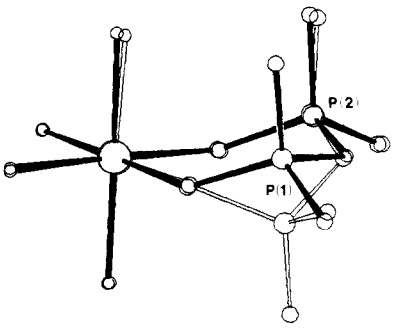


FIGURE 4: Overlay diagram of the (imidodiphosphato)cobalt complex (solid bonds) superimposed with the cobalt–pyrophosphate complex (open bonds) at all ring atoms except for P(1). Observe that the equatorial O3(P1) atom of the imidodiphosphate complex overlaps the O2(P1) atom of the pyrophosphate complex.

The ring bond angle at the cobalt atom of 94.4° for the acid form is significantly greater than the corresponding angle of 91.8° for the neutral form. On the other hand, the N(P) bond angle of 122.7° for the acid form is slightly less than the corresponding angle of 124.2° in the neutral form. The bond angles for the neutral form are symmetric with nearly equal ring angles at O1(P1) and O1(P2) as well as at P(1) and P(2) with the torsion angles being equal and opposite as expected for a chair structure. In contrast, the acid form has a significantly greater bond angle at O1(P1) (130.5°) than at O1(P2) (124.2°) and a somewhat greater bond angle at P(1) (107.2°) than at P(2) (105.0°).

A comparison of the CoPNP and CoPP crystal structures shows that the average P–N bond length in the neutral structure is about 0.05 Å longer than the average P to bridge oxygen bond length of 1.614 Å observed for the CoPP structure (Merritt & Sundaralingam, 1980). The P–N–P bond angle in the neutral structure is about 3° smaller than the corresponding P–O–P angles of 127.1°. In the uncomplexed imidodiphosphate crystal structure (Larsen & Willett, 1974), the P–N bond lengths are slightly longer (1.678 Å) and the P–N–P angle is wider (127.2°) since the phosphate groups are not restrained by the chelating metal.

Hydrogen Bonding. All hydrogen atoms in the neutral structure except H3(N3), H2(N4), H1(W1), and H2(W2) participate in hydrogen bonding (Table IIA). The imido proton on the bridge nitrogen atom is hydrogen bonded to the phosphate oxygen O3(P2) of an adjacent molecule: the N(P) to O3(P2) distance is 3.055 Å. The shortest hydrogen bond is O2(P2)–H(O2P2)···O2(P1) with a distance between heavy atoms of 2.458 Å. The acid form is characterized by just six strong hydrogen bonds (Table IIB). The imido proton H(NP) is hydrogen bonded to a Cl^- ion with an N(P) to Cl(1) distance of 3.196 Å. Cl(2) is hydrogen bonded to both O3(P2) and N(3). The strongest hydrogen bond in the acid structure is between O2(P2) and O3(P1) with a length of 2.416 Å which is 0.04 Å shorter than that found in the neutral complex. The proton shared by the double-bonded oxygen atoms is probably engaged in a tunneling effect (De la Vega, 1982).

Apyrase Reaction. Apyrase was found to catalyze the hydrolysis of PP (turnover number 70 min^{-1}) and of PNP (turnover number 19 min^{-1}). The phosphoroamidate found in the hydrolysis of PNP was taken directly to orthophosphate.[2] The relative rates of the apyrase-catalyzed hydrolysis of Co-

[2] The phosphoroamidate undergoes hydrolysis in aqueous solution at pH 6.7 at a rate (k = 0.5 h^{-1} at 38 °C) (Chanley & Feageson, 1963) much slower than we observe with apyrase, and therefore, we conclude that apyrase catalyzes this process.

Table I: Fractional Positional Parameters[a] for All Atoms of Tetraammine(imidodiphosphato)cobalt(III) Trihydrate and Tetraammine(imidodiphosphato)cobalt(III) Dihydrochloride

atom	x	y	z	B_{eq} or B (Å²)	atom	x	y	z	B_{eq} or B (Å²)
	Trihydrate Parameters					Dihydrochloride Parameters			
Co	1084 (2)	2607 (1)	4359 (2)	1.16 (5)	Co	7325 (2)	−154 (1)	−1400 (1)	1.66 (3)
P(1)	4087 (3)	1454 (2)	7170 (3)	1.61 (8)	P(1)	4820 (3)	304 (2)	44 (1)	1.81 (4)
P(2)	4048 (3)	1434 (2)	4411 (3)	1.66 (8)	P(2)	8757 (3)	−323 (2)	223 (1)	2.06 (5)
O1(P1)	2184 (7)	1939 (4)	6172 (6)	1.87 (21)	O1(P1)	5204 (7)	−332 (5)	−711 (3)	2.17 (12)
O2(P1)	5758 (7)	2080 (4)	8148 (6)	2.31 (21)	O2(P1)	4024 (9)	1707 (5)	−65 (3)	3.58 (17)
O3(P1)	4054 (7)	799 (4)	8128 (7)	2.48 (21)	O3(P1)	3507 (8)	−417 (6)	567 (3)	2.70 (14)
N(P)	4434 (9)	988 (5)	5962 (8)	1.92 (25)	N(P)	6857 (9)	491 (7)	470 (4)	2.61 (17)
O1(P2)	2134 (7)	1907 (4)	3588 (7)	2.03 (20)	O1(P2)	9188 (7)	−113 (5)	−601 (3)	2.27 (13)
O2(P2)	5712 (7)	2083 (4)	5040 (7)	2.44 (22)	O2(P2)	10222 (8)	110 (6)	763 (3)	3.66 (18)
O3(P2)	4056 (8)	791 (4)	3430 (7)	2.69 (22)	O3(P2)	8302 (10)	−1795 (5)	292 (3)	3.32 (16)
N(1)	−82 (9)	3252 (5)	2502 (8)	2.22 (26)	N(1)	9401 (9)	−60 (6)	−2103 (4)	2.52 (17)
N(2)	−60 (9)	3313 (5)	5069 (8)	1.84 (23)	N(2)	5498 (10)	−174 (7)	−2213 (3)	2.53 (17)
N(3)	3330 (9)	3357 (5)	5414 (8)	1.95 (25)	N(3)	7271 (9)	1751 (6)	−1339 (3)	2.41 (16)
N(4)	−1159 (9)	1883 (5)	3361 (8)	2.25 (27)	N(4)	7406 (9)	−2050 (6)	−1409 (4)	2.92 (18)
O(W1)	7330 (8)	479 (5)	11021 (8)	3.75 (25)	C1(1)	7456 (3)	2699 (2)	1718 (1)	2.62 (5)
O(W2)	704 (10)	−120 (5)	1750 (10)	6.43 (35)	C1(2)	7321 (3)	−2460 (2)	1894 (1)	2.70 (5)
O(W3)	−1432 (13)	458 (9)	4938 (11)	12.49 (47)	H(NP)	700 (11)	102 (8)	84 (4)	4.0
H(NP)	498 (9)	43 (5)	620 (8)	4.0	H(O2P1)	333 (12)	170 (7)	−51 (4)	4.0
H(O2P2)	562 (9)	243 (5)	430 (8)	4.0	H(O2P2)	1186	−15	66	4.0
H1(N1)	−31 (9)	395 (5)	264 (8)	4.0	H(O3P2)	758 (9)	−193 (7)	67 (4)	4.0
H2(N1)	53 (9)	332 (5)	215 (8)	4.0	H1(N1)	1033 (11)	−42 (8)	−180 (4)	4.0
H3(N1)	−126 (9)	313 (5)	172 (8)	4.0	H2(N1)	989 (11)	62 (8)	−223 (4)	4.0
H1(N2)	−15 (10)	402 (5)	483 (8)	4.0	H3(N1)	902 (10)	−62 (7)	−240 (4)	4.0
H2(N2)	38 (10)	327 (5)	587 (8)	4.0	H1(N2)	434 (11)	6 (7)	−200 (4)	4.0
H3(N2)	−127 (9)	306 (5)	469 (8)	4.0	H2(N2)	507 (11)	−93 (7)	−233 (4)	4.0
H1(N3)	347 (9)	362 (5)	493 (8)	4.0	H3(N2)	611 (10)	24 (7)	−256 (4)	4.0
H2(N3)	331 (9)	379 (5)	610 (8)	4.0	H1(N3)	782 (9)	213 (7)	−85 (4)	4.0
H3(N3)	423 (9)	312 (5)	580 (8)	4.0	H2(N3)	601 (10)	222 (7)	−130 (4)	4.0
H1(N4)	−138 (9)	161 (5)	267 (8)	4.0	H3(N3)	784 (10)	205 (8)	−171 (4)	4.0
H2(N4)	−109 (9)	151 (5)	397 (8)	4.0	H1(N4)	627 (10)	−245 (8)	−110 (4)	4.0
H3(N4)	−233 (9)	212 (5)	304 (8)	4.0	H2(N4)	744 (9)	−240 (7)	−175 (4)	4.0
H1(W1)	655 (9)	73 (5)	1028 (8)	4.0	H3(N4)	842 (11)	−233 (8)	−110 (4)	4.0
H2(W1)	713 (8)	−1 (5)	1134 (7)	4.0					
H1(W2)	5 (9)	16 (5)	175 (8)	4.0					
H2(W2)	196 (9)	4 (5)	217 (8)	4.0					
H1(W3)	−69 (9)	24 (5)	454 (9)	4.0					
H2(W3)	−278	46	413	4.0					

[a] Values are multiplied by 10^4 for nonhydrogen atoms and 10^3 for hydrogen atoms.

$(NH_3)_4PP$ and $Co(NH_3)_4PNP$ was ca. 1 to 1. The product of the $Co(NH_3)_4PP$ reaction was determined by ^{31}P NMR to be $Co(NH_3)_4(P)_2$. In the presence of apyrase, $Co(NH_3)_4(P)_2$ undergoes hydrolysis to $Co(NH_3)_4(P)$ at a rate of 3.9%/h, and $Co(NH_3)_4(P)$ undergoes further phosphate loss at a rate of 2.0%/h. In the absence of apyrase, the two rates were determined to be 2.1%/h and 0.96%/h.

The product of the apyrase-catalyzed hydrolysis of $Co(NH_3)_4PNP$ appeared, based upon its ^{31}P chemical shift, to be $Co(NH_3)_4PP$. The product identification was confirmed by demonstrating that PP was liberated from this complex when the mixture was heated with EDTA. Under those conditions $Co(NH_3)_4PNP$ was known to undergo conversion to P and $Co(NH_3)_4PP$ to PP. Once formed in the reaction mixture, $Co(NH_3)_4PP$ underwent hydrolysis to $Co(NH_3)_4(P)_2$. The ^{31}P NMR resonance from this species (−11.2 ppm) was just barely resolved from that of $Co(NH_3)_4PNP$ (−11.5 ppm). Longer incubation periods resulted in P loss from the $Co(NH_3)_4(P)_2$ complex as evidenced by the appearance of resonances from bidentate $Co(NH_3)_4(PO_4)$ and monodentate $Co(NH_3)_4(H_2O)(HPO_4)$ in the ^{31}P NMR spectrum measured at 13 h.

Discussion

The present study was undertaken to determine whether polyphosphoroamidates such as AMP-PNP or PNP might be useful as probes of proton transfer steps in enzyme-catalyzed phosphoryl transfer reactions. Under normal circumstances P–N bonds are stable to cleavage; therefore, only those enzymes capable of protonating the nitrogen atom would be expected to cleave the P–N–P linkage. In order for a "negative" result (no activity with the phosphoroamidate) to be meaningful, the structure of the phosphoroamidate must be isosteric with that of the natural substrate. Thus, we first examined the geometric and conformational perturbations which result from the substitution of a nitrogen atom (NH) for a bridge oxygen atom of a metal-complexed polyphosphate substrate.

The X-ray structure of the exchange inert P^1,P^2-bidentate $Co(NH_3)_4PP$ complex had been previously solved (Merritt & Sundaralingam, 1980) so we prepared and analyzed the P^1,P^2-bidentate $Co(NH_3)_4PNP$ crystal structure. As shown in Figure 4, the two crystal structures are essentially identical except for the puckering of one atom. Given the flexibility of the chelate ring, it would appear that the chelate ring of the imidodiphosphate complex could assume the conformation required for catalysis at the enzyme active site. The nitrogen atom would then occupy essentially the same position in the enzyme active site as the bridge oxygen atom of the corresponding PP complex.

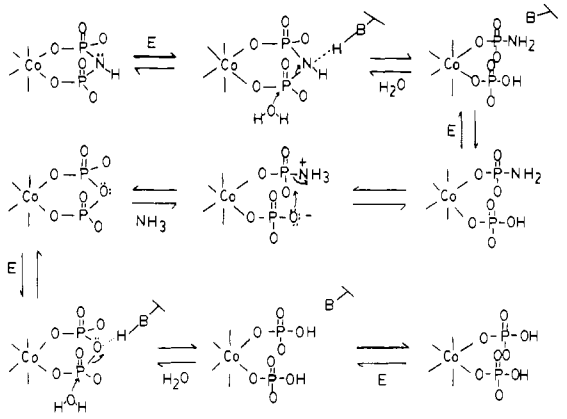
$Co(NH_3)_4PNP$ should serve as substrate for an enzyme reaction if the enzyme is capable of protonating the imido nitrogen atom to the quarternary charged state. Although *E. coli* alkaline phosphatase catalyzes the hydrolysis of PNP, it does not accept bidentate metal complexed CoPP or CoPNP as substrate (W. B. Knight and D. Dunaway-Mariano, unpublished results). Potato apyrase, on the other hand, hy-

Table II: List of Hydrogen Bonding Interactions for Tetraammine(imidodiphosphato)cobalt(III) Trihydrate and Tetraammine(imidodiphosphato)cobalt(III) Dihydrochloride

A-H···B	symmetry code	translation x	translation y	translation z	bond lengths (Å) A···B	bond lengths (Å) A-H	bond lengths (Å) H···B	angle (deg), A-H···B
(A) Trihydrate[a]								
N(P)-H(NP)···O3(P2)	3	1	0	1	3.055	0.98	2.08	176
O2(P2)-H(O2P2)···O2(P1)	2	0	0	−1	2.458	0.94	1.53	171
N(1)-H1(N1)···O(W2)	4	0	0	0	2.887	1.17	1.75	164
N(1)-H2(N1)···O1(P1)	2	0	0	−1	3.033	0.82	2.25	162
N(1)-H3(N1)···O2(P2)	2	−1	0	−1	3.004	0.87	2.14	170
N(2)-H1(N2)···O(W2)	4	0	0	0	3.038	1.16	2.02	144
N(2)-H2(N2)···O1(P2)	2	0	0	0	3.086	0.71	2.38	175
N(2)-H3(N2)···O2(P1)	2	−1	0	−1	2.964	0.95	2.08	154
N(3)-H1(N3)···O3(P1)	2	0	0	−1	3.171	0.73	2.45	167
N(3)-H2(N3)···O3(P2)	2	0	0	0	3.219	1.02	2.29	151
N(4)-H1(N4)···O(W1)	1	−1	0	−1	3.044	0.78	2.32	155
N(4)-H3(N4)···O2(P1)	2	−1	0	−1	3.022	0.93	2.14	157
O(W1)-H2(W1)···O3(P1)	3	1	0	2	2.788	0.92	1.91	159
O(W2)-H1(W2)···O(W1)	1	−1	0	−1	2.693	0.72	2.04	151
O(W3)-H1(W3)···O(W3)	3	0	0	1	2.803	1.02	1.92	143
O(W3)-H2(W3)···O3(P2)	1	−1	0	0	3.353	0.95[b]	2.41	145
(B) Dihydrochloride[c]								
O2(P1)-H(O2P1)···Cl(1)	3	−1	0	0	3.173	0.92	2.30	157
N(P)-H(NP)···Cl(1)	1	0	0	0	3.196	0.86	2.35	172
O2(P2)···H(O2P2)···O3(P1)	1	1	0	0	2.416	1.21[d]	1.21	180
O3(P2)-H(O3P2)···Cl(2)	1	0	0	0	2.983	0.85	2.23	148
N(3)-H1(N3)···O2(P1)	3	0	0	0	3.192	1.02	2.18	169
N(3)-H3(N3)···Cl(2)	2	1	0	−1	3.206	0.83	2.49	145

[a] Symmetry codes: (1) x, y, z, (2) $-x, y + 0.5, -z$, (3) $-x, -y, -z$, and (4) $x, -y + 0.5, z$. [b] H2(W3) was fixed at this distance. [c] Symmetry codes: (1) x, y, z, (2) $0.5 - x, -y, 0.5 + z$, (3) $0.5 + x, 0.5 - y, -z$, and (4) $-x, 0.5 + y, 0.5 - z$. [d] H(O2P2) was fixed midway between O2(P2) and O3(P1).

Scheme I



drolyzes the CoPNP complex at a rate only 3–4-fold slower than for CoPP. The reaction product is orthophosphate, suggesting that apyrase, like alkaline phosphatase (W. B. Knight and D. Dunaway-Mariano, unpublished results), catalyzes the hyrolysis of phosphoroamidate very efficiently. $Co(NH_3)_4PP$ is converted to $Co(NH_3)_4(P)_2$ by apyrase as it is by yeast inorganic pyrophosphatase (Haromy et al., 1982). Unlike pyrophosphatase,[3] apyrase also catalyzes the hydrolysis (although very slowly) of *cis*-$[Co(NH_3)_4(P)_2]_2$ to $Co(NH_3)_4(H_2O)(P)$ and $Co(NH_3)_4(H_2O)(P)$ to $Co(NH_3)_4(H_2O)_2$.

The expected product of the apyrase-catalyzed hydrolysis of $Co(NH_3)_4PNP$ was $Co(NH_3)_4(P)(PN)$. The species, however, was not observed at any time during the course of the reaction. Instead, the observed product was $Co(NH_3)_4PP$.

As indicated in Scheme I, once the product is released into solution, it can exist in equilibrium with several protonated forms. The small amount of complex which is protonated at the nitrogen atom will spontaneously displace NH_3 concomitant with ring closure.[4] The $Co(NH_3)_4PP$ thus formed is then reabsorbed by apyrase and converted to $Co(NH_3)_4(P)_2$.

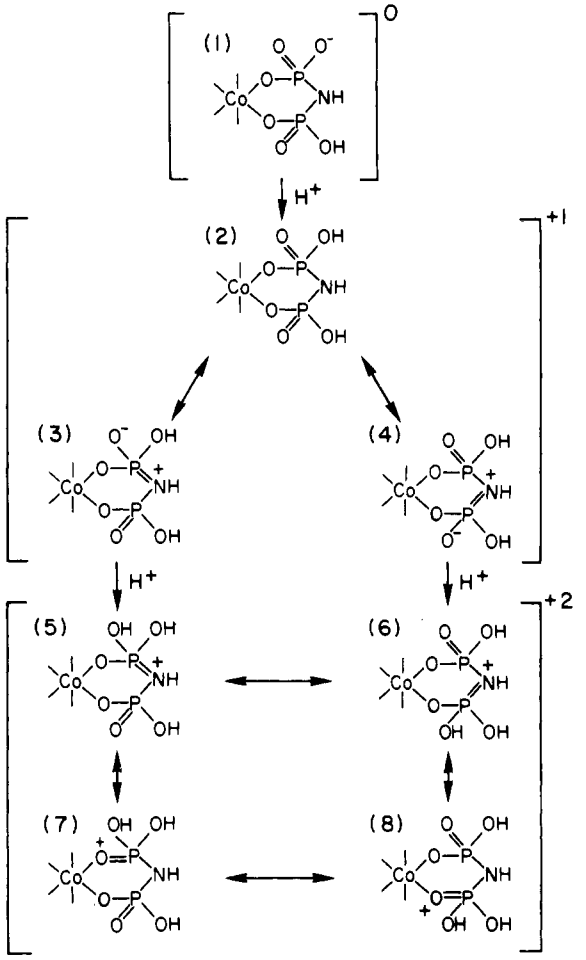
It is interesting to note that while PNP and $Co(NH_3)_4PNP$ behave similarly toward apyrase, their properties in solution are significantly different. In acid solution uncomplexed PNP undergoes rapid hydrolysis as a result of nitrogen protonation (Quimby et al., 1960) while our X-ray and ^{31}P NMR analyses demonstrate that $Co(NH_3)_4PNP$ is stable in HCl. The hydrolysis of uncomplexed PNP in solution may be due to the availability of all phosphate oxygen atoms for potential protonation since none of the oxygen atoms are involved in metal ligation. This hyperprotonation allows sufficient positive charge to build up at the phosphorus and nitrogen atoms to result in bond cleavage.

The CoPNP hydrochloride crystals display protonation at the phosphate oxygen atoms but not at the nitrogen atom. When the anionic phosphate oxygen atom of **1** in Scheme II is protonated, the net charge on the complex is +1 (**2**) which would be expected to preclude further protonation. However, the X-ray structure shows that in the crystal an additional proton can be accepted which is shared between the two double-bonded phosphate oxygen atoms of neighboring molecules. This additional protonation may be visualized to occur in solution via the valency bond structures **3** and **4** which yield **5** and **6**, respectively. The positive charge on the nitrogen atom can also migrate to the liganded oxygen atoms as represented by **7** and **8**. The occurrence of this resonance is demonstrated by the shortening of the average P to O1 distances by 0.03

[3] The release of P_i from $Co(NH_3)_4(P)_2$ and $Co(NH_3)_4(P)$ was measured in the presence and absence of pyrophosphatase. The release rates, which overall were faster than those observed in the present study (they were measured at a higher pH and temperature), were unaffected by the pyrophosphatase (Haromy et al., 1982).

[4] Hydrolysis of PNP in acid solution leads to PP formation (Quimby et al., 1960). We have observed slow PP formation from PNP at pH 6.6 in the presence of Mg^{2+}.

Scheme II



Å and the elongation of the average Co to O1 distances by 0.02 Å. The hyperprotonation of the phosphate oxygen atoms of the CoPNP complex does not result in cleavage of the P–N–P linkage, but instead the resulting partial positive charge on the bridge nitrogen atom prevents further protonation of the nitrogen atom. Apparently, apyrase hydrolyzes the $Co(NH_3)_4PNP$ complex by selectively protonating the bridge nitrogen atom rather than the phosphate oxygen atoms.

Supplementary Material Available

Listing of anisotropic thermal parameters and of structure factors for both crystal structures (11 pages). Ordering information is given on any current masthead page.

Registry No. $Co(NH_3)_4PNP \cdot 3H_2O$, 86942-14-5; $Co(NH_3)_4PNP \cdot 2HCl$, 86942-15-6; $Co(NH_3)_4PP$, 63915-24-2; $Co(NH_3)_4(P)_2$, 86942-13-4; $[Co(NH_3)_4CO_3]NO_3$, 15040-52-5; PNP, 27590-04-1; PP, 2466-09-3; apyrase, 9000-95-7.

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