Investigation of the Regiospecificity and Stereospecificity of Proton Transfer in the Yeast Inorganic Pyrophosphatase Catalyzed Reaction†

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ABSTRACT: The regiospecificity and stereospecificity of proton transfer in the yeast inorganic pyrophosphatase (PPase) catalyzed hydrolysis of P^1,P^2-bidentate $Mg(H_2O)_4(PP_i)^{2-}$ were probed with exchange-inert metal complexes of imidodiphosphate (PNP) and thiopyrophosphate (PPS). PPase was unable to catalyze the hydrolysis of $Mg(H_2O)_4PNP$ and P^1,P^2-bidentate $Co(NH_3)_4PNP$ under conditions that resulted in rapid hydrolysis of the corresponding metal–PP_i complexes. PPase was found to catalyze the hydrolysis of $Mg(H_2O)_4PPS$ at 17% the rate of $Mg(H_2O)_4PP_i$ hydrolysis. The K_m of $Mg(H_2O)_4PPS$ was determined to be 300 μM, which is a value 10-fold greater than that observed for $Mg(H_2O)_4PP_i$. P^1,P^2-Bidentate $Cr(H_2O)_4PPS$ and $Co(NH_3)_4PPS$ (prepared from PPS) were both found to be substrates for PPase. The enzyme specifically catalyzed the hydrolysis of the R_P enantiomers of these complexes and not the S_P enantiomers. These results are accommodated by a reaction mechanism involving enzyme-mediated proton transfer to the *pro-R* oxygen atom of the incipient phosphoryl leaving group of the bound P^1,P^2-bidentate $Mg(H_2O)_4PP_i^{2-}$ complex.

Yeast inorganic pyrophosphatase (PPase)[1] catalyzes the hydrolysis of inorganic pyrophosphate (PP_i) to orthophosphate (P_i) in the presence of Mg(II). Previous studies in our laboratory have identified the active substrate in the PPase-catalyzed reaction as the P^1,P^2-bidentate Mg(II) complex of the pyrophosphate tetraanion (Knight et al., 1981, 1983). During the initial catalytic step, the $MgPP_i$ complex is cleaved to the *cis*-$[Mg(P_i)_2]$ complex (Haromy et al., 1982). In the absence of enzyme-mediated proton transfer to the phosphoryl group that is displaced during the course of the hydrolytic cleavage of $MgPP_i^{2-}$, Mg(II)-coordinated phosphate trianion would be generated. The high basicity of the Mg(II) complex of HPO_4^{2-} (pK_a = 10.4; Smith & Martell, 1976) suggests that the Mg(II) (PO_4^{3-}) would not be a suitable leaving group in the PPase-catalyzed reaction. The participation of an enzyme active site residue as an acid catalyst in the reaction is therefore highly probable. In fact, the essential role of an active site acid residue has been implicated by the dependency of the reaction rate on the pH of the reaction solution (Knight et al., 1981). Specifically, the results from the pH studies have shown that the catalytic activity of the enzyme is destroyed upon deprotonation of an enzyme residue having an apparent pK_a of 7.8.

The object of the studies reported in this paper is the examination of the regiochemistry and stereochemistry of the putative proton transfer to the incipient leaving group of the $MgPP_i$ complex. For this purpose, the substrate activities of exchange-inert metal complexes of the pyrophosphate analogues imidodiphosphate (PNP) and thiopyrophosphate (PPS) were tested. The results obtained from these studies are consistent with the proposal that catalysis proceeds with enzyme-mediated proton transfer to the *pro-R* oxygen atom of the departing phosphoryl group of the P^1,P^2-bidentate $Mg(H_2O)_4PP_i$ substrate.

Materials and Methods

PPase was purified according to the modified method (Bond, 1979) of Cooperman et al. (1973). The enzyme used in these experiments migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (7.5% acrylamide) and had an activity of 690 μmol of P_i min^{-1} (mg of protein)$^{-1}$ at pH 7.5. All PPase concentrations are reported in terms of active site concentrations. P^1,P^2-Bidentate $Co(NH_3)_4PP$ and P^1,P^2-bidentate $Co(NH_3)_4PNP$ were prepared according to the methods of Cornelius et al. (1977) and Haromy et al. (1983), respectively. ^{31}P NMR spectra were measured on an IBM WP200SY (operating at 81.02 Hz) NMR spectrometer. Chemical shifts are reported in ppm downfield (+) or upfield (−) from a 0.1 M D_3PO_4 external standard.

Thiopyrophosphate (PPS). PPS was prepared by a modified version of the Tridot and Tudo (1960) procedure. Accordingly, 7.5 g of Na_3SPO_3 (Akerfelt, 1960) was dissolved in a minimal amount of water. The solution was adjusted to 10.2 with HCl and then evaporated to dryness in vacuo. The resulting powder was transfered to a drying pistol and dried over P_2O_5 for 3 h at room temperature and then for 4 h at 110 °C. The ^{31}P NMR spectrum taken of the reaction mixture dissolved in D_2O at pH 8.5 indicated the presence of P_i, PSO_3, PP_i, and PPS. The reaction mixture was dissolved in 2 L of water and absorbed onto a Dowex 1-Cl (100–200-mesh) column (80 × 2.5 cm). The column chromatography was carried out using a linear gradient (2 L of 0.06 M → 2 L of

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[1] Abbreviations: PPase, yeast inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; P_i, orthophosphate; PNP, imidodiphosphate; PPS, thiopyrophosphate; PS, thiophosphate; K^+PIPES, potassium 1,4-piperazinediethanesulfonate; K^+MES, potassium 2-(*N*-morpholino)-ethanesulfonate; K^+HEPES, potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid.

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1.0 M) of K_2CO_3 and was monitored by carrying out phosphate analysis (Fiske & Subbarow, 1925) on the column fractions. Peak fractions were combined, concentrated, and then analyzed by ^{31}P NMR techniques. The PPS [34.0 (d) and −9.8 ppm (d), J = 28 Hz at pH 4.4] eluted with ca. 0.45 M K_2CO_3. In order to remove most of the K_2CO_3 from the PPS sample, the sample was adjusted to pH 7.0 with $HClO_4$, and the resulting $KClO_4$ was separated by filtration. The yield of PPS varied between 20 and 40% from preparation to preparation.

[^{32}P]Thiopyrophosphate ([^{32}P]PPS). A total of 1.7 mL of 85% phosphoric acid (15 mmol) and 5 mCi of [^{32}P]P_i was added to 7.2 mL (30 mmol) of tri-*n*-butylamine in 100 mL of pyridine. The mixture was concentrated in vacuo, redissolved in anhydrous pyridine, and reconcentrated. The pyridine evaporation step was repeated 3 times. In a separate flask, 1.55 mL (15 mmol) of $PSCl_3$ was equilibrated with 25 mL of anhydrous pyridine (under an Ar atmosphere) at 0 °C for 20 min. The anhydrous tri-*n*-butylammonium salt of phosphoric acid was dissolved in 25 mL of anhydrous pyridine and added to the stirred $PSCl_3$/pyridine solution. After being stirred for 1 h at 0 °C, the reaction solution was decanted away from a polymeric substance into an aqueous solution containing 60 mmol of KOH at 0 °C. The resulting solution (pH 8.5) was evaporated to dryness, dissolved in D_2O, and then analyzed by ^{31}P NMR techniques. The reaction products (P_i, PSO_3, PPS) were resolved on an anion-exchange column in the same manner described above. The yield of the [^{32}P]PPS was highly variable (5–40%) as a result of competing polymerization reactions. Adjustments made in reaction conditions failed to significantly reduce the relative efficiency of these competing side reactions.

Cr(III) and Co(III) Complexes of PPS. P^1,P^2-Bidentate $Co(NH_3)_4$PPS and P^1,P^2-bidentate $Cr(H_2O)_4$PPS were prepared by heating solutions 10 mM in PPS and 10 mM in [$CrCl_3 \cdot 6H_2O$] (Baker Chemical Co.) or [$Co(NH_3)_4Cl_2$]Cl (Cornelius et al., 1977) (pH 4) at 80 °C for 4 min. The reaction mixtures were purified by Dowex 50-H^+ (2% crosslinkage) column chromatography at 4 °C. The reaction solutions were absorbed onto the column (ca. 10 mL of solution/1 mL of resin), and the desired complex was eluted with water. The yield of the $Cr(H_2O)_4$PPS ranged from 10 to 20% and that of the $Co(NH_3)_4$PPS from 1 to 5%. Structural assignments were based upon comparison of the chromatographic and spectral properties of the complex with those of the corresponding authentic sample prepared by a different synthetic approach (Lin & Dunaway-Mariano, 1984).

PPase Reactions with Mg(II) and Co(III) Complexes of PP and PNP. The hydrolysis reactions of MgPP and MgPNP were monitored using the spectrophotometric assay described previously (Knight et al., 1981). PNP (0.4 mM) was incubated at 25 °C with 4 μM PPase, 20 mM $MgCl_2$, and 100 mM potassium 1,4-piperazinediethanesulfonate (K^+PIPES) (pH 7.0) for 25 h. An assay for P_i revealed that no reaction had taken place during this period. In contrast, PP_i (0.4 mM) incubated under these same conditions was hydrolyzed within 0.5 s.

The hydrolysis reactions of $Co(NH_3)_4PP_i$ and $Co(NH_3)_4$-PNP were monitored with the ^{31}P NMR techniques described previously (Haromy et al., 1983). $Co(NH_3)_4$PNP (4.8 mM) was incubated at 25 °C with 0.12 mM pyrophosphatase, 20 mM $MgCl_2$, and 100 mM K^+ PIPES (pH 6.7). The ^{31}P NMR spectra of the reaction mixture measured over a 54-h period revealed that no hydrolysis of the $Co(NH_3)_4$PNP had taken place. Under these same reaction conditions, 4.2 mM $Co(NH_3)_4PP_i$ was hydrolyzed to $Co(NH_3)_4(P_i)_2$ within a 5-min incubation period.

PPase Reaction with MgPPS. The V_{max} and K_m values of MgPPS were measured with [^{32}P]PPS as substrate. The product [^{32}P]P_i was assayed by the phosphomolybdate extraction procedure described previously (Knight et al., 1981). Reactions containing enzyme, 10 mM $MgCl_2$, 50 mM K^+-PIPES, and varying amounts of [^{32}P]PPS were allowed to proceed to 8–12% conversion. The V_{max} and K_m values were evaluated from the inverse plots of the initial velocity vs. [PPS]. The K_m and V_{max} values of $MgPP_i$ were measured under identical reaction conditions (using [^{32}P]PP_i).

PPase Reaction with $Cr(H_2O)_4$PPS. *(A) Radioisotopic Assay.* A solution 10 mM in $MgCl_2$, 50 mM in potassium 2-(*N*-morpholino)ethanesulfonate (K^+MES) (pH 6.0), 0.6 μM in [^{32}P]$Cr(H_2O)_4$PPS, and 2.3 μM in PPase was incubated at 25 °C. The reaction was assayed for product in the manner described previously for the reaction of [^{32}P]$Cr(H_2O)_4PP_i$ after 30 min of incubation and after 1 h of incubation. A control reaction not containing enzyme was treated in the same manner.

(B) CD Assay. $Cr(H_2O)_4$PPS (2.3 mM) was incubated with 10 mM $MgCl_2$, 30 μM PPase, and 100 mM K^+MES at pH 6 and 0 °C. The circular dichroism (CD) spectrum of the reaction solution was measured over a 4-h period with a JASCO 500-C spectropolarimeter. The rate of $Cr(H_2O)_4$PPS racemization was measured by first incubating 10 mM $Cr(H_2O)_4$PPS with 10 mM $MgCl_2$, 100 mM K^+MES (pH 6), and 79 μM PPase at 4 °C for 15 min and then quickly passing the reaction solution through a Sephadex G-50 column at 4 °C (10 mM K^+MES, pH 6, as eluant). The fractions containing chromium complexes were pooled, adjusted to pH 4, and concentrated in vacuo. The rate of $Cr(H_2O)_4$PPS racemization at pH 6.0 was calculated from the rate of the decrease in the $Cr(H_2O)_4$PPS/$Cr(H_2O)_4(P_i)$(PS) solution ellipticity upon incubation at 25 °C in the presence and absence of PPase.

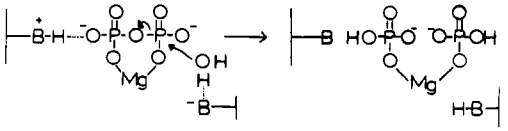
The K_i of $Cr(H_2O)_4$PPS was measured by testing the complex as a competitive inhibitor vs. $MgPP_i$ in the PPase reaction. The velocity of the PPase-catalyzed hydrolysis of $MgPP_i$ was measured with the spectrophotometric assay procedure described previously (Knight et al., 1981). The K_i value was determined from inverse plots of the initial reaction velocity measured at varying $Cr(H_2O)_4$PPS concentrations (0, 1, and 4 mM) vs. [$MgPP_i$] (10–100 μM). The reaction solutions contained 10 mM $MgCl_2$ and 50 mM K^+MES (pH 5.9).

PPase Reaction of $Co(NH_3)_4PPS$. $Co(NH_3)_4$PPS (0.56 mM) was incubated at 25 °C with 10 mM $MgCl_2$, 50 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (K^+HEPES) (pH 7) and 15 μM PPase. The reaction was monitored over a 17-h period by measuring changes in solution ellipticity. The K_m and V_{max} values for $Co(NH_3)_4$PPS were determined from inverse plots of the initial velocity of the reaction vs. [$Co(NH_3)_4$PPS] (0.3–5.0 mM).

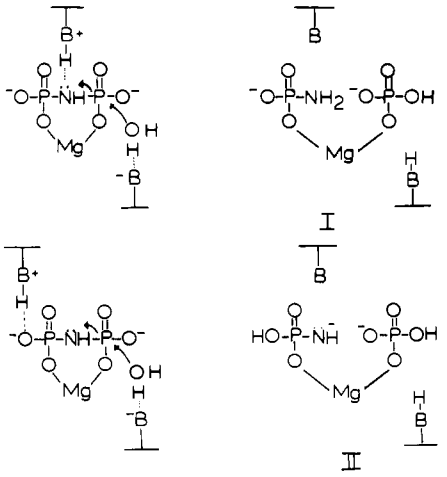
Results and Discussion

Stereochemical, positional isotope exchange, and kinetic studies have provided evidence that enzyme-catalyzed phosphoryl-transfer reactions proceed by "S_N2-like" mechanisms [see Knowles, (1980) and Frey (1982)]. Accordingly, the enzyme may activate the phosphoryl group of the bound substrate for transfer by delocalizing electron density from the electrophilic phosphorus center and from the leaving group. During the past few years wer have been engaged in the study of the catalytic mechanism of the phosphoryl transferring enzyme yeast inorganic pyrophosphatase. During the course

Scheme I



Scheme II

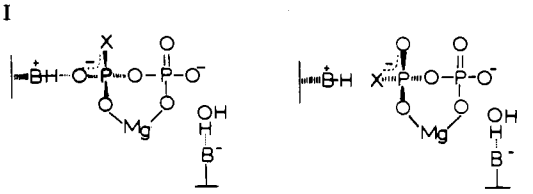


of these studies we discovered that this enzyme recognizes the P^1,P^2-bidentate $Mg(H_2O)_4PP_i^{2-}$ complex as substrate (Knight et al., 1981, 1983). The basicity of the putative leaving group in the catalyzed hydrolysis reaction, Mg^{2+}-complexed phosphate trianion, is such to warrant enzyme-mediated proton transfer during the course of catalysis (vide infra). The purpose of the studies described below was to examine the regiochemistry and stereochemistry of the proton transfer from the enzyme to the substrate (see Scheme I).

Proton Transfer to Bridge Oxygen Atom of $MgPP_i$. In an earlier publication (Haromy et al., 1983) we had suggested that imidophosphate derivatives of phosphate esters or anhydrides might be used to test for enzyme-mediated proton transfer to the leaving group in a phosphoryl-transfer reaction. In this study we have utilized Mg(II) and Co(III) complexes of PNP as probes for PPase-mediated proton transfer to the bridge oxygen atom of PP_i. As illustrated below, in Scheme II, proton transfer to the bidentate metal–PNP complex bound in the PPase active site may occur at the bridging oxygen atom or at one of the two distal oxygen atoms. The tautomeric form of the product (I) generated via protonation of the atom at the bridge position is stable while that (II) generated via protonation at the distal position is quite high in energy. Thus, we predicted that PPase could catalyze the hydrolysis of the bidentate metal–PNP complex if and only if it could transfer a proton to the nitrogen atom during or prior to catalysis.

Previous studies (Knight et al., 1981; Haromy et al., 1982, 1983) have shown that (1) MPNP is a tight competitive inhibitor vs. MPP (and hence that is binds to the PPase substrate binding site), (2) P^1,P^2-bidentate $Co(NH_3)_4PP_i$ is a substrate for PPase, (3) P^1,P^2-bidentate $Co(NH_3)_4PNP$ and P^1,P^2-bidentate $Co(NH_3)_4PP_i$ are isosteric, and (4) phosphoryl transferring enzymes that mediate proton transfer to the oxygen atom undergoing bond cleavage can do so to the nitrogen atom of an imidophosphate substrate analogue. In this study we found that PPase was unable to catalyze the hydrolysis of MgPNP and P^1,P^2-bidentate $Co(NH_3)_4PNP$ under conditions that lead to rapid conversion of $MgPP_i$ and P^1,P^2-bidentate $Co(NH_3)_4PP_i$. Taken together, these results do not unambiguously exclude the possibility of proton transfer to the bridge oxygen of the substrate; however, they did cast significant doubt and prompted us to consider an alternate regiochemistry of proton transfer (vide infra).

Chart I



Proton Transfer to Distal Oxygen Atom of $MgPP_i$. Our attention was next focused on examining possible hydrogen-bonding interactions between a PPase active site residue and the distal oxygen atoms of the leaving group of the $MgPP_i$ reaction. The basic strategy that we took was to substitute one of the distal oxygen atoms of the leaving group with an atom having inferior hydrogen-bonding capabilities and to test the stereospecificity of PPase toward the two enantiomers of the analogue. As illustrated in Chart I, the enantiomer which places the distal oxygen atom juxtaposed to the putative hydrogen-bonding group will bind productively while that which places the substitute atom in this position may not bind productively.

Ultimately, sulfur was chosen to substitute for the distal oxygen atom because (1) MgPPS, a known substrate for PPase, is cleaved by addition of H_2O to the phosphosphoryl center rather than to the thiophosphoryl center (Webb & Trentham, 1980), (2) the thiophosphoryl group displaced as the leaving group in the MgPPS reaction possesses the same charge as the phosphoryl group displaced in the MgPP reaction, and (3) the distal oxygen atom of the thiophosphoryl moiety of the PPase-bound MgPPS is expected to form a stronger hydrogen bond with the putative active site acid group than would the sulfur atom.[2] While the thiophosphoryl center of the active substrate form P^1,P^2-bidentate MgPPS is chiral, the two enantiomers present in solution undergo rapid equilibration as a result of the rapid of ligand exchange that takes place at the Mg(II) center. Thus, in order to generate stable stereochemical probes for our studies, the exchange-inert metal ions Cr(III) and Co(III) were used in place of the Mg(II) ion to form the metal–PPS complex. Both P^1,P^2-bidentate Cr(III)PP_i and Co(III)PP_i have been previously shown to be substrates for PPase (Knight et al., 1981; Haromy et al., 1982), and it was expected that the Cr(III) and Co(III) complexes of PPS would likewise be substrates.

Preparation and Substrate Activity of MgPPS. Although MgPPS had been shown to be a substrate for PPase, its K_m and turnover rate had not been reported. In order to evaluate the substrate activity of MgPPS, ^{32}P-labeled PPS was prepared by reaction of [^{32}P]P_i and $PSCl_3$. Using the radiolabeled PPS as substrate, we were able to conveniently monitor the progress of the PPase-catalyzed reaction by assaying for the [^{32}P]P_i generated as product. The K_m and V_{max} values for the Mg(II) complexes of [^{32}P]PPS and [^{32}P]PP_i were evaluated at pH 6.5 under identical reaction conditions. Substitution of the sulfur atom for the distal oxygen atom resulted in a 10-fold increase in the K_m value (300 vs. 30 μM) and a 6-fold decrease in the V_{max} value of the substrate. The diminution in the V_{max} value and elevation in the K_m value that we observed with the PPS are quite similar to the changes in K_m and V_{max} values that one observes in switching adenosine 5′-(2-thiotriphosphate)

[2] Frey and Sammons (1985) have examined the charge localization on phosphorothioates in some detail. Although the charge appears concentrated on the sulfur vs. oxygen atom, in the crystalline state the oxygen atom rather than the sulfur atom of the phosphorothioate is found hydrogen bonded to the countercation (Mikolajczky et al., 1976; Saenger & Eckstein, 1970).

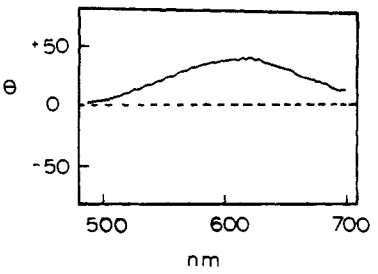


FIGURE 1: CD spectrum of a reaction mixture initially containing 2.3 mM $Cr(H_2O)_4PPS$, 10 mM $MgCl_2$, 100 mM K^+MES (pH 6), and 30 μM PPase after incubation at 0 °C for 15 min.

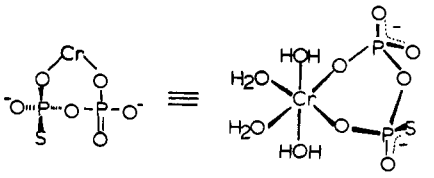
for adenosine 5′-triphosphate as substrate for kinases [see Frey (1982)].

Preparation and Substrate Activity of $Cr(H_2O)_4PPS$. The P^1,P^2-bidentate $Cr(H_2O)_4PPS$ complex was prepared by reaction of $[CrCl_3{\cdot}6H_2O]$ with PPS. The $Cr(H_2O)_4PPS$ was purified by chromatographing the crude reaction mixture on a Dowex 50 (H^+) ion-exchange column with H_2O. $Cr(PPS)_2$ eluted first from the column followed by Cr(PPS). The other product of the reaction, $Cr_2(PPS)$, remained on the column. The K_i value of the $Cr(H_2O)_4PPS$ prepared in this manner was determined from initial velocity data obtained from studies in which the complex was tested as a competitive inhibitor vs. $MgPP_i$. The K_i value obtained (1.1 mM) is approximately twice the value of the K_i measured for $Cr(H_2O)_4PP_i$ under similar conditions (Knight et al., 1981).

The substrate activity of $[^{32}P]Cr(H_2O)_4PPS$ was evaluated by assaying the reaction mixture for $[^{32}P]P_i$ released upon treatment of the product of the enzymatic reaction $Cr(H_2O)_4(P_i)(PS)$ with EDTA. Under reaction conditions where the PPase:$Cr(H_2O)_4PPS$ ratio was initially 4:1, approximately 40% of the $Cr(H_2O)_4PPS$ was converted to product upon extended incubation (30 and 60 min). This result is consistent with one of the two $Cr(H_2O)_4PPS$ enantiomers absorbed to the enzyme undergoing catalysis; however, in the absence of knowledge of the purity of the $Cr(H_2O)_4PP$ sample, stereospecificity could not be assumed. For this reason we turned to CD techniques to examine the stereospecificity of PPase toward $Cr(H_2O)_4PPS$. The time course for the PPase-catalyzed hydrolysis of $Cr(H_2O)_4PPS$ at pH 6 was examined over a 4-h period by monitoring the molar ellipticity of the reaction solution at the λ_{max}, 600 nm. The ellipticity measured at a reaction period of 5 min was 24 deg cm^2/dmol. At 15 min, the molar ellipticity had increased to 25 deg cm^2/dmol (see Figure 1). At 30 min, the molar ellipticity decreased to 22 deg cm^2/dmol, and after 1.5 h, it dropped to 12 deg cm^2/dmol. After 2 h, the ellipticity of the solution was too small to measure. Since the CD spectrum of the $Cr(H_2O)_4PPS$ enantiomer derived from the Λ-$Cr(H_2O)_4ADP\alpha S$ isomer [generated from (S_P)-ADPαS] (Lin & Dunaway-Mariano, 1984) displays a positive Cotton effect (θ = 100 deg cm^2/dmol at 610 nm) and that derived from Δ-$Cr(H_2O)_4ADP\alpha S$ [generated from (R_P)-ADPαS] shows a negative Cotton effect of the same wavelength and intensity, the CD spectrum of the PPase–$Cr(H_2O)_4PPS$ reaction mixture must derive from the $Cr(H_2O)_4PPS$ enantiomer having S_P configuration. Thus, the $Cr(H_2O)_4PPS$ enantiomer consumed during the PPase-catalyzed reaction must as indicated in Chart II have the R_P configuration.

The initial increase and then decrease observed in the ellipticity of the PPase–$Cr(H_2O)_4PPS$ reaction mixture could arise from competing enzymic reaction of the (S_P)-$Cr(H_2O)_4PPS$ enantiomer or from isomerization of the S_P enantiomer to the R_P enantiomer. Since the presence of PPase

Chart II



is necessary for the reaction of the S_P enantiomer but not necessary for the isomerization of the enantiomer (providing that the isomerization is not enzyme catalyzed), these two modes of ellipticity diminution were deemed distinguishable. Accordingly, PPase was incubated with racemic $Cr(H_2O)_4PPS$ until the reaction solution had acquired an ellipticity of 25 deg cm^2/dmol at which time the enzyme was separated and the pH of the resulting solution was adjusted to 4.0. The ellipticity of the resulting solution was 23 deg cm^2/dmol. After 14.5 h, the ellipticity of the solution had only dropped to 18 deg cm^2/dmol; however, when this solution was adjusted to pH 6.0 and allowed to warm to ambient temperature in order to measure the CD spectrum, the ellipticity dropped below the level of detection. Thus, racemization of $Cr(H_2O)_4PPS$ takes place at a rapid rate at pH 6 in the absence of the enzyme. The pH dependency of the racemization rate is consistent with the pH dependency observed for the epimerization rate of β,γ-bidentate $Cr(H_2O)_4ATP$ (Dunaway-Mariano & Cleland, 1980) and α,β-bidentate $Cr(H_2O)_4ADP$ (Gruys et al., 1983). The actual rate of racemization of $Cr(H_2O)_4PPS$ at pH 6 is however, considerably greater than the epimerization rates observed for the Cr(III)–nucleotide complexes.

Preparation and Substrate Activity of $Co(NH_3)_4PPS$. Because of the instability of the $Cr(H_2O)_4PPS$ enantiomers under the PPase reaction conditions (viz., pH 6), P^1,P^2-bidentate $Co(NH_3)_4PPS$ was prepared as a potential PPase substrate. Unlike Cr(III)–aqua complexes, Co(III)–amine complexes are quite stable toward ligand exchange at pH 6, 25 °C. The $Co(NH_3)_4PPS$ enantiomers were therefore deemed potentially useful in determining the extent of the PPase stereospecificity.

The O,O-coordinated P^1,P^2-bidentate $Co(NH_3)_4PPS$ complex was prepared in very low yield (1–5%) by reaction of $[Co(NH_3)_4Cl_2]Cl$ with PPS. The low yield of the $Co(NH_3)_4PPS$ complex results from the occurrence of a number of competing reactions [leading to the very labile O,S-coordinated P^1,P^2-bidentate $Co(NH_3)_4PPS$ complex and to $Co(NH_3)_4(PPS)_2$ and $(Co(NH_3)_4)_2PPS$ complexes] and from the thermal decomposition of the desired complex once it is formed in the reaction mixture. Attempts to generate the $Co(NH_3)_4PPS$ complex under milder reaction conditions (25–40 °C vs. 80 °C and pH 5–6 vs. pH 3) failed. The $Co(NH_3)_4PPS$ complex is most conveniently purified by chromatographing it on a Dowex 50 (H^+) column with H_2O as eluant. Under these conditions $(Co(NH_3)_4)_2PPS$ and $Co(NH_3)_4PP$ will remain bound to the column while $Co(NH_3)_4(PPS)_2$ passes directly through the column. The $Co(NH_3)_4PPS$ gradually elutes from the column with continued washing.

The time course for the PPase-catalyzed hydrolysis of $Co(NH_3)_4PPS$ at pH 7 was monitored over a 17-h period by CD spectral techniques. After a 5-min period, the molar ellipticity of the reaction at λ_{max} 525 nm reached 160 deg cm^2/dmol. At a 30-min reaction period, the molar ellipticity of the reaction had increased to 536 deg cm^2/dmol, where it remained over the next 17-h period (see Figure 2). The CD spectrum of the $Co(NH_3)_4PPS$ enantiomer derived from the Λ isomer of O,O-coordinated α,β-bidentate $Co(NH_3)_4ADP\alpha S$ complex [generated from (S_P)-ADPαS] displays a positive Cotton effect

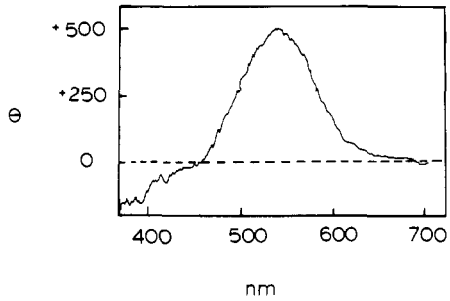
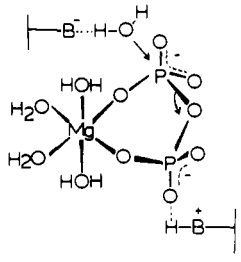


FIGURE 2: CD spectrum of a reaction mixture initially containing racemic $Co(NH_3)_4PPS$ (0.56 mM), $MgCl_2$ (10 mM), PPase (15 μM), and K^+HEPES (50 mM, pH 7.0) after incubation at 25 °C for 1 h.

Chart III



at 525 nm of 1068 deg cm^2/dmol while that derived from the Δ isomer of the O,O-coordinated α,β-bidentate $Co(NH_3)_4ADP\alpha S$ complex [generated from the (R_P)-ADPαS] displays a negative Cotton effect of −1068 deg cm^2/dmol (Lin & Dunaway-Mariano, 1981). Thus, as was observed to be the case with the active $Cr(H_2O)_4PPS$ enantiomer, the active $Co(NH_3)_4PPS$ enantiomer has the *R* configuration. Moreover, since the apparent ellipticity of the reaction mixture generated from PPase and racemic $Co(NH_3)_4PPS$ stabilized at half the value of the pure (S_P)-$Co(NH_3)_4PPS$ enantiomer, it can be concluded that (i) PPase does not catalyze the reaction of the (S_P)-$Co(NH_3)_4PPS$ enatiomer and (ii) unlike the Cr-$(H_2O)_4PPS$ complex the $Co(NH_3)_4PPS$ complex does not undergo racemization under the reaction conditions.

The K_m and V_{max} values for the $Co(NH_3)_4PPS$–PPase reaction were determined by using CD techniques to monitor the rate of disappearance of the (R_P)-$Co(NH_3)_4PPS$ enantiomer present in the racemic $Co(NH_3)_4PPS$ mixture. We found the racemic $Co(NH_3)_4PPS$ to have a K_m of 8 mM and a turnover number of 40 min^{-1} at pH 7.0.

Conclusions

The results from the present studies demonstrate that PPase cannot accommodate substitution of the bridge oxygen atom of its $MgPP_2$ substrate with an NH group nor can it accommodate substitution of the *pro-R* oxygen atom with a sulfur atom. These findings are consistent with a reaction mechanism that involves enzyme-mediated proton transfer to the *pro-R* oxygen atom of the bound $MgPP_i$ prior to or in concert with the hydrolytic cleavage step (see Chart III). Current studies are focused on testing this mechanistic model and on identifying the structure of the putative acid catalyst.

Registry No. PPase, 9024-82-2; PPS, 68488-87-9; [^{32}P]PPS, 103203-04-9; $Co(NH_3)_4PPS$, 103301-98-0; $Cr(H_2O)_4PPS$, 103301-99-1; $Mg(H_2O)_4PP_i$, 103203-01-6; $Mg(H_2O)_4PNP$, 103203-02-7; $Co(NH_3)_4PNP$, 103203-03-8; $Co(NH_3)_4PP_i$, 63915-34-4; $Mg(H_2O)_4PPS$, 103203-05-0; Na_3SPO_3, 10101-88-9; $MgPP_i$, 20768-12-1; MgPNP, 94194-77-1; MgPPS, 103203-06-1.

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