Investigation of the Catalytic Mechanism of Yeast Inorganic Pyrophosphatase†

Wilson B. Knight, Steven W. Fitts,‡ and Debra Dunaway-Mariano*

ABSTRACT: P^1,P^2-Bidentate $Co(NH_3)_4PP$ was found to be a competitive inhibitor of pyrophosphatase vs. MgPP (K_{is} = 8.7 mM, pH 7) and, in the presence of Mg^{2+}, an active substrate as well. P^1,P^2-Bidentate Cr(III) complexes of pyrophosphate, imidodiphosphate, and methylenediphosphonate were also competitive inhibitors vs. MgPP (pH 5.9; K_{is} = 0.2, 0.2, and 0.4 mM, respectively). In the presence of Mg^{2+}, P^1,P^2-bidentate $Cr(H_2O)_4PP$ was found to have a K_m 10-fold greater and a turnover number 36-fold smaller than MgPP at pH 5.9. Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, and Fe^{2+} activate the CrPP–pyrophosphatase reaction, while Ca^{2+} and Ba^{2+} are not activators but serve as competitive inhibitors vs. Mg^{2+} (K_{is} = 0.35 and 2.3 mM). At levels above 0.1 mM, Mn^{2+}, Co^{2+}, and Zn^{2+} show activator inhibition. Kinetic studies with CrPP and Mg^{2+} suggest that the kinetic mechanism is rapid equilibrium ordered, with CrPP adding before Mg^{2+}. pH studies of the MgPP/Mg^{2+} reaction and the CrPP/Mg^{2+} reaction suggest that the active form of the substrate is $(MgPP)^{2-}$ and that the uncomplexed metal ion cofactor interacts with at least two active-site residues, one possibly via H bonding and the other by direct coordination. The former group (pK_a = 5.6) appears on the basis of temperature and solvent perturbation studies to be a carboxylic acid. The MgPP reaction also requires that an active-site residue (pK_a = 7.5) be protonated. Temperature and solvent perturbation studies suggest that this residue is an amine. A mechanism accounting for these observations is presented.

In recent years much attention has been focused on the determination of mechanisms of enzymatic phosphoryl transfer reactions. Yeast inorganic pyrophosphatase catalyzes the most fundamental of such reactions, yet, surprisingly, its mechanism of action remains undetermined despite the large number of reported studies dealing with this topic. Previous studies have suggested that two divalent metal ions per enzyme subunit are required for maximal activity (Cooperman & Chiu, 1973; Rapoport et al., 1973). In vivo, this requirement is satisfied by Mg^{2+}. Kinetic studies of Mg^{2+} activation are consistent with a model requiring formation of an active enzyme–Mg^{2+} complex which can bind free PP_i, a competitive inhibitor of the reaction, or MgPP, the substrate of the reaction (Moe & Butler, 1972; Rapoport et al., 1972; Shafranskii et al., 1977). Whether or not both metal ions participate directly in catalysis is not known. However, recent NMR studies indicate that, while one of the metal ions is bound very close to one of the P_i binding sites, the second metal ion is removed from both P_i sites and, thus, may be serving simply a structural role (Hamm & Cooperman, 1978). Sperow & Butler (1976) have proposed, on the basis of studies with Cr^{3+}, that the pyrophosphate is bound to the enzyme via a metal ion bridge, while Bond et al. (1980) have provided evidence that an active-site arginine residue plays an essential role in the binding of the pyrophosphate.

There have been several kinetic mechanisms proposed for the pyrophosphatase reaction. Avaeva et al. (1977) have suggested that the reaction proceeds via a covalent enzyme–pyrophosphate intermediate. A double-displacement mechanism involving the intermediacy of phosphoenzyme, as suggested by the rapid $P_i \rightleftarrows HOH$ exchange observed by Cohn (1958), has also been considered. Recent studies, however, have failed to support either of these two mechanisms (Rapoport et al., 1973; Sperow et al., 1973). In fact, Janson et al. (1979) have demonstrated that the rapid $P_i \rightleftarrows HOH$ exchange catalyzed by pyrophosphatase need not involve the formation of a phosphorylated enzyme.

It is clear that before any firm conclusions can be drawn about the mechanism of action of pyrophosphatase, the structure of the substrate bound in the active site and the active-site groups involved in binding and catalysis must be identified. The results of studies which provide information on both of these points are presented below.

Materials and Methods

General. NMR spectra were recorded by using a Varian XL-100 spectrometer (25 °C, D_2O solvent). Absorption spectra were measured by using a Perkin-Elmer 552 spectrophotometer. Spectrophotometric kinetic studies were carried out by using a Gilford 250 spectrophotometer. ^{32}P-Labeled pyrophosphate (PP) was purchased from Amersham Chemical Co. Pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], Mes [2-(*N*-morpholino)ethanesulfonic acid], Hepes [*N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], Ches [2-(*N*-cyclohexylamino)ethanesulfonic acid], Taps [3-[[tris-(hydroxymethyl)methyl]amino]propanesulfonic acid], NADP (nicotinamide adenine dinucleotide phosphate), and all of the enzymes and pyrophosphate analogues used were purchased from Sigma Chemical Co. The buffers were adjusted to the desired pH by using KOH. $Co(NH_3)_4PP$ (Cornelius et al., 1977) and $Cr(H_2O)_4PP$ (Merritt et al., 1981) were prepared according to published procedures. $Cr(H_2O)_4$(imidodiphosphate) and $Cr(H_2O)_4$(methylenediphosphonate) were prepared from chromium chloride and imidodiphosphate or methylenediphosphonate by using the same general method used to prepare $Cr(H_2O)_4PP$. Pyrophosphatase was dialyzed at 4 °C against a solution 50 mM in Pipes (pH 7), 1 mM in EDTA (ethylenediaminetetraacetic acid), and 0.5 mM in

† From the Department of Chemistry, University of Maryland, College Park, Maryland 20742. *Received January 27, 1981.* This work was supported, in part, by grants from the Research Corporation and the National Institutes of Health (Biomedical Research Support Grant, University of Maryland). Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. A preliminary report on this work has been presented (Knight et al., 1981).

‡ A NSF–URP Fellow (SPI 7926820).

0006-2960/81/0420-4079$01.25/0

dithiothreitol and then against a solution 50 mM in Pipes (pH 7).

Kinetic Assays. The pH of the final reaction mixtures was measured at the temperature of the reaction with a Corning 112 pH meter. The pH values used for the calculation of the kinetic data derived from reactions run in 30% propylene glycol were measured from the corresponding fully aqueous reaction mixtures. All assays were run in Mes, Pipes, Hepes, Taps, or Ches buffer. Two different types of assay methods were used in the kinetic studies.

Spectrophotometric Method. All pH studies involving MgPP as substrate and all inhibition studies involving the inhibitors $Co(NH_3)_4PP$, $Cr(H_2O)_4PP$, $Cr(H_2O)_4$(imidodiphosphate), and $Cr(H_2O)_4$(methylene diphosphonate) were carried out by using this assay method. The rate of P_i (orthophosphate) formation from MgPP was determined from the increase in reaction solution absorptivity at 340 nm resulting from the formation of reduced NADPH via the following coupling system: phosphorylase *a*/glycogen (10–30 units/mL; 2 mg/mL), phosphoglucomutase (10–50 units/mL), and glucose-6-phosphate dehydrogenase/NADP (10 units/mL; 0.5 mM). The reactions were initiated by the addition of pyrophosphatase to quartz cells containing 1-mL reaction mixtures 80 mM in buffer and 20 mM in magnesium acetate and maintained at the specified temperature. The stability of pyrophosphatase to the reaction conditions was checked by preincubation experiments. The coupling enzymes were maintained at high enough concentrations in the reaction mixtures so that only the rate of the pyrophosphatase reaction was limiting.

^{32}P Radioisotopic Method. This assay procedure was used for all reactions of $Cr(H_2O)_4PP$ and for the reactions of MgPP which were used to determine the relative reactivity of MgPP and $Cr(H_2O)_4PP$ or to determine the kinetic mechanism of the $MgHPP/Mg^{2+}$–pyrophosphatase system at pH 5.3. The $[^{32}P]Cr(H_2O)_4PP$–pyrophosphatase reactions (1 mL) were terminated by addition of 60 μL of $HClO_4$. The mixture was filtered through a glass wool plug (in a disposable pipet) to remove denatured enzyme and $KClO_4$ and then adjusted to pH 2 by using 1 M NaOH; 0.3 mL of 250 mM EDTA was added and the final pH adjusted to 5. After incubation at 100 °C for 2 min, the solution was cooled. A 1-mL aliquot was transferred to 0.3 mL of 250 mM ammonium molybdate and 70 μL of 6 N HCl. After mixing, a 1-mL aliquot was removed from the solution and added to 1 mL of an isobutyl alcohol/benzene solution (1:1). The mixture was rigorously vortexed for 30 s and then allowed to stand until the two layers separated. The aqueous layer (bottom) was transferred to a test tube; 0.75-mL aliquots of the aqueous and the organic layers were added to vials containing 10 mL of Beckman Ready-solv MP or New England Nuclear Aquasol scintillation fluid. The samples were counted by using an Intertechnique scintillation counter. The fraction of substrate converted during the reaction was calculated by dividing the counts of the organic layer by the sum of the counts of the aqueous and organic layer. Color quenching by the yellow phosphomolybdate complex formed from the molybdate present in the aqueous sample and P_i present in the scintillation fluid resulted in no greater than 3% systematic error. Reactions involving $Cr(H_2O)_4PP$ at concentrations above 1 mM were carried out at a volume of 0.25 mL. The reactions were terminated by the addition of 20 μL of $HClO_4$ and the resulting mixtures diluted with water to ensure that the aliquot extracted did not contain enough phosphomolybdate to saturate the organic layer. We found that a 0.25-mL reaction mixture 5 mM in $Cr(H_2O)_4PP$ should be diluted to 4 mL before taking a 1-mL aliquot to extract. The same method of analysis was used to assay the $[^{32}P]$MgPP reactions with the exception that the EDTA step is omitted and the volume of molybdate reduced to 70 μL.

The time courses for the reactions of $Cr(H_2O)_4PP$ and MgPP were linear up to 25% conversion. For the fixed time assays, the maximum conversion was limited to 15%. All reactions were carried out at 25 °C. The metal ions were added to the reaction mixtures as their chloride salts. A fixed chloride concentration was achieved by addition of KCl.

Data Processing. All kinetic data were computer fitted by using the Fortran programs of Cleland (1979) to

$$V_0 = VA/[K(1 + I/K_i) + A] \quad (1)$$

$$V_0 = VA/(K + A + A^2/K_i) \quad (2)$$

$$V_0 = VA/(K + A) \quad (3)$$

$$V_0 = VAB/(K_BA + AB + K_{iA}K_B) \quad (4)$$

$$\log Y = \log [c/(1 + H/K_1 + K_2/H)] \quad (5)$$

$$\log Y = \log [c/((H/K_1)^2 + K_2/H)] \quad (6)$$

$$\log Y = \log [c/(1 + H/K_1)] \quad (7)$$

$$\log Y = \log [c/(1 + H/K_1 + H^2/(K_1K_2))] \quad (8)$$

$$\log Y = \log [c/(1 + K_1/H)] \quad (9)$$

where $Y = 1/K_i$, V, or V/K, c = pH-independent value of Y, K_1 and K_2 = dissociation constants of the groups that ionize, K_{iA} = dissociation constant of EA, K_B = Michaelis constant of B, A and B = substrate concentrations, I = inhibitor concentrations, H = hydrogen ion concentration, V_0 = initial velocity, and V = maximal velocity.

Results

Determination of the Structure of the Active Substrate for Pyrophosphatase. P^1,P^2-Bidentate $Co(NH_3)_4PP$ and $Cr(H_2O)_4PP$ were found to be competitive inhibitors vs. MgPP (20 mM free Mg^{2+}). The K_{is} values determined by computer fitting the data to eq 1 are 8.7 ± 0.6 mM (at pH 7) and 0.20 ± 0.01 mM (at pH 5.9), respectively. The bidentate Cr(III) complexes of imidodiphosphate and methylenediphosphonate [prepared in 30–40% yield using a procedure similar to that reported for the $Cr(H_2O)_4PP$ complex (Merrit et al., 1981)] were also found to be competitive inhibitors of pyrophosphatase with K_{is} values (at pH 5.9) of 0.21 ± 0.02 mM and 0.42 ± 0.03 mM.

The $Co(NH_3)_4PP$ and $Cr(H_2O)_4PP$ complexes were tested as substrates for pyrophosphatase in the presence of Mg^{2+}. Reaction of $Co(NH_3)_4PP$ was followed by using both ^{31}P NMR spectrometry and absorption spectrophotometry. The ^{31}P NMR spectra of the enzymatic reaction mixture [10 mM $Co(NH_3)_4PP$, 1 mM Mg^{2+}, and 25 mM Pipes, pH 7.2] taken at varying conversions showed a steady decrease in the $Co(NH_3)_4PP$ resonance (4.3 ppm downfield from H_3PO_4) and the buildup of the $Co(NH_3)_4(P_i)_2$ resonance (11.1 ppm downfield from H_3PO_4). When the reaction mixture was allowed to stand at room temperature overnight, the lavender product complex crystallized in near-quantitative yield. The proton NMR spectrum of the product complex in 0.1 M DCl/D_2O was characterized by two singlets in a 1:1 ratio at shifts of 0.78 and −0.25 ppm from that of $Co(NH_3)_6^{3+}$. In analogy to the NMR spectral properties reported previously for $Co(NH_3)_4PP$ (Cornelius et al., 1977), the geometry of the

Table I: Kinetic Constants for Divalent Cation Activators of the $Cr(H_2O)_4PP$–Pyrophosphatase Reaction (50 mM Mes, pH 5.9, 25 °C)

metal ion	K_m (mM)	K_i (mM)	relative V
Co^{2+}	0.05 ± 0.02	0.6 ± 0.3	0.6
Zn^{2+}	0.07 ± 0.02	0.23 ± 0.07	2.0
Mn^{2+}	0.036 ± 0.007	3.7 ± 0.8	3.2
Mg^{2+}	2.2 ± 0.2		1.0

$Co(NH_3)_4(P_i)_2$ complex was given a cis assignment.

The absorption changes which accompany the reaction of $Co(NH_3)_4PP$ are very evident in the visible region of the electromagnetic spectrum. At pH 7,[1] the absorption maxima of a $Co(NH_3)_4PP$ reaction mixture shifted from 371 to 375 nm and 520 to 528 nm and the absorptivity at these new maxima is increased 16% and 20%.[2] The most convenient wavelength range for following the reaction is between 585 and 590 nm (where the absorptivity doubles).

Using the ^{32}P radioisotopic assay described under Materials and Methods, we found $Cr(H_2O)_4PP$ to have a K_m of 0.20 mM [under the same conditions (10 mM Mg^{2+} and 50 mM Mes, pH 5.9), MgPP gave K_m of 18 μM] and a turnover number 36-fold smaller than that of MgPP. Like the $Co(NH_3)_4PP$ reaction, the $Cr(H_2O)_4PP$ reaction (pH 5.9) proceeds to ~95% completion.

Divalent Cation Activation of the $Cr(H_2O)_4PP$–Pyrophosphatase Reaction. Three $Cr(H_2O)_4PP$–pyrophosphatase control reactions were performed. The first reaction mixture (pH 5.9) consisted of $Cr(H_2O)_4PP$ and pyrophosphatase but did not contain divalent cations. The second contained $Cr(H_2O)_4PP$ and Mg^{2+} but no pyrophosphatase. The third contained $Cr(H_2O)_4PP$, Mg^{2+}, and the pyrophosphatase. The first two reaction mixtures were analyzed for $Cr(H_2O)_4(P_i)_2$ by extracting P_i from the reaction mixture after EDTA and molybdate treatment (see Materials and Methods). The first control reaction was determined to contain 2–3% product or product-like substance and the second control reaction, 1–2% of this substance. The third control reaction, treated directly with molybdate and extracted [omitting the EDTA treatment which removes the Cr(III) from the reactant and product complexes], contained 1% extractable material. When this same reaction mixture was first treated with EDTA, 40% of the original $Cr(H_2O)_4PP$ was extracted as product. Thus, the $Cr(H_2O)_4PP$ used in our experiments was not significantly contaminated by free pyrophosphate, and the $Cr(H_2O)_4PP$ is not a substrate of pyrophosphatase unless in the presence of suitable divalent cations.

A series of metal cations were tested as activators of the $Cr(H_2O)_4PP$–pyrophosphatase reaction (50 mM Mes, pH 5.9). At a concentration of 10 mM metal ion and 0.5 mM $Cr(H_2O)_4PP$, the relative activities of the metal ions were determined to be Mg^{2+} 100%, Co^{2+} 28%, Zn^{2+} 22%, Fe^{2+} 8%, Cd^{2+} 5%, and Ni^{2+} 3%. Li^+, K^+, Hg^{2+}, Pb^{2+}, Pd^{2+}, Be^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+}, Al^{3+}, and Cr^{3+} did not activate the reaction.[3] The K_m and relative turnover number were measured for Mg^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} and are reported in Table I. These last three cations showed, as indicated in Figure 1, inhibition at levels exceeding 0.1 mM, and, therefore, their K_m and K_i values were calculated by using eq 3. Equation 2 was used for calculation of K_m and V for Mg^{2+}. Ca^{2+} was found to be a competitive inhibitor vs. Mg^{2+}, with a K_{is} value of 0.35 ± 0.02 mM at both nonsaturating and saturating levels of $Cr(H_2O)_4PP$ (0.05 and 5.0 mM). Ba^{2+}, also a competitive inhibitor vs. Mg^{2+}, was determined to have a K_{is} value of 2.3 ± 0.2 mM (0.5 mM CrPP). The effect of Zn^{2+} concentration on the kinetic constants of the pyrophosphatase reaction was measured at varying $Cr(H_2O)_4PP$ concentrations (pH 5.9).

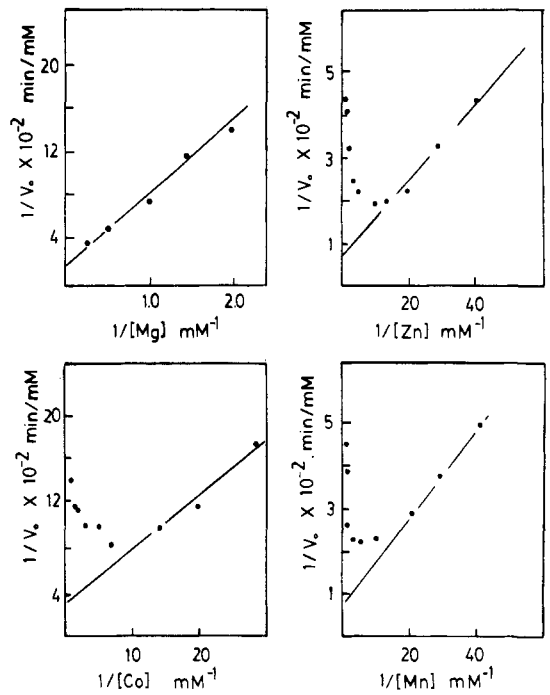

FIGURE 1: Double-reciprocal plots of metal ion activation of the $Cr(H_2O)_4PP$ (0.5 mM)–pyrophosphatase reaction. These studies were conducted with 75 mM Mes buffer at pH 5.9.

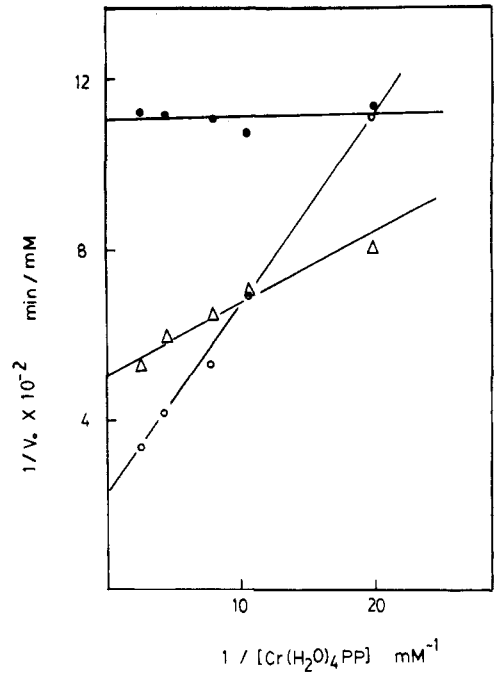

FIGURE 2: Double-reciprocal plots of the $Cr(H_2O)_4PP$–pyrophosphatase reaction at (○) 0.05 mM Zn^{2+}, (△) 1.0 mM Zn^{2+}, and (●) 3.5 mM Zn^{2+}. These studies were carried out with 75 mM Mes buffer at pH 5.9.

[1] The change is pH dependent.

[2] The spectral changes are quite similar to those noted by Cornelius & Cleland (1978) for the hexokinase-catalyzed conversion of $Co(NH_3)_4ATP$ to $Co(NH_3)_4(G6P)(ADP)$.

[3] Most of these metal ions are ionized or are not monomeric at this pH. Of the metal ions which do activate, Mn^{2+}, Zn^{2+}, and Co^{2+} are inhibitory at this concentration; thus the reported relative activities of these metal ions are not reflective of the maximum reaction velocities that would be measured for the $Cr(H_2O)_4PP$ reaction at varying metal concentration.

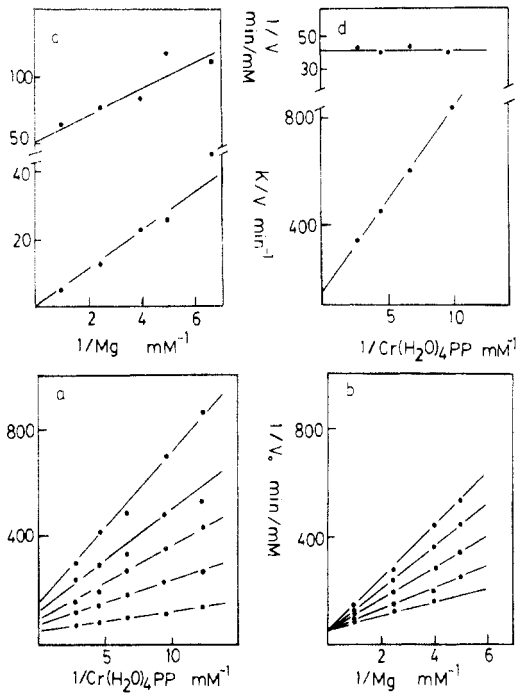

FIGURE 3: Kinetics of the $Cr(H_2O)_4PP/Mg^{2+}$–pyrophosphatase reaction at pH 6.0. (a, b) Double-reciprocal plots of initial velocity vs. $Cr(H_2O)_4PP$ at fixed Mg^{2+} concentrations of 4.0, 2.5, 2.0, and 1.5 mM or vs. Mg^{2+} at fixed $Cr(H_2O)_4PP$ concentrations of 0.353, 0.217, 0.149, 0.104, and 0.081 mM. (c, d) Slope (K/V) and intercept ($1/V$) replots of the data from (a) and (b) fitted to eq 4 (see Materials and Methods).

Table II: Kinetic Constants for the $Cr(H_2O)_4PP$–Mg^{2+}–Pyrophosphatase System Calculated for a Rapid Equilibrium Ordered System

	pH 5.1	pH 6.2
$Cr(H_2O)_4PP$, K_i (mM)	0.45 ± 0.1	0.54 ± 0.1
Mg^{2+}, K_m (mM)	25 ± 7	3.5 ± 0.8
V (mM/min)	0.03 ± 0.002	0.09 ± 0.007

The results obtained are shown in Figure 2.

Kinetic Mechanism. The kinetic mechanism of the pyrophosphatase reaction was examined by measuring the variation of reaction velocity as a function of substrate [$Cr(H_2O)_4PP$] and metal cofactor (Mg^{2+}) concentration. A typical data set is shown in Figure 3. The data from reactions carried out at pH 5.1 and 6.2 were computer fitted to eq 4 which describes the dependence of reaction rate on substrate concentration for a rapid equilibrium ordered system [in the present case, $Cr(H_2O)_4PP$ adds to the enzyme before Mg^{2+}]. The kinetic constants obtained in this manner are summarized in Table II. A similar kinetic study was used to measure the kinetic constants for the MgHPP/Mg^{2+} system at pH 5.3. As with $Cr(H_2O)_4PP/Mg^{2+}$, this system obeys rapid equilibrium ordered kinetics. The K_i for MgHPP was determined to be 0.23 ± 0.08 mM and the K_m for Mg^{2+} to be 5 ± 2 mM.

pH Studies. The pH variation of V and V/K for the MgPP–pyrophosphatase reaction (20 mM Mg^{2+}) was measured by using the coupled assay system described under Materials and Methods. The data obtained and shown in Figure 4 were computer fitted to eq 5 and 6 which describe the ionization of one group in the acid range and one group in the basic range of the pH scale (V profile) and the ionization of two groups in the acid range and one group in the basic range of the pH scale (V/K profile), respectively. pH profiles were determined at several different temperatures and at a 30% by volume propylene glycol concentration. The pK_a values

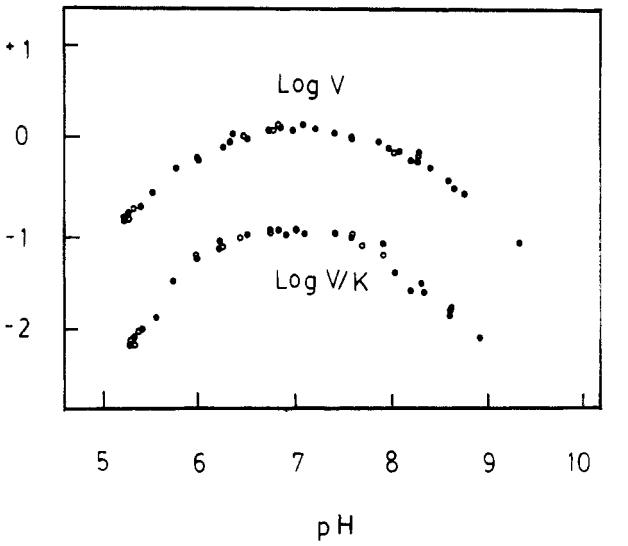

FIGURE 4: pH profile of the V (μM PP consumed per s) and V/K (s^{-1}) of the pyrophosphatase reaction carried out at a free Mg^{2+} concentration of 20 mM.

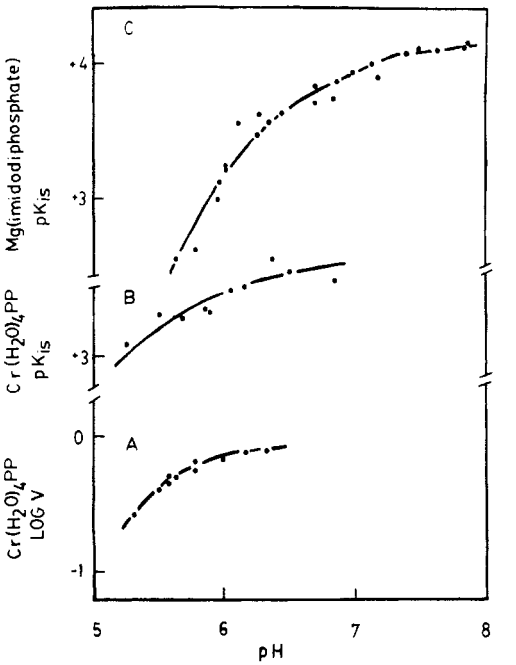

FIGURE 5: (A) pH profile of the $Cr(H_2O)_4PP$ V (μM $Cr(H_2O)_4PP$ consumed per s) measured at 10 mM free Mg^{2+}. (B) pH profile of the $Cr(H_2O)_4PP$ K_{is} (M) vs. MgPP measured at 20 mM free Mg^{2+}. (C) pH profile of the Mg(imidodiphosphate) K_{is} (M) measured at 20 mM free Mg^{2+}. The theoretical curves are shown.

calculated are provided in Table III. The pK_a values determined from the V and V/K profiles measured between pH 5 and 7.5 showed little or no temperature dependence, as did that determined from the V profile measured between pH 6.5 and 9. The pK_a determined from the V/K profile spanning the base pH range was, however, temperature dependent. The van't Hoff equation was used to determine a ΔH of 7 ± 1 kcal/mol for ionization of this group.

The pH variation of the K_{is} values of the competitive inhibitors Mg(imidodiphosphate) and $Cr(H_2O)_4PP$ (vs. MgPP at 20 mM Mg^{2+}) was measured, and the results obtained are presented in Figure 5. These data along with that measured for the pH variation in the V of the $Cr(H_2O)_4PP$ reaction at fixed, nonsaturating Mg^{2+} (20 mM) (see Figure 5) were fitted to eq 7 [K_{is} and V for $Cr(H_2O)_4PP$] and 8 which describe the ionization of a single acid group and two acid groups [K_{is} for

Table III: Acid Dissociation Constants Determined from the V, V/K, or K_{is} Profiles for Substrates or Inhibitors of Pyrophosphatase[a]

substrate or inhibitor	profile	temp of reaction (°C)	no. of groups ionizing	pK_a values[d]	eq fitted
			(I) Acid Range		
MgPP[b]	V	15.5	1	6.27 ± 0.03	7
	V	25.0	1	6.25 ± 0.02, 6.23 ± 0.05	7
	V	36.0	1	6.22 ± 0.05	7
	V/K	15.5	2	5.71 ± 0.02	8
	V/K	25.0	2	5.64 ± 0.04, 5.65 ± 0.02	8
	V/K	36.0	2	5.61 ± 0.03	8
	V/K	25.0	2	5.46 ± 0.03	6
	V/K	25.0 (solvent)[f]	2	6.14 ± 0.04	6
MgPNP[b]	K_{is}	25.0	2	6.29 ± 0.02	8
$Cr(H_2O)_4PP$[b]	K_{is}	25.0	1	5.79 ± 0.07	7
$Cr(H_2O)_4PP$[c]	V	25.0	1	5.50 ± 0.04	7
Mg^{2+}[d]	V	25.0	1	5.70 ± 0.02	7
Mg^{2+}[d]	V/K	25.0	2	6.15 ± 0.03	8
			(II) Base Range		
MgPP[b]	V	13	1	8.48 ± 0.04, 8.64 ± 0.03	9
	V	20	1	8.39 ± 0.03	9
	V	25	1	8.55 ± 0.02, 8.48 ± 0.04	9
	V	33	1	8.66 ± 0.02	9
	V	38	1	8.41 ± 0.02, 8.59 ± 0.04	9
	V/K	13	1	7.94 ± 0.03, 8.18 ± 0.02	9
		20	1	8.07 ± 0.02	9
		25	1	7.85 ± 0.03, 7.74 ± 0.02	9
		33	1	7.89 ± 0.02	9
		38	1	7.69 ± 0.04, 7.66 ± 0.02, 7.48 ± 0.04	9
		25	1	7.60 ± 0.06	6
		25 (solvent)[f]	1	7.60 ± 0.08	6

[a] All reactions were carried out in Mes, Pipes, Hepes, Taps, or Ches buffers. [b] Carried out in the presence of 20 mM Mg^{2+}. PNP stands for imidodiphosphate. [c] Carried out in the presence of 10 mM Mg^{2+}. [d] Carried out in the presence of 5 mM $Cr(H_2O)_4PP$. [e] Results from duplicate and triplicate experiments are shown. [f] Reactions were run in 30% by volume propylene glycol.

Mg(imidodiphosphate)], respectively.[4] The pK_a values determined in this manner are reported in Table III. The pH variation of V and V/K for Mg^{2+} [5 mM $Cr(H_2O)_4PP$] was determined by using the ^{32}P radioisotopic assay (see Materials and Methods). The data obtained and shown in Figure 6 were fitted to eq 7 (V) and 8 (V/K) to give the pK_a values reported in Table III.

Discussion

Previous kinetic studies of the pyrophosphatase reaction provided evidence which suggested that two divalent cations per active site are required for maximal enzymatic activity (Cooperman & Chiu, 1973; Rapoport et al., 1973). In vivo pyrophosphate exists as a Mg^{2+} coordination complex of which there exists several structural isomers in rapid equilibrium. The most abundant of these isomers is the P^1,P^2-bidentate isomer, and, as such, we felt that this might be the most probable candidate for the pyrophosphatase substrate. To test this hypothesis, we examined the binding properties and substrate activities of the exchange-inert complexes P^1,P^2-bidentate $Cr(H_2O)_4PP$ and $Co(NH_3)_4PP$. Both complexes are competitive inhibitors vs. MgPP, but only the Cr(III) complex binds tightly to the free enzyme (K_i = 0.45 mM). Interestingly, the binding affinity of the electronically equivalent Mg^{2+} species, MgHPP, is almost identical with that of $Cr(H_2O)_4PP$ (K_i = 0.23 mM). The poor binding affinity of the $Co(NH_3)_4PP$ complex (K_{is} = 8.7 mM) could be attributed to the geometry of its chelate ring (Merrit & Sundaralingam, 1980); however, the observed similarity in binding affinities of the P^1,P^2-bidentate Cr(III) complexes of pyrophosphate, imidodiphosphate, and methylenediphosphonate suggests that substrate binding is relatively insensitive to chelate ring geometry. A reasonable explanation of the weak $Co(NH_3)_4PP$ binding is that the substrate water ligands, which are absent in the $Co(NH_3)_4PP$ complex, serve to anchor the substrate in the active site via H bonding. By comparison, the NH_3 ligands are weaker H-bond donors and, of course, cannot accept H bonds from active-site group donors.

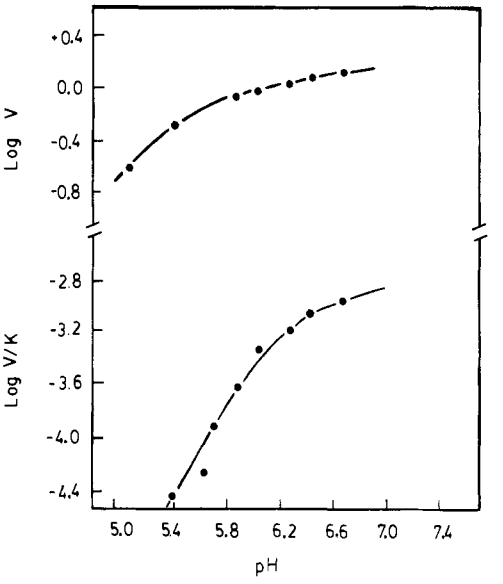

FIGURE 6: pH profiles of the V (μM $Cr(H_2O)_4PP$ consumed per s) and V/K (s^{-1}) of Mg^{2+} measured at 5 mM $Cr(H_2O)_4PP$. The theoretical curves are shown.

We determined both $Cr(H_2O)_4PP$ and $Co(NH_3)_4PP$ to be substrates for pyrophosphatase in the presence but not in the absence of added divalent cations. This finding demonstrates

[4] The magnesium imidodiphosphate K_{is} data give a better fit to eq 8 than to eq 7 (6.20 ± 0.02, δ = 0.107, vs. 6.99 ± 0.05, δ = 0.150). Sigma is the square root of the residual least square.

that the natural substrate is indeed P^1,P^2-bidentate MgPP[5] and that there is a requirement for at least one additional metal ion cofactor. In order to learn more about the catalytic role of this second metal, we carried out kinetic studies using $Cr(H_2O)_4PP$ as substrate. First, the kinetic mechanism of the $Cr(H_2O)_4PP/Mg^{2+}$ reaction was examined. As indicated by the data presented in Figure 3, the mechanism is rapid equilibrium ordered where $Cr(H_2O)_4PP$ binds before Mg^{2+}. This mechanism was also observed for the $MgHPP/Mg^{2+}$/pyrophosphatase reaction at pH 5.3. The stickiness of the $(MgPP)^{2-}$ substrate (Janson et al., 1979) is lost upon protonation. Findings which are consistent with the notion of an ordered mechanism of substrate/activator addition derive from the Ca^{2+} inhibition studies. Specifically, Ca^{2+} was found to be a linear competitive inhibitor vs. Mg^{2+}, with a K_{is} value which is independent of the level of $Cr(H_2O)_4PP$ substrate present in the reaction mixture. This suggests that Ca^{2+} and, by analogy, Mg^{2+} bind to only one form of the enzyme, i.e., the enzyme–$Cr(H_2O)_4PP$ complex.[6]

The $Cr(H_2O)_4PP$ also proved useful as a substrate for the study of the specificity of pyrophosphatase for the metal ion activator. Of the metal ions tested, only the divalent cations Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, and Fe^{2+} are activators. The kinetic constants of these first four metal ions are provided in Table I. Interestingly, Mg^{2+}, which shows catalytic activity comparable to that of Zn^{2+}, Co^{2+}, and Mn^{2+}, binds much more loosely to the enzyme. Even Ca^{2+} and Ba^{2+}, which are not activators, bind as tight or tighter than Mg^{2+} (K_{is} = 0.35 and 2.3 mM, respectively). Another major difference between the binding properties of Mg^{2+} and those of Zn^{2+}, Co^{2+}, and Mn^{2+} is that at levels exceeding 0.1 mM the latter three metals, as indicated by Figure 1, become inhibitory. Using Zn^{2+} as the activation ion, we briefly explored the mechanism of the observed activator inhibition.[7] As shown in Figure 2, increasing Zn^{2+} levels results in tighter $Cr(H_2O)_4PP$ binding to the enzyme, but also in decreased V. Such a result may, in fact, be expected for an ordered mechanism where metal ion dissociation must precede product release. The natural activator, Mg^{2+}, binds relatively loosely to the enzyme–substrate complex (K_m is close to physiological levels) and presumably even more loosely to the enzyme–product complex. Zn^{2+}, Co^{2+}, and Mn^{2+}, on the other hand, bind very tightly to the enzyme–substrate complex and perhaps tight enough to the enzyme–product complex to inhibit product release at relatively low metal ion levels (i.e., 0.1 mM). Alternatively, the observed activator inhibition could be accounted for if it is assumed that Zn^{2+}, Co^{2+}, and Mn^{2+} (and not Mg^{2+} or Ca^{2+}) selectively bind to a second site on the enzyme which either directly or indirectly traps product in the active site. While we favor the first explanation, we are currently conducting studies designed to distinguish between the two possible inhibition mechanisms.

For derivation of information regarding the participation of active-site residues in substrate binding and catalysts, pH studies were conducted. Initial investigations were carried out by using MgPP as substrate in the presence of 20 mM Mg^{2+}. From the V and V/K pH profiles shown in Figure 4, it can be deduced that at least three residues (enzyme groups or substrate) must be in the proper ionization state for substrate binding and catalysis to occur. One of these groups must be protonated for activity. The apparent pK_a of this group (7.6) is well out of the range of that of Mg^{2+} bound water (11.4) and MgHPP [6.4, Frey & Stuehr (1972)] and, hence, is an enzyme group. The value of the ΔH of ionization determined for this group (7 kcal/mol), along with the observed lack of effect of added organic solvent on its pK_a value (see Table III), suggests that the group is an amine. While Bond et al. (1980) have provided evidence for an essential active-site arginine, the present data are not sufficient so as to allow us to rule out the possibility that the active-site residue is a histidine or lysine.

The MgPP V/K profile (Figure 4) measured over the acid pH range indicates that there are two groups that must be ionized for substrate binding and/or catalysis to occur. Only one of these ionizations appears in the MgPP V profile. The pK_a values determined from these profiles (see Table III) fall close to that of MgHPP. In order to determine if one of the ionizations seen in the V/K profile is in fact the ionization of MgHPP to MgPP, we examined the pH profiles of the binding of substrate analogues to the enzyme. The K_{is} profile of Mg(imidodiphosphate) shown in Figure 5 reflects the ionization of two groups over the acid pH range which have an averaged pK_a value (6.29) higher than the average of the two pK_a values determined from the MgPP V/K profile. A shift in pK_a to a higher value is expected when the pK_a is measured by using a competitive inhibitor as compared to a substrate (when the substrate is sticky), but the present shift in pK_a may also be due to the higher pK_a of monoprotonated Mg(imidodiphosphate) relative to MgHPP (~8 vs. 6.4).[8] The K_{is} profile of $Cr(H_2O)_4PP$ (Figure 5), an inhibitor which remains fully ionized over the pH range in study, revealed the ionization of a single group having a pK_a of 5.7. Taken together, the results from these two studies strongly suggest that the binding between pyrophosphatase and substrate is determined by the protonation state of both the substrate and an enzyme group. The results from the temperature and solvent perturbation studies (Table III) are supportive of the assignment of one of the ionizations to the substrate and indicate that the named enzyme group is a carboxylic acid. Specifically, the enthalpies of ionizations of both MgHPP (Irani, 1961) and carboxylic acids (Cleland, 1977) are known to be quite small ($\leq \pm 1$ kcal/mol), as was observed for the groups in question. In addition, the results from the solvent perturbation study indicate that these groups are neutral rather than cationic acids.

Our attention was next focused on determining the role of this enzyme group in the MgPP hydrolysis. Because the ionization of the enzyme residue is seen in both the $Cr(H_2O)_4PP$ V– and K_{is}–pH profiles, it was concluded that this group was also the source of the ionization observed in the MgPP V–pH profile. Since each of the pH profiles described above was measured at a fixed free Mg^{2+} concentration (10–20 mM), it occurred to us that an anionic enzyme residue might affect both substrate binding and catalysis if it were to bind the second metal ion activator. Accordingly, the pH dependence of the $Cr(H_2O)_4PP$ and Mg^{2+} binding was examined. As indicated by the results shown in Table II, at saturating Mg^{2+} the binding of $Cr(H_2O)_4PP$ is pH independent (over the acid range) while at saturating $Cr(H_2O)_4PP$ the binding affinity of Mg^{2+} drops dramatically at low pH. The Mg^{2+} levels used

[5] It may be argued that this result does not rule out free pyrophosphate as a substrate. However, the fact that $NiPP/Ni^{2+}$ is not a substrate (Butler & Sperow, 1977) but $Cr(H_2O)_4PP/Ni^{2+}$ is suggests at least two roles for the metal ion cofactor, one of which is to complex the pyrophosphate.

[6] It may be argued that Ca^{2+} binds to both E and ES with equal affinity, but we feel that this is improbable.

[7] We ruled out inhibition by contaminating heavy metal ions on the basis that Mn^{2+} which was freed from traces of heavy metals by dithiazone/CCl_4 extraction still showed activator inhibition.

[8] Coordination of Mg to HPP decreases the pK_a of the latter from 8.4 to 6.6 (Frey & Stuehr, 1972). Coordination of Mg^{2+} to imidodiphosphate should drop the pK_a from 10.2 (Irani, 1961) by at least this amount.

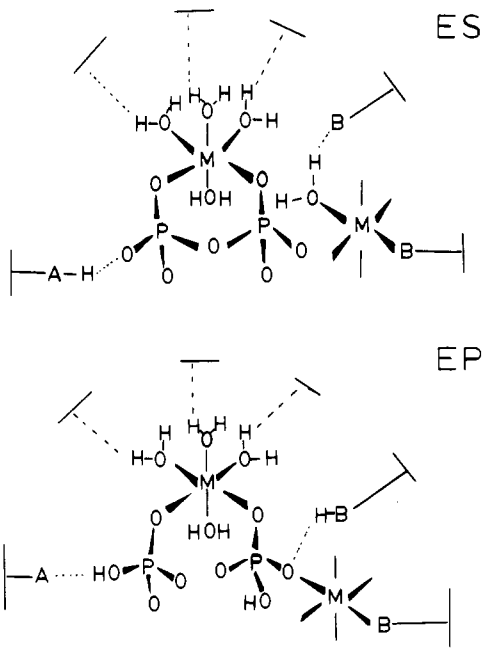

FIGURE 7: Proposed mechanism of action of pyrophosphatase.

for measurement of the $Cr(H_2O)_4PP$ K_{is}- and V-pH profiles were not high enough for Mg^{2+} to be saturating at the low end of the pH range. Similarly, it was found that while 20 mM Mg^{2+} is saturating in the MgPP reaction at pH 7, it is not saturating below pH 6. In the presence of saturating MgHPP, the K_m of Mg^{2+} is 5 mM at pH 5.3.[9]

In order to further probe for enzyme residues which may be responsible for binding the activator metal ion, the effect of pH on the pyrophosphatase reaction at varying Mg^{2+} concentrations and fixed, saturating $Cr(H_2O)_4PP$ concentration was measured. As indicated by the results shown in Figure 6, there exists at least two enzyme groups which affect Mg^{2+} binding. By comparison of the pK_a values obtained from the V and V/K profiles, it is clear that binding of Mg^{2+} to the enzyme has a dramatic effect on ionization of these two groups. The pK_a of one of the two groups is too low to be observed in the V profile while the second appears to be decreased by at least 0.8 pH unit. It is likely that the first group coordinates to the Mg^{2+} while the second H-bonds to the Mg^{2+} water ligands.[10]

On the basis of the present data, we feel that pyrophosphatase may follow the mechanism shown in Figure 7. For this mechanism, it is assumed that the active substrate is the fully ionized P^1,P^2-bidentate $Mg(H_2O)_4PP$ complex and that it is anchored in the active site via H bonds between active-site residues and the Mg water ligands. The phosphate that will be displaced during the hydrolytic step is H bonded to an active-site residue which is probably an amine. Presumably proton transfer from this residue will occur during the cleavage of the phosphoanhydride bond. The second metal ion binds very close to the phosphoryl moiety that will undergo the nucleophilic attack. Since this metal does not bind to the free enzyme, it is likely that the binding of the MgPP to the enzyme triggers a conformational change which aligns active-site groups for coordination of the metal. H-bonding interactions occur between the coordinated metal and an active-site residue and perhaps to the phosphoryl moiety itself. As indicated in Figure 7, deprotonation of one of the activator metal water ligands accompanies its attack on the phosphorus. Upon formation of the *cis*-$Mg(H_2O)_4(Pi)_2$ product complex, the enzyme may undergo another conformational change which exposes the coordinated metal to solvent water, allowing hydrolysis to occur and, ultimately, product release.[11] According to this mechanism, Ca^{2+} and Ba^{2+} do not serve as activators because they are not acidic enough to undergo the deprotonation step. Zn^{2+}, Co^{2+}, and Mn^{2+} may cause activator inhibition by maintaining a high affinity for the binding site provided by the bound $Mg(H_2O)_4(Pi)_2$ and active-site group ligands. Mg^{2+} presumably binds very loosely to this site. An interesting feature of the proposed mechanism concerns the apparent roles of the two metal ion cofactors. By coordination to the pyrophosphate, one metal ion aids in anchoring the substrate to the active site via H-bond interactions with the water ligands and at the same time aligns and via e^- induction activates the phosphoryl group for attack. The second metal ion serves to overcome the large entropic barrier required to freeze a water molecule in a precise orientation in the active site by fixing the water via a coordination bond.[12]

References

Avaeva, S. M., Bakuleva, N. P., Baratova, C. A., Nazarova, T. I., & Fink, N. Yu. (1977) *Biochim. Biophys. Acta 482*, 173–184.

Bond, M. W., Chiu, N. Y., & Cooperman, B. S. (1980) *Biochemistry 19*, 94–102.

Butler, L. G., & Sperow, J. W. (1977) *Bioinorg. Chem. 7*, 141–150.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273–387.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 84–103.

Cohn, M. (1958) *J. Biol. Chem. 230*, 369–379.

Cooperman, B. S., & Chiu, N. Y. (1973) *Biochemistry 12*, 1670–1676.

Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279–3285.

Cornelius, R. D., Hart, P. A., & Cleland, W. W. (1977) *Inorg. Chem. 16*, 2799–2805.

Frey, C. M., & Stuehr, S. J. (1972) *J. Am. Chem. Soc. 94*, 8898–9204.

Hamm, D. J., & Cooperman, B. S. (1978) *Biochemistry 17*, 4033–4040.

Irani, R. R. (1961) *J. Phys. Chem. 65*, 1463–1465.

Janson, C. A., Degani, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 3743–3749.

Knight, W. B., Fitts, S. W., & Dunaway-Mariano, D. (1981) 15th Middle Atlantic Regional Meeting of the American

[9] We have not accurately measured the K_m for Mg^{2+} by using $(MgPP)^{2-}$ as substrate at pH 7 but, from preliminary data, estimate that it is ~0.2 mM.

[10] A reviewer of this manuscript has suggested that the second enzyme group may also be directly coordinated to Mg^{2+}. We however, feel that such coordination would result in a decrease in the pK_a of this group by more than the observed 0.8 pH unit. This reviewer also suggested that the enzyme group in question may be located at an allosteric site.

[11] The mechanism shown in Figure 7 might be modified to allow uncomplexed "hydroxide" to attack the phosphorus. Accordingly, binding of the second metal activator (second sphere) is accompanied by a conformational change which inserts an active-site group into the metal coordination, displacing a water ligand (H bonded to an active-site residue) against the phosphoryl phosphorus to which it will bond. The active site would be closed, the water trapped between the phosphate, and the metal given no choice but to add to the phosphorus or add back to the metal and thereby reverse the process.

[12] Preliminary ESR studies carried out in collaboration with J. J. Villafranca have indicated that the metal–metal distance between active site bound $Cr(H_2O)_4$(imidodiphosphate) and Mn^{2+} is 9 Å, which is the distance one might expect to occur between the metal center of the substrate and the second metal ion cofactor bound in the active site in the manner shown in Figure 7.

Chemical Society, Washington, D.C., Abstr. BIOL 109.
Merrit, E. A., & Sundaralingam, M. (1980) *Acta Crystallogr., Ser. B B36*, 2576-2584.
Merrit, E. A., Sundaralingam, M., & Dunaway-Mariano, D. (1981) *J. Am. Chem. Soc.* (in press).
Moe, O. A., & Butler, L. G. (1972) *J. Biol. Chem. 247*, 7308-7314.
Rapoport, T. A., Hohne, W. E., Reich, J. Heitman, P., & Rapoport, S. J. (1972) *Eur. J. Biochem. 26*, 237-246.
Rapoport, T. A., Hohne, W. E., Heitman, P., & Rapoport, S. J. (1973) *Eur. J. Biochem. 33*, 341-347.
Shafranskii, V. A., Baikov, A. A., Andrikovich, P. F., & Avaeva, S. M. (1977) *Biokhimiya (Moscow) 42*, 1244-1250.
Sperow, J. W., & Butler, L. G. (1976) *J. Biol. Chem. 251*, 2611-2612.
Sperow, J. W., Moe, O. A., Ridlington, J. W., & Butler, L. G. (1973) *J. Biol. Chem. 247*, 7308.

Investigation of the Nature of Enzyme-Coenzyme Interactions in Binary and Ternary Complexes of Liver Alcohol Dehydrogenase with Coenzymes, Coenzyme Analogues, and Substrate Analogues by Ultraviolet Absorption and Phosphorescence Spectroscopy†

S. Subramanian,* J. B. Alexander Ross,‡ Philip D. Ross, and L. Brand

ABSTRACT: The difference spectra of binary and ternary complexes of horse liver alcohol dehydrogenase with oxidized and reduced nicotinamide adenine dinucleotides, nicotinamide 1,N^6-ethenoadenine dinucleotide, and adenosine diphosphate ribose along with a number of substrate analogues have been measured. These spectra bear a very close resemblance to those obtained by perturbation of the coenzyme(s) and their analogues by acid, NaCl, dioxane, or *tert*-butyl alcohol. It is inferred that the coenzymes experience a combination of ionic and nonpolar environments at the adenine binding site of the enzyme. This is borne out by published X-ray crystallographic results. The phosphorescence spectra do not indicate the presence of ionized tyrosine in ternary complexes involving enzyme, coenzyme, and substrate analogues. The ultraviolet spectra can be explained as arising from the perturbation of the coenzyme chromophores upon binding to the enzyme without having to invoke tyrosine ionization.

The extensive X-ray crystallographic studies of liver alcohol dehydrogenase (LADH)[1] and several of its binary and ternary complexes (Brändén et al., 1975; Brändén, 1977; Eklund & Brändén, 1979) indicate that LADH undergoes a conformational change upon formation of complexes containing the nicotinamide adenine dinucleotide coenzymes, NAD^+ and NADH. In solution studies, Subramanian & Ross (1977, 1978) found that the thermodynamic parameters for the binding of NAD^+ and NADH to LADH differed dramatically from those obtained for the binding of NAD^+ and NADH to other dehydrogenases. In addition, the thermodynamic parameters for the binding of ADP-ribose to all the dehydrogenases studied (Subramanian & Ross, 1978), including LADH, were very similar. These results were also consistent with the occurrence of a conformational change in LADH upon binding NAD^+ or NADH but not ADP-ribose. Further thermodynamic studies (Subramanian & Ross, 1979) demonstrated that the binding of NAD^+ to LADH was accompanied by a proton dissociation from a zinc-bound water on the enzyme while no proton release was detected upon binding NADH to LADH in the pH range 6-8.6.

The tryptophan fluorescence of LADH is partially quenched upon binding of NAD^+ and NADH (Luisi & Favilla, 1970) as well as a number of other reagents (Theorell & Tatemoto, 1971). The quenching produced by NADH is usually attributed to energy transfer (Theorell & Tatemoto, 1971). The quenching by NAD^+ has been ascribed to both energy transfer and a conformational change by Abdallah et al. (1978). In the binary and ternary complexes of LADH with NAD^+, it was argued (Laws & Shore, 1978) that the fluorescence quenching could be due neither to resonance energy transfer nor to collisional quenching by virtue of the fact that the tryptophan residues are far from the active center of the enzyme. Tryptophans-314 which are in a hydrophobic milieu are reported not to change their environment as a result of the conformational change while tryptophans-15 already in an aqueous environment are reported to become more exposed to the solution (Eklund & Brändén, 1979). Hence, it was postulated by Laws & Shore (1978) that ternary complex formation causes a conformational change and a consequent ionization of tyrosine-286 at the surface of the enzyme. They further suggested that resonance energy transfer from Trp-314 to ionized Tyr-286 was responsible for the quenching of protein fluorescence.

It is the purpose of this communication to study the LADH-coenzyme interactions by ultraviolet difference

† From the Laboratory of Nutrition and Endocrinology (S.S.) and the Laboratory of Molecular Biology (P.D.R.), National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205, and the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218 (J.B.A.R. and L.B.). *Received January 23, 1981.* This research was supported in part by NIH Grant GM 11632.

‡ Present address: Department of Laboratory Medicine, University of Washington, Seattle, WA 98195.

[1] Abbreviations used: LADH, liver alcohol dehydrogenase (EC 1.1.1.1); ϵNAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; TFE, 2,2,2-trifluoroethanol; Me_2SO, dimethyl sulfoxide; IBA, isobutyramide; Pyr, pyrazole.

0006-2960/81/0420-4086$01.25/0