Yeast Inorganic Pyrophosphatase. Functional and $^{113}Cd^{2+}$ and ^{31}P Nuclear Magnetic Resonance Studies of the Cd^{2+}–Enzyme†

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ABSTRACT: We have exploited the nuclear spins of $^{113}Cd^{2+}$ and ^{31}P to characterize Cd^{2+} binding sites on yeast inorganic pyrophosphatase (PPase) and to elucidate the nature of the Cd^{2+}–inorganic phosphate (P_i) interaction on the enzyme surface. We have also carried out functional studies on the Cd^{2+}–enzyme to test the relevance of our results for the more active Mg^{2+}–enzyme. Our major results and conclusions are as follows: (1) Equilibrium dialysis (using $^{115}Cd^{2+}$) and ^{113}Cd NMR agree in showing the presence of one high-affinity and several low-affinity Cd^{2+} sites per enzyme subunit in the absence of P_i and three high-affinity sites per subunit in the presence of P_i. This latter result agrees with previous results found with Mg^{2+}, Mn^{2+}, and Co^{2+} (Springs et al., 1981; Cooperman et al., 1981). (2) By their chemical shifts, all three tightly bound $^{113}Cd^{2+}$ ions in the presence of P_i have predominantly or exclusively oxygen ligands in their inner spheres. This is consistent both with chemical modification studies, indicating the absence of lysines, histidines, and cysteines, and with our inability to oxidize enzyme-bound Co^{2+} to Co^{3+}, a process which would require inner-sphere nitrogen coordination to Co^{2+}. (3) In the presence of three bound $^{113}Cd^{2+}$ ions per subunit, we observe distinct P_i resonances corresponding to P_i bound to the high-affinity site (site 1) and to the low-affinity site (site 2). Both of these resonances show large downfield shifts from P_i in solution ($\sim$8 ppm and $\sim$3 ppm, respectively). Interestingly, binding of P_i to site 2 modulates the chemical shift in site 1, causing a shift of some 0.8 ppm upfield. This modulation supports the idea that the two sites are close to one another. (4) The line width for P_i bound to site 1 narrows when $^{112}Cd^{2+}$ ($I = 0$) replaces $^{113}Cd^{2+}$ ($I = {}^1/_2$), thus providing very strong evidence for scalar coupling and inner-sphere binding between $^{113}Cd^{2+}$ and $^{31}P_i$. By contrast, little narrowing is seen for P_i bound to site 2, a result consistent with, but not proving, a lack of inner-sphere binding at this site. (5) Distinct ^{31}P resonances corresponding to enzyme-bound P_i are only seen in the presence of *three* bound Cd^{2+} ions per subunit, despite the fact that P_i is bound to PPase even in the absence of Cd^{2+}. This observation is consistent with other evidence for a conformational change accompanying the binding of a third metal ion per subunit. (6) Measures of catalytic function (PP_i hydrolysis, H_2O–P_i oxygen exchange, enzyme-bound PP_i formation) obtained in the presence of Cd^{2+} allow calculation of the equilibrium constants and rate constants for overall PPase catalysis. The major result is that whereas the affinities for PP_i and both P_i bindings are not very different from the values found in the presence of Mg^{2+} (constants within an order of magnitude), the rate constants corresponding to PP_i hydrolysis and re-formation on the enzyme are drastically lower, $\sim 10^5$-fold. (7) Because of the slow rate constants involved, the pre-steady-state rate of EPP_i formation could be measured without need of a rapid quench apparatus. The rate constant obtained agrees within experimental error with that predicted from the values of k_3 and k_4 obtained from equilibrium and steady-state measurements, demonstrating the adequacy of our minimal scheme in accounting for PPase catalysis, at least in the presence of Cd^{2+}.

In recent years several groups, including our own, have devoted a substantial effort toward the goal of obtaining a detailed understanding of the mechanism of action of yeast inorganic pyrophosphatase (EC 3.6.1.1) (PPase)[1] [for a recent review, see Cooperman (1982)]. This enzyme is a dimer of identical subunits (Heinrikson et al., 1973). In our previous work with PPase, we have presented evidence for two P_i sites per subunit, one of high affinity, denoted site 1, and one of lower affinity, denoted site 2. We have also shown that in the presence of P_i, and as a requirement for enzyme activity, each subunit binds three divalent metal ions (Hamm & Cooperman, 1978; Cooperman et al., 1981; Springs et al., 1981). A major mechanistic question posed by these results is the extent to which the bound divalent ions interact with either of the two P_is bound in sites 1 and 2. A possible approach to this question is suggested by results recently obtained with bacterial alkaline phosphatase (Armitage & Otvos, 1982). This enzyme also binds three divalent metal ions per subunit. Substitution of $^{113}Cd^{2+}$, having nuclear spin ${}^1/_2$, for the native divalent metal ions (two Zn^{2+} and, most probably, one Mg^{2+}) led not only to the observation of three different chemical shifts for enzyme-bound Cd^{2+}, corresponding to the three binding sites (Otvos & Armitage, 1980), but also to direct evidence for inner-sphere binding of enzyme-bound Cd^{2+} to enzyme-bound P_i, based on the observation of scalar coupling between $^{113}Cd^{2+}$ and $^{31}P_i$ (Otvos et al., 1979).

In this paper, we exploit the probe properties of $^{113}Cd^{2+}$ to characterize the metal ion binding sites on PPase and to determine the nature of the divalent metal ion–P_i interaction on the enzyme surface, using both ^{113}Cd and ^{31}P NMR. We also determine both the affinity and stoichiometry of Cd^{2+} binding to the enzyme and the kinetic and thermodynamic parameters

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[1] Abbreviations: FID, free induction decay; Mes, 4-morpholineethanesulfonate; PPase, yeast inorganic pyrophosphatase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; Tris, tris(hydroxymethyl)-aminomethane; PPO, 2,5-diphenyloxazole; Me_2POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene. The subscript T indicates the total stoichiometric concentration of a species added to a solution.

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for PPase catalysis of both PP_i hydrolysis and H_2O–P_i oxygen exchange, in the presence of Cd^{2+} as the required divalent metal ion cofactor. These determinations not only are important for interpretation of the NMR results but also allow comparison with similar results previously obtained with other divalent metal ions which confer higher activity on PPase, such as Mg^{2+} (Springs et al., 1981) or Co^{2+} or Mn^{2+} (Cooperman et al., 1981).

Experimental Procedures

Materials

The following materials were obtained from the sources indicated: ^{113}CdO and ^{112}CdO at 96% atom enrichment (Oak Ridge National Laboratory); $^{115m}CdCl_2$ and carrier-free $^{32}PP_i$ (New England Nuclear); carrier-free $^{32}P_i$ (ICN); Cd atomic absorption standard, ultrapure HCl, and KOH (Alfa); ^{18}O-enriched water (≥99%, Norsk-Hydro); D_2O (99.8%, Aldrich); monobasic potassium phosphate, primary standard grade (Fisher); Dowex 1-X2 (200 mesh), Dowex 50-X8 (200 mesh), and Chelex-100 (200–400 mesh) (Bio-Rad). All other chemicals were reagent grade and used without further purification.

PPase was prepared as described previously (Cooperman et al., 1973) with modifications (Bond, 1979) and ranged in specific activity from 480 to 720 μmol min^{-1} mg^{-1} as determined by the standard titrimetric assay (Cooperman et al., 1973). As appropriate, protein could be regenerated free of divalent metal ion by equilibration against Chelex-100 (Na^+ form) in 50 mM Tris at pH 7.0, followed by removal of the resin. Enzyme was routinely concentrated under nitrogen with an Amicon stir cell containing a PM-10 filter. Equilibration of the enzyme with D_2O was also accomplished by repetitive dilution and concentration in the Amicon stir cell. Protein concentration was determined by A_{280}, using an extinction coefficient of a 0.1% solution equal to 1.45 (Kunitz, 1952). A subunit molecular weight of 35 000 was assumed in these calculations (Bond et al., 1980; Cooperman et al., 1981).

Methods

Equilibrium Dialysis. Dialysis experiments were performed essentially as described previously (Cooperman et al., 1981) at 22 ± 1 °C in buffer A [100 mM Tris-HCl (pH 7.0, measured at room temperature) and 100 mM KCl]. Cd^{2+} concentration was determined by liquid scintillation counting of the ^{115m}Cd β decay, using an Ansitron with a wide-open window. The scintillation cocktail contained 4 mL of a 3:1 toluene (0.5% PPO, 0.01% Me_2POPOP):Triton X-100 mixture.

^{113}Cd NMR. NMR spectra were taken on either a Bruker HFX-90-MHz spectrometer operating at 19.96 MHz (Yale) or a Bruker CXP-200-MHz spectrometer operating at 44.37 MHz (NIH Middle Atlantic NMR Facility at the University of Pennsylvania) at 25 °C. The operating parameters, spectral width (5000 Hz), and exponential line broadening applied to the free induction decay (FID) (30 Hz) were common for both spectrometers. Several additional parameters were specific for each spectrometer. The pulse angle, acquisition time, and pulse delay were, respectively, 40°, 0.2 s, and 0.8 s for the HFX-90 and 90°, 1.64 s, and 1.4 s for the CXP-200. $^{113}Cd^{2+}$ and P_i concentrations were determined by atomic absorption spectroscopy and the method of Josse (1966), respectively. All spectra were taken in buffer B [50 mM Tris-HCl (pH 7.0 at room temperature) and 90% D_2O] unless otherwise noted.

^{31}P NMR. NMR spectra were taken on a Bruker 360-MHz spectrometer (NIH Middle Atlantic NMR Facility at the University of Pennsylvania) operating at 145.7 MHz at 4 °C. Spectral parameters were the following: spectral width, 8064 Hz; pulse angle 90°; acquisition time, 1 s; pulse delay, 3 s. The T_1 values for observed phosphate resonances ranged from approximately 3 to 5 s. As the total repetition time was 4 s, differences in intensities due to incomplete relaxation could not exceed 30%. Methylphosphonic acid was used as an external standard in a 3-mm coaxial tube. Broad-band proton decoupling was employed throughout these measurements, except when noted in the text. An exponential line broadening of 5 Hz was applied to the observed FID. All spectra were taken in buffer B unless otherwise noted.

^1H NMR. Approximate T_1 values for water protons in H_2O–D_2O mixtures (6:94) in the presence of 10 mM $^{113}CdCl_2$ or $^{112}CdCl_2$ were determined by repetition of 90° scans with a decreasing repetition rate. Spectra were taken on the Bruker 360-MHz instrument. Operating parameters were the following: spectral width, 4000 Hz; pulse angle, 90°; acquisition time, 2 s; pulse delay, 1–60 s.

PP_i Hydrolysis. Initial rates were measured as described in Springs et al. (1981) except that reactions were initiated by the addition of PP_i. All reactions used buffer C (50 mM Tris-HCl, pH 7.0 at room temperature, and 200 mM KCl) unless otherwise noted.

Water–Phosphate ^{18}O Exchange. ^{18}O exchange was measured by ^{31}P NMR (Cohn & Hu, 1978) as previously reported (Springs et al., 1981) with some modifications. Reaction mixtures were made up in buffer C. Aliquots were quenched with a final concentration of 0.17 N HCl and 0.17% sodium dodecyl sulfate. In typical analyses, quenched samples (3.0 mL) containing 0.025 mM PPase, 8.5 mM P_i, and 0.08–0.4 mM Cd^{2+} were incubated for at least 3 h at room temperatures and each applied to a Dowex 50 column (0.8 cm × 6 cm). The P_i, eluted with 1 mL of 1 N HCl, was adjusted to pH ~8, applied to a Chelex-100 column (0.8 cm × 7 cm), and eluted with H_2O. Samples were then lyophilized, redissolved in 50% D_2O containing 50 mM ethylenediaminetetraacetic acid (EDTA), and adjusted to pH 8.2 ± 0.2.

Enzyme-Bound Pyrophosphate Formation. PPase (0.50 mM) in buffer C, 0.5–3 mM $CdCl_2$, and 2.0–30 mM P_i were incubated at 25 or 4 °C for the indicated time in rate studies and for at least 1 h in equilibrium studies. A control, identical except that it contained only 1.4 μM PPase and 0.26 mM bovine serum albumin, was run in parallel. Equilibration solutions were 0.1 mL in total volume. Aliquots (0.02 mL) at each [P_i] were quenched with 0.03 mL of 4 M trichloroacetic acid. The remainder of the experimental procedure was as previously described (Springs et al., 1981), except that the equilibrium determinations were made in quadruplicate.

Results

Equilibrium Dialysis Measurement of Cd^{2+} Binding. Equilibrium dialysis experiments were used to measure binding of $^{115}Cd^{2+}$ to PPase in the presence and absence of P_i. The observed data are plotted in Scatchard form in Figure 1. In the absence of P_i (Figure 1a), nonequivalent sites are indicated. The data clearly suggest the presence of one high-affinity site per subunit as well as some larger number of low-affinity sites. In previous work, we and others have presented evidence for a total of three to four divalent metal ions per subunit (Moe & Butler, 1972; Cooperman et al., 1981; Springs et al., 1981). Accordingly, data were fit to a theoretical curve described by eq 1, where n represents the number of equivalent sites of type

$$\nu = \sum_i \frac{n_i[L]}{K_i + [L]} \quad (1)$$

i, K represents the corresponding dissociation constant, and

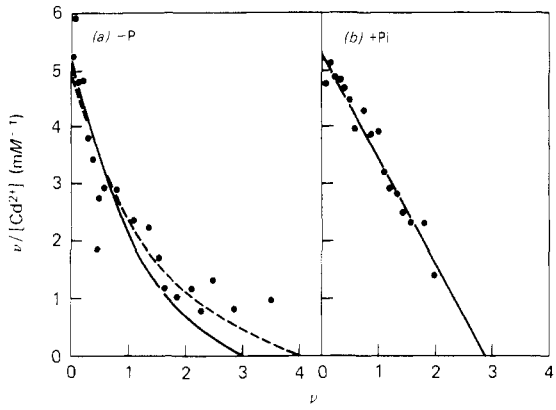


FIGURE 1: Scatchard plots of Cd^{2+} binding to PPase measured by equilibrium dialysis. (a) No P_i added, [PPase] = 50–500 μM; theoretical curve to eq 1 with K_1 = 0.225 mM, n_1 = 1 and K_2 = 2.5 mM, n_2 = 2 (solid line); with K_1 = 0.25 mM, n_1 = 1 and K_2 = 3.0 mM, n_2 = 3 (dashed line). (b) In the presence of 0.5 mM P_i, [PPase] = 200 μM. Line is linear least-squares fit to data with n = 3 and K_D = 0.55 mM.

FIGURE 2: ^{113}Cd NMR spectra at 19.96 MHz of solutions of PPase, $^{113}Cd^{2+}$, and P_i, 25 °C.

	$[PPase]_T$ (mM)	$[^{113}Cd^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	pH	no. of scans
(a)	1.54	1.38		7.3	80 000
(b)	2.73	8.18		6.4	70 700
(c)	2.73	8.18	2.73	6.5	160 000
(d)	2.73	8.18	8.18	7.15	134 400

[L] represents the free ligand concentration. Assuming one high-affinity site, and either two or three low-affinity sites, yielded the dissociation constants listed in the legend to Figure 1. The Scatchard plot changes dramatically on addition of P_i (Figure 1b) and is most straightforwardly interpreted as showing the presence of three tight sites of approximately equal affinity, whose dissociation constant is also listed in the legend to Figure 1. Measurement of ν at higher Cd^{2+} and P_i concentrations were prevented by cadmium phosphate precipitation.

NMR Spectra of Enzyme-Bound $^{113}Cd^{2+}$. Representative $^{113}Cd^{2+}$ NMR spectra of solutions of $^{113}Cd^{2+}$ and PPase, in the absence and presence of P_i, are presented in Figure 2, and the peak positions and line widths are listed in Table I. Only a single peak is observed for $[Cd^{2+}]_T < [PPase]_T$ (Figure 2a).

Table I: $^{113}Cd^{2+}$ Chemical Shifts and Line Widths[a]

conen (mM) PPase	$^{113}Cd^{2+}$	P_i	δ[b]	$\Delta\nu_{1/2}$ (Hz)	pH
1.5	1.35	0.00	−22.6	75	7.3
2.7	2.7	0.00	−23.0	99	6.5
2.07	8.2	0.00	17.7	150	6.4
			−4.8	290	
			−25.6	247	
2.7	8.2	2.7	18.8	86	6.5
			−2.5	322	
			−32.5	279	
2.7	8.2	8.2	8.9 ± 0.3	79 ± 26	7.1
			−18.7 ± 2.1	153 ± 27	
			−34.0 ± 1.6	87 ± 28	

[a] Variations in chemical shifts and line widths for identical samples are ≤10% and ≤30%, respectively. [b] Relative to 0.1 M $Cd(ClO_4)_2$.

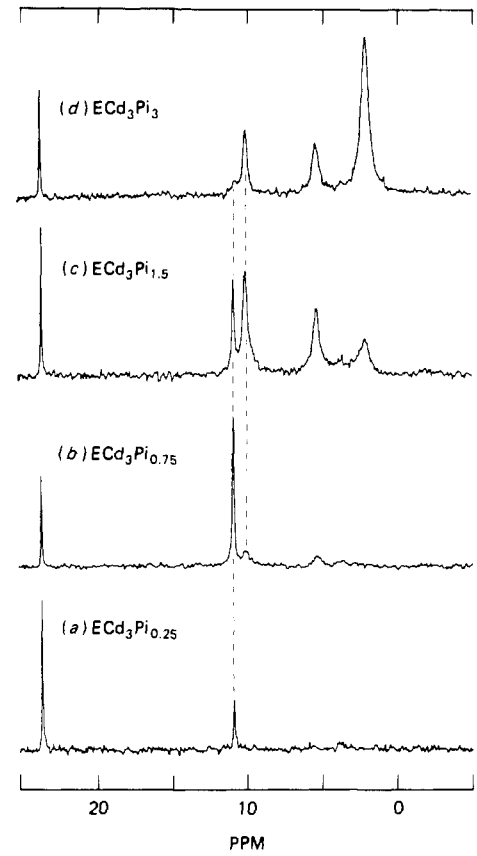


FIGURE 3: ^{31}P NMR spectra at 145.7 MHz of solutions of fixed PPase and $^{113}Cd^{2+}$ concentrations and varying $[P_i]$, pH 7.0, 4 °C. The peak at +23.4 ppm is methylphosphonic acid.

	$[PPase]_T$ (mM)	$[^{113}Cd^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	no. of scans
(a)	2.68	7.47	0.67	6416
(b)	2.52	7.47	1.88	6416
(c)	2.52	7.47	3.76	6664
(d)	1.75	4.78	5.26	6160

The chemical shift for this peak is insensitive to pH in the range 6.4–7.3. At $[Cd^{2+}]_T$ equal to 3$[PPase]_T$, three broad resonances are observed (Figure 2b). Addition of 1 equiv of P_i causes an apparent upfield shift of 7 ppm in the peak which had been at −25.6 ppm (Figure 2c). Further addition of P_i causes an overall upfield shift in the three peaks as well as considerable narrowing (Figure 2d).

NMR Spectra of Enzyme-Bound $^{31}P_i$. Representative ^{31}P NMR spectra of solutions of $^{113}Cd^{2+}$, PPase, and P_i are

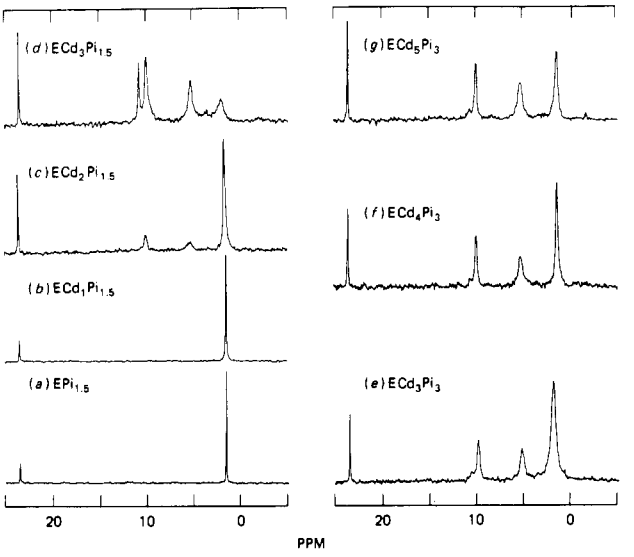


FIGURE 4: ^{31}P NMR spectra at 145.7 MHz of solutions of fixed PPase–P_i concentration and varying $^{113}Cd^{2+}$ concentration, pH 7.0, 4 °C. The peak at +23.4 ppm is methylphosphonic acid. Note that spectra d and e are identical with spectra c and d, respectively, of Figure 3.

PPase:P_i		$[PPase]_T$ (mM)	$[^{113}Cd^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	no. of scans
	(a)	1.69		2.56	2000
	(b)	1.69	1.69	2.56	2400
1:1.5	(c)	1.69	3.37	2.56	7200
	(d)	2.52	7.47	3.76	6664
1:3	(e)	1.75	4.78	5.26	6160
	(f)	1.67	6.68	5.01	3256
	(g)	1.67	8.35	5.01	7280

presented in Figures 3 and 4. In Figure 3, the $^{113}Cd^{2+}$:PPase ratio is maintained constant at 3:1, and the spectra are recorded as a function of increasing $[P_i]$. In Figure 4, the spectra are shown as a function of increasing Cd^{2+} concentration, with two fixed enzyme:P_i ratios, 1:1.5 (Figure 4a–d) and 1:3 (Figure 4e–g). A total of four major peaks are seen, whose relative intensities are a sensitive function of the PPase:Cd^{2+}:P_i proportions. As discussed below in greater detail (see Discussion), the resonances at 10.6, ~9.8, and ~5.2 ppm correspond to enzyme-bound P_i, while the resonance at ~2 ppm corresponds to P_i in solution at pH 7.0. A minor resonance at −1.6 ppm is also seen in Figure 4g. We believe this represents enzyme-bound PP_i, since it both corresponds in relative amount (about 5% of the peak at either ~5.2 or ~9.8 ppm) to the value obtained by direct measurement (see Enzyme-Bound PP_i Formation below) and is 7–11 ppm upfield from enzyme-bound P_i (at pH 7.0, PP_i in solution is 9.1 ppm upfield from P_i).[2]

The dependence of the observed ^{31}P line width on Cd^{2+} nuclear spin was investigated by determining the line widths in the presence, separately, of either $^{113}Cd^{2+}$ or $^{112}Cd^{2+}$. Measurements were made at a [PPase]:$[Cd^{2+}]$:$[P_i]$ relative composition of 1:3:1.5 such that all four major ^{31}P peaks are visible, and the results are presented in Figure 5 and Table II. Great care was taken to prepare identical samples, which differed only in the stock $CdCl_2$ solution utilized. As a precaution to test for possible paramagnetic impurities, approximate water proton relaxation rates were determined for both $CdCl_2$ solutions and found to be equal $[1/T_1 = 0.093\ s^{-1}$ ($^{113}CdCl_2$) and $0.087\ s^{-1}$ ($^{112}CdCl_2$)] within experimental error.

[2] K. M. Welsh and B. S. Cooperman, unpublished results.

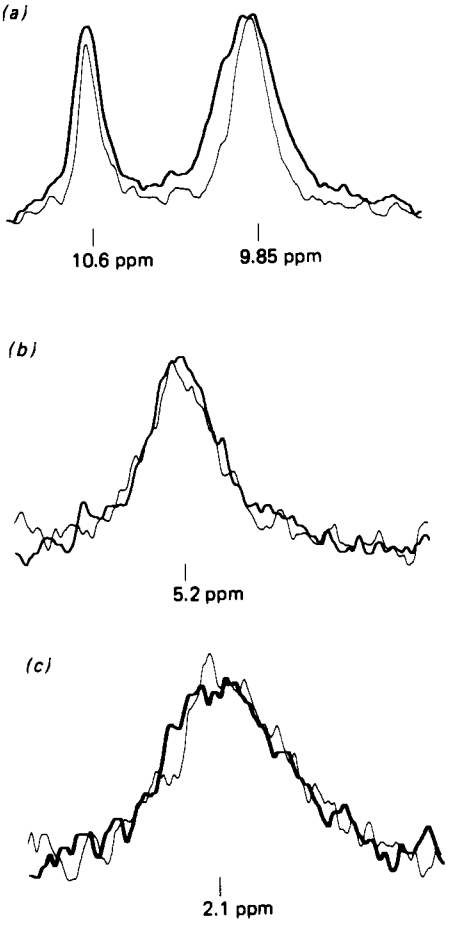


FIGURE 5: Direct comparison of ^{31}P NMR spectra of solutions of PPase–$^{113}Cd^{2+}$–P_i and PPase–$^{112}Cd^{2+}$–P_i, pH 7.0, 4 °C. Both solutions were 2.09 mM in PPase and 3.05 mM in P_i. Heavy line, 6.44 mM $^{113}Cd^{2+}$; narrow line, 6.60 mM $^{112}Cd^{2+}$. Portions of the spectra shown in panels a–c each have a spectral width of 2 ppm. Broad-band proton decoupling was not employed during the acquisition of these spectra.

Table II: ^{31}P Chemical Shifts and Line Widths[a]

δ[b]	$\Delta\nu_{1/2}$ (Hz) ^{113}Cd	$\Delta\nu_{1/2}$ (Hz) ^{112}Cd	$[\Delta\nu_{1/2}(^{113}Cd)]/[\Delta\nu_{1/2}(^{112}Cd)]$[c]
2.09	106	111	0.98 ± 0.04
5.20	71	67	1.04 ± 0.04
9.79	54	34	1.62 ± 0.07
10.61	30	23	1.31 ± 0.03

[a] PPase:Cd^{2+}:P_i, 1:3:1.5; PPase concentration, 2.1–2.5 mM. [b] Relative to 0.1 M H_3PO_4. [c] Values reported are average of two independent experiments.

As may be seen, the peak at ~9.8 ppm is clearly narrower in the presence of $^{112}Cd^{2+}$ than in the presence of $^{113}Cd^{2+}$. A clear but smaller narrowing effect is also apparent for the peak at 10.6 ppm. There appears to be no effect on either the peak at ~5.2 ppm or the peak at ~2 ppm.

Enzyme-Bound PP_i Formation. Enzyme-bound (EPP_i) formation at equilibrium was measured as a function of P_i concentration in the presence of Cd^{2+}. Under our conditions, P_i is bound essentially stoichiometrically to site 1 (as shown by the NMR results in Figure 3, similar tight binding has also been found for the Mn^{2+}–enzyme; Cooperman et al., 1981) so that over the P_i concentration range studied, the total enzyme concentration, $[E]_T$, is given by eq 2 (i.e., there is essentially no P_i-free enzyme). The dependence of EPP_i for-

$$[E]_T = [EP_i] + [E(P_i)_2] + [EPP_i] \quad (2)$$

Table III: Values for Empirical Parameters

parameter	Cd^{2+}	Mg^{2+} [a]	Cd^{2+}	Mg^{2+} [b]	Cd^{2+}	Mg^{2+} [b]	Cd^{2+}
pH	7.0	7.0	8.1	8.0	9.0	9.0	7.5
temp (°C)	25	25	25	25	25	25	4
$k_{cat,hyd}$ (s^{-1})	0.015	212	0.053	103	0.134	28	
$K_{m,hyd}$ (μM)	2.5	4.3	4.4	6.1	31	8.1	
$k_{cat,ex}$ (s^{-1})	8×10^{-4}	171					
P_c	≤ 0.05 [c]	0.23 ± 0.07					
K_3	19	4.8					42
$K_{p,app}$ (mM)	2.1	27					2.8

[a] From Springs et al. (1981). [b] Estimated from Knight et al. (1981). [c] Upper limit; see Table IV.

Table IV: Water-P_i Oxygen Exchange

concn (mM)			rate constants		
PPase	P_i	$Cd^{2+}{}_T$	overall oxygen exchange [μM atoms of ^{18}O (μmol of enzyme)$^{-1}$ s^{-1}]	$P^{18}O_4$ disappearance [4 × μmol of $P^{18}O_4$ (μmol of enzyme)$^{-1}$ s^{-1}]	calcd P_c
0.093	2	1	7.6×10^{-4}		
0.280	6	1	8.6×10^{-4}		
0.467	10	1	7.4×10^{-4}		
0.031	10	0.5	6.2×10^{-4}	26.9×10^{-4}	<0
0.031	10	0.25	6.2×10^{-4}	24.6×10^{-4}	0.01
0.031	10	0.1	6.5×10^{-4}	26.7×10^{-4}	<0

mation on $[P_i]_T$ can then be expressed according to the Scatchard-type equation (eq 3), where $K_3 = [E(P_i)_2]/[EPP_i]$

$$\frac{[EPP_i]}{[E]_T[P_i]_T} = \frac{K_3 + 1}{K_3 K_{p,app}}\left(\frac{1}{K_3 + 1} - \frac{[EPP_i]}{[E]_T}\right) \quad (3)$$

and $K_{p,app} = [EP_i][P_i]/[E(P_i)_2]$. It should be noted that the enzyme and P_i concentration terms employed in these equations refer to all forms of a particular species without regard to the stoichiometry of bound Cd^{2+} or the protonation state.

Data obtained at two temperatures gave good straight lines when plotted according to eq 3, yielding the values for K_3 and $K_{p,app}$ presented in Table III. Corresponding values for these two parameters obtained in the presence of Mg^{2+} are also presented in Table III.

The rate of EPP_i formation was also measured and found to follow first-order kinetics, as shown in Figure 6. Rate constants measured at $[P_i]$ equal to 10 mM showed no trend over a $[Cd^{2+}]$ range of 0.5–3.0 mM. The average value was 0.0088 ± 0.0014 s^{-1}.

Enzyme Catalysis of PP_i Hydrolysis. Eadie–Hofstee plots of steady-state rate data for PPase catalysis of PP_i hydrolysis in the presence of Cd^{2+}, at several pH values, yielded values of $k_{cat,hyd}$ and $K_{m,hyd}$ which are presented in Table III alongside those previously determined in the presence of Mg^{2+}. PPase catalysis of PP_i hydrolysis in the presence of Cd^{2+} was also studied at times corresponding to less than a single turnover (≤1 min). As is clear from Figure 7, there is no burst of P_i formation.

Enzyme Catalysis of H_2O–P_i Oxygen Exchange. Rate constants for H_2O–P_i oxygen exchange at different Cd^{2+} and P_i concentrations are listed in Table IV. The values obtained are all apparently close to saturation, permitting estimation of a value for $k_{cat,ex}$ of approximately 8×10^{-4} s^{-1} (Table III). Controls, lacking either PPase or Cd^{2+}, showed no exchange within the same time range.

Hackney & Boyer (1978) have defined a partition coefficient, P_c, for enzyme-catalyzed H_2O–P_i oxygen exchange as the rate at which enzyme-bound P_i loses water in the exchange step divided by the sum of this rate and the rate of release of

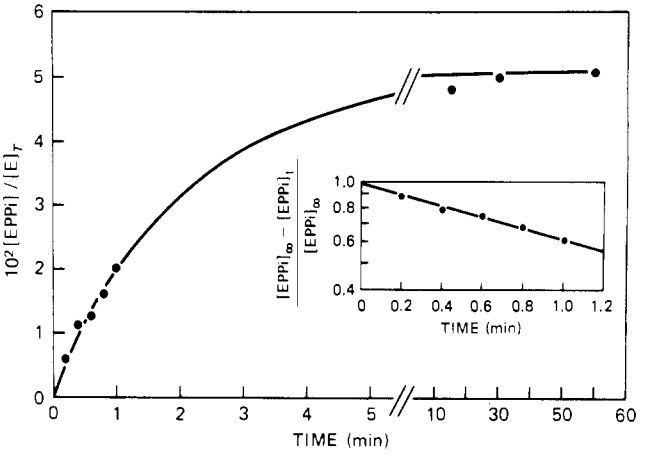


FIGURE 6: Kinetics of EPP_i formation, pH 7, 25 °C. [PPase], 0.5 mM; $[Cd^{2+}]$, 3.0 mM; $[P_i]$, 10 mM. Inset: Semilog plot of EPP_i formation data.

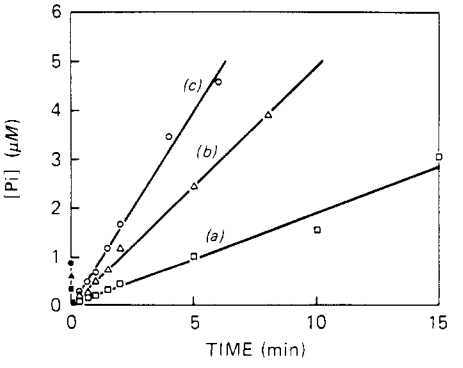


FIGURE 7: Time dependence of PPase-catalyzed hydrolysis of PP_i at times approximating single turnover and beyond, pH 7.0, 25 °C. Solutions were 50 μM in $CdCl_2$ and 10 μM in PP_i. $[PPase]_T$ equals (a) 0.33, (b) 0.65, and (c) 0.98 μM. Solid symbols on the y axis correspond to stoichiometries of P_i equal to $[PPase]_T$. The lines drawn have slopes equal to steady-state velocities.

P_i to the medium and have shown that P_c can be calculated from the ratio, R, of the rate constant for $P^{18}O_4$ disappearance

to the rate constant for overall oxygen exchange (as defined in Table IV) via eq 4. As seen in Table IV, the values of P_c

$$P_c = \frac{4 - R}{3} \quad (4)$$

calculated from the measured R values are indistinguishable from zero.

Discussion

Cd^{2+} Binding and $^{113}Cd^{2+}$ NMR. Cd^{2+} binding to PPase in the presence of P_i is similar to that previously observed with Mn^{2+} and Co^{2+} in that three divalent metal ions are bound per subunit with relatively high affinity,[3] and such binding is adequately characterized by a single intrinsic binding constant. However, in the absence of P_i, there is a difference in that only a single high-affinity site is seen, in contrast to the two observed for Mn^{2+} and Co^{2+} [and for Mg^{2+} and Zn^{2+} as well (Rapoport et al., 1973)]. There are, in addition, a number of low-affinity sites. Although it is reasonable to estimate the number of such sites as between two and three per subunit based on the data in Figure 1a and previous studies of divalent metal ion binding to PPase, it may be that the number of low-affinity sites is even larger, since the calculated K_D values ($\geq$2.5 mM) are not so different from what one would expect for rather nonspecific binding. What is clear from the equilibrium dialysis results is that the presence of P_i induces an increase from one to three of the number of high-affinity Cd^{2+} sites per subunit.

The $^{113}Cd^{2+}$ NMR results (Figure 2, Table II) are consistent with the binding results. Thus, addition of 1 equiv of $^{113}Cd^{2+}$ per subunit yields a single, narrow resonance as expected for a unique site of high affinity (Figure 2a). Further addition of $^{113}Cd^{2+}$ leads to a shift and broadening of this resonance and the appearance of additional broader resonances (Figure 2b). The broader line widths of these latter resonances are consistent with weaker binding of Cd^{2+} at these sites. The concomitant broadening of the resonance assigned to the high-affinity site could reflect its sensitivity to the binding of Cd^{2+} to the lower affinity sites, similar to what has been observed for alkaline phosphatase (Otvos & Armitage, 1980). The addition of sufficient P_i to saturate both P_i sites on the enzyme (Figure 2d; also, see below) leads to the appearance of three narrower lines, consistent with stronger binding, and to three distinct sites.

Chemical shifts of $^{113}Cd^{2+}$ bound to proteins have been shown to vary over a range of 900 ppm, and there is good evidence that the chemical shift value for a particular $^{113}Cd^{2+}$ is useful in identifying the ligand atoms bound to it (Armitage & Otvos, 1982). All of the chemical shifts reported in Table I fall within a very limited region (<45 ppm) of the accessible spectral range, one which is suggestive of predominantly or exclusively oxygen liganding to enzyme-bound $^{113}Cd^{2+}$, either six- or seven-coordinate (Sudmeier et al., 1981; Rodesiler & Amma, 1982). This suggestion is consistent with previous demonstrations of the lack of essential lysines or histidines in PPase (Cooperman & Chiu, 1973; Heitmann & Uhlig, 1974), as well as with recent unsuccessful attempts in this laboratory to oxidize PPase-bound Co^{2+} to Co^{3+} with added hydrogen peroxide.[4] It is well-known that such oxidations require the presence of nitrogen ligands in the inner coordination sphere of Co^{3+}. In fact, for many of the previously reported successful oxidations of enzyme-bound Co^{2+}, the divalent metal ion is known to be directly bound to nitrogen ligands [as reviewed in Legg (1978)]. Mg^{2+} shows a strong preference for binding to an oxygen rather than to a nitrogen ligand. It is the likely physiological divalent metal ion activator of PPase, based both on its availability and on the fact that is confers maximal catalytic activity (Butler & Sperow, 1977; Janson et al., 1979; Welsh et al., 1983). Thus, the suggestion of an all-oxygen ligand inner sphere for each of the divalent metal ion binding sites is not unexpected. In this connection, it is interesting to note that $^{113}Cd^{2+}$ bound to the single Mg^{2+} site on an alkaline phosphatase subunit (there are, in addition, two Zn^{2+} sites) has a chemical shift similar to those seen for PPase (Otvos & Armitage, 1980). This prompts the tentative suggestion that a chemical shift near zero relative to $^{113}Cd(ClO_4)_2$ in solution might be characteristic of $^{113}Cd^{2+}$ binding to a Mg^{2+} binding site on a protein.

^{31}P NMR. The ^{31}P NMR results presented in Figures 3–5, and Table II, taken together with other results obtained earlier, provide clear evidence (a) that there are two P_i sites present per PPase subunit which differ in affinity from one another in the presence of Cd^{2+}, similar to previous results obtained in the presence of Mn^{2+}, Co^{2+}, and Mg^{2+} (Hamm & Cooperman, 1978; Cooperman et al., 1981; Springs et al., 1981), (b) that the P_is in these sites are in slow chemical exchange with each other and with P_i in solution only in the presence of several (probably three) Cd^{2+} ions bound per PPase subunit, (c) that the two P_i sites are mutually interactive, and (d) that at least one of the enzyme-bound Cd^{2+} ions is in inner-sphere contact with P_i bound in the higher affinity site. These points are discussed in turn below.

The spectra in Figure 3a–c are easily understood in terms of the sequential filling of two P_i sites of differing affinity. Thus, we interpret the single peak at 10.6 ppm seen in Figure 3a ($[P_i]_T/[PPase]_T = 0.25$) as P_i binding in site 1 when site 2 is empty, and the peaks at $\sim$9.8 and $\sim$5.2 ppm, which are just visible in Figure 3b ($[P_i]_T/[PPase]_T = 0.75$), and prominent in Figure 3c ($[P_i]_T/[PPase]_T = 1.5$), as simultaneous P_i binding to sites 1 and 2, respectively. The observed decrease in peak intensity at 10.6 ppm which accompanies the rise in intensity at $\sim$9.8 ppm is obviously in accord with this interpretation. One would expect that as $[P_i]_T$ were raised further, the 10.6-ppm peak would disappear altogether as site 2 was occupied. This expectation is fulfilled as shown in Figure 3d ($[P_i]_T = 3[PPase]_T$), but, somewhat puzzlingly, the peak at $\sim$2 ppm, corresponding to P_i in solution, is seen to be more intense relative to those at $\sim$5.2 and $\sim$9.8 ppm than would have been expected if the 3 equiv of P_i per enzyme subunit were distributed in a 1:1:1 proportion among sites 1 and 2 and solution.

The answer to this puzzle lies in the recognition that the observation of peaks at 10.6, $\sim$9.8, and $\sim$5.2 ppm depends not only on P_i occupancy of sites 1 and 2 but also on the stoichiometry of bound Cd^{2+}. Indeed, from the spectra in Figure 4, we conclude (1) that P_i bound either to site 1 or to site 2 in the presence of 0, 1, or 2 Cd^{2+} per PPase subunit has a chemical shift similar to or identical with that of free P_i in solution and (2) that large changes in the chemical shifts of enzyme-bound P_i, sufficient to achieve the slow exchange limit among the various P_i species in solution, depend upon the binding of a third Cd^{2+} per enzyme subunit. Our first con-

[3] The observed value of K_D for Cd^{2+} binding in the presence of 0.5 mM P_i is 0.55 mM. A lower K_D value would be expected at higher $[P_i]$, in accord with the results presented in Table VI, but precipitation problems constrained us to work at the lower concentration. Moe (O. A. Moe, unpublished results) has recently measured a K_I for Cd^{2+} of 0.22 μM, as measured by inhibition of PPase catalysis of Mg^{2+}-dependent PP_i hydrolysis.

[4] A. Banerjee and B. S. Cooperman, unpublished results.

clusion is based upon the results presented in Figure 4a–c showing ^{31}P NMR spectra for solutions of PPase, Cd^{2+}, and P_i in which $[Cd^{2+}]_T \leq 2[PPase]_T$. These spectra show a single or dominant ^{31}P peak, having a chemical shift identical with that of P_i in solution, even though P_i is bound to PPase in the absence of added divalent metal ion [K_D is equal to 0.8 mM (presumably to site 1); Cooperman et al., 1981], and added divalent metal ion is known to increase P_i affinity for the enzyme. This result excludes the possibility of a fast exchange between free and enzyme-bound P_i having substantially different chemical shifts. Our second conclusion is based on the NMR spectrum in Figure 4d ($[Cd^{2+}]_T = 3[PPase]_T$), in which the characteristic peaks for enzyme-bound P_i, visible at low intensity in Figure 4c, become prominent.

These conclusions provide an explanation of the ^{31}P NMR spectrum in Figure 3d via eq 5 and 6. That is, raising the

$$EM_3P_i + P_i \rightleftharpoons EM_3(P_i)_2 \quad (5)$$

$$EM_3(P_i)_2 + P_i \rightleftharpoons EM_2(P_i)_2 + MP_i \quad (6)$$

P_i concentration would lead to the formation of $EM_2(P_i)_2$ and to a decrease in peak intensity at ~9.8 and ~5.2 ppm (here it should be pointed out that doubling $[P_i]_T$ between spectra c and d of Figure 3 leads to a much larger change, more like 10-fold, in free P_i concentration, which is the relevant quantity in eq 6). A test of this explanation is that raising the free Cd^{2+} concentration, would, via eq 7, result in an increase in peak

$$EM_2(P_i)_2 + M \rightleftharpoons EM_3(P_i)_2 \quad (7)$$

intensity at ~9.8 and ~5.2 ppm and a decrease in peak intensity at ~2 ppm. This is exactly what is observed in Figure 4e–g (PPase:Cd^{2+}:P_i ratios of 1:3:3, 1:4:3, and 1:5:3, respectively).

That three bound Cd^{2+} ions are required for large changes in the chemical shift and the resulting slow chemical exchange is consistent with several lines of evidence obtained previously for a conformational change accompanying the binding of a third divalent metal ion per PPase subunit restricting solvent access to the active site (Cooperman et al., 1981). It also is consistent with our current finding of three high-affinity Cd^{2+} binding sites per subunit in the presence of P_i by either equilibrium dialysis or $^{113}Cd^{2+}$ NMR (Figures 1 and 2). In contrast to our results with Cd^{2+}, we find only a single ^{31}P resonance (at ~2 ppm)[2] in solutions of enzyme, Mg^{2+}, and P_i in which three Mg^{2+} ions and two P_is are bound per subunit (Springs et al., 1981). However, since saturation of site 2 and binding of a third Mg^{2+} both require a high P_i concentration, it is not clear whether the failure to see enzyme-bound P_i peaks is due to a lack of change in chemical shift or to rapid exchange of enzyme-bound and free P_i.

According to our interpretation of the spectra in Figure 3, the chemical shift of P_i in site 1 undergoes a 0.8-ppm upfield shift when site 2 is also filled with P_i. This change is an indication of site–site interaction and is consistent with the notion that the two sites we are monitoring by ^{31}P NMR are occupied by the two P_is formed on PP_i hydrolysis, since they should be close to one another on the enzyme surface.

Studies with alkaline phosphatase containing bound $^{113}Cd^{2+}$ have provided unambiguous evidence for a Cd^{2+}–P_i inner-sphere complex on the enzyme surface as discussed above. In our own spectra, we fail to observe a resolvable $^{113}Cd^{2+}$–^{31}P scalar coupling. However, such failure cannot be taken as negative evidence against inner-sphere binding. Model studies show that J values for ^{113}Cd–O–^{31}P binding can vary from 2.4 to 46 Hz (Dean, 1981; Reed & Bock, 1979), and J values ≤10 Hz would have been insufficient to generate an observable doublet in any of the enzyme-bound ^{31}P peaks in Figures 3 and 4. In the absence of an observable doublet, we sought to obtain evidence for or against inner-sphere binding by comparison of ^{31}P line widths in enzyme–Cd^{2+}–P_i solution which are otherwise identical but differ as to whether $^{113}Cd^{2+}$ ($I = {}^1/_2$) or $^{112}Cd^{2+}$ ($I = 0$) is used. Substitution of $^{113}Cd^{2+}$ for $^{112}Cd^{2+}$ leads to clear broadenings of the peaks at ~9.8 and 10.6 ppm (Table II), which are well beyond experimental error. Since these broadenings are not due to paramagnetic impurities in the $^{113}Cd^{2+}$ sample utilized (see Results), the most plausible explanation for them is that they reflect ^{113}Cd–^{31}P scalar coupling. They therefore provide evidence for inner-sphere binding between at least one enzyme-bound Cd^{2+} and P_i in site 1 whether or not site 2 is also filled with P_i. If it is assumed that the broadenings are due to a single Cd^{2+} in the coordination sphere of P_i in site 1, J values of 20 ± 5 Hz and 10 ± 2 Hz may be estimated for the peaks at ~9.8 and 10.6 ppm, respectively. These best-fit values of J, applied to the spectral lines measured in the presence of $^{112}Cd^{2+}$, give line shapes that, when calculated as the sums of the Lorentzian line shapes for the doublets produced, are essentially identical with those observed in the presence of $^{113}Cd^{2+}$. The peak at ~5.2 ppm shows no apparent broadening by $^{113}Cd^{2+}$. This suggests a lack of inner-sphere Cd^{2+} coordination to P_i in site 2. However, it must be noted that because of the inherent uncertainty in our measurement of $\Delta\nu_{1/2}$ and because of the broadness of the ~5.2-ppm peak even in the presence of $^{112}Cd^{2+}$, a J value <10 Hz would be undetectable. An additional result not inconsistent with inner-sphere binding by enzyme-bound divalent metal ion to P_i in site 1 but not site 2 is the much larger change in chemical shift, relative to P_i in solution, seen for P_i binding in site 1 (7.7–8.5 ppm) as compared with P_i binding in site 2 (3.1 ppm). We note that the chemical shift observed for P_i in site 1 is similar to that seen for noncovalent binding of P_i to Cd^{2+}–alkaline phosphatase (Otvos et al., 1979).

Equilibria and Kinetics Related to PPase Functions. Our previous analysis of PPase kinetics in the presence of Mg^{2+} (Springs et al., 1981) showed that the simple scheme shown below, where M is a divalent metal ion (eq 8), was sufficient

$$M_2E + MPP_i \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M_3EPP_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} M_3E(P_i)_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} M_2EP_i \underset{k_8}{\overset{k_7}{\rightleftharpoons}} M_2E + P_i \quad (8)$$

(H_2O^* on the k_3 step; MP_i^* on the k_5 step)

to quantitatively account for the observed rates of PP_i hydrolysis, P_i–H_2O oxygen exchange, and PP_i–P_i exchange. As indicated, the first P_i released from enzyme following hydrolysis is the one which exchanges oxygen with water. Assuming the same scheme applies for Cd^{2+} permits evaluation of kinetic and equilibrium constants for eq 8. From our earlier analysis, k_4 is given by eq 9. Since $K_3 \gg 1$ and $P_c \approx 0$ (Table III), k_4 is essentially equal to $k_{cat,ex}$ (8×10^{-4} s^{-1}); i.e., synthesis of enzyme-bound PP_i is exclusively rate determining for enzyme-catalyzed oxygen exchange. This is also approximately true for enzyme-catalyzed oxygen exchange in the presence of Mg^{2+} (Springs et al., 1981). The rate constant for PP_i hydrolysis on the enzyme, k_3, is given by K_3k_4 and is equal to 1.5×10^{-2} s^{-1} at pH 7. As this is indistinguishable from the value for $k_{cat,hyd}$ (Table III), the indication is that PP_i hydrolysis on the enzyme is exclusively rate determining in overall enzyme catalysis of PP_i hydrolysis. The complete

$$k_4 = k_{cat,ex}\frac{K_3 + 1}{K_3}\frac{4 - 3P_c}{4(1 - P_c)} \quad (9)$$

failure to see any burst of P_i release in a time corresponding to single turnover (Figure 7) is consistent with this notion. This result is in marked contrast to enzyme catalysis of PP_i hydrolysis in the presence of Mg^{2+}, where the hydrolysis and both P_i release steps (characterized by rate constants k_3, k_5, and k_7) were all partially rate determining. According to eq 8, the rate constant for EPP_i formation should be given by $k_3 + k_4$, or 1.6×10^{-2} s^{-1}. Given the slight differences in conditions between the oxygen exchange, PP_i hydrolysis, and EPP_i formation experiments, we consider the measured value of 0.9×10^{-2} s^{-1} (Figure 7) to be essentially indistinguishable from this number. This agreement is strong evidence for the adequacy of eq 8 in accounting for PPase catalysis in the presence of Cd^{2+}.

Cd^{2+} had previously been reported to inhibit Mg^{2+}-dependent PPase activity, without itself conferring any activity (Butler & Sperow, 1977). Our present observation of Cd^{2+}-dependent activity is not inconsistent with this since the relative activity we see is low enough that it would have been missed in earlier work. However, the very low rate constants measured in the presence of Cd^{2+}, as well as the differences in pH dependence for V_{max} in the presence of Cd^{2+} as compared with Mg^{2+} (Table III), do raise the question as to whether the activity we observe is really due to PPase, rather than to a trace contamination with a nonspecific alkaline phosphatase. The similarities in equilibrium constants and the good agreement between the observed and independently calculated rate of EPP_i formation, as discussed above, provide strong evidence that the activity we observe is due to PPase. The unimportance of alkaline phosphatase activity is also demonstrated by our results (not shown) examining *p*-nitrophenyl phosphate as a substrate in the presence of Cd^{2+} at pH 8. Although some enzyme-dependent hydrolysis was found, the apparent V_{max} was quite slow ($\leq$10% compared to PP_i).

Relevance of the Cd^{2+}–Enzyme Studies and Related Results for the Active-Site Geometry of the Mg^{2+}–Enzyme. The ^{31}P NMR results summarized in Table II provide clear evidence for inner-sphere Cd^{2+} binding to P_i in site 1 and suggest a lack of such binding to P_i in site 2. Given the low activity of the Cd^{2+}–enzyme as compared to the Mg^{2+}–enzyme, a question arises as to the relevance of these results for the Mg^{2+}–P_i interaction on the enzyme surface. Although no definitive answer can be given to this question at this time, there are two grounds for believing in the relevance of the Cd^{2+} results. First, they are fully consistent with results obtained in previous and ongoing work with the Mn^{2+}–enzyme. Thus, we have shown that the effect of enzyme-bound Mn^{2+} on the longitudinal relaxation rate (T_1) for ^{31}P bound in site 2 is consistent only with outer-sphere complex formation between P_i in this site and enzyme-bound Mn^{2+}, regardless of whether one, two (Hamm & Cooperman, 1978), or three Mn^{2+} ions are bound per subunit. No clear effect was found on the T_1 for P_i in site 1, a result that does not distinguish between the possibilities that P_i in site 1 is far from Mn^{2+} or that P_i is quite close to enzyme-bound Mn^{2+} but not in rapid exchange with P_i in solution. The Mn^{2+}–enzyme is approximately 5% as active as the Mg^{2+}–enzyme (Hohne & Heitmann, 1974; Butler & Sperow, 1977; Janson et al., 1979; Welsh et al., 1983). However, viewed on a logarithmic (or free energy of activation) scale, the Mn^{2+}–enzyme may be considered to have high activity, since k_{cat} for PP_i hydrolysis by the Mg^{2+}–enzyme (212 s^{-1}, Table III) is some 10^{10} times higher than the rate constant for uncatalyzed PP_i hydrolysis under the same conditions. The consistency of the Mn^{2+}–enzyme and Cd^{2+}–enzyme results regarding the Mn^{2+}–P_i interaction thus strongly supports the relevance of the Cd^{2+} results for the Mg^{2+}–enzyme. Second, a comparison of the equilibrium constants for eq 8, where M is either Cd^{2+} or Mg^{2+} (Table V), clearly shows similarities with respect to the position of the equilibria for hydrolysis of EPP_i to $E(P_i)_2$ and to the binding of both PP_i and of P_i to site 2. From the ^{31}P NMR experiment in Figure 3, it is also clear that P_i in site 1 is bound with much higher affinity than P_i in site 2, as is the case with Mg^{2+}. Although K_7 in the presence of Cd^{2+} has not been determined, the NMR data in Figure 3 clearly indicate a value of <0.2 mM for this constant. These comparisons also support the relevance of the Cd^{2+} results, since similar binding constants suggest formation of structurally similar complexes.

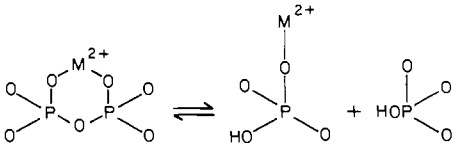
Table V: Equilibrium and Rate Constants for Equation 8[a]

constant	Cd^{2+}	Mg^{2+} [b]
K_1 ($=k_1/k_2$) ($\times 10^6$ M)	$\leq$2.5[c]	0.2
K_3 ($=k_3/k_4$)	19	4.8
K_5 ($=k_5/k_6$) ($\times 10^3$ M)	1[d]	4.5
K_7 ($=k_7/k_8$) ($\times 10^3$ M)	<0.2[e]	0.52
k_3 (s^{-1})	0.015	1070
k_4 (s^{-1})	8×10^{-4}	222

[a] pH 7.0, 25 °C. [b] Springs et al. (1981). [c] Assuming $K_{m,hyd} \leq K_D$. This is reasonable because k_3 is rate limiting for catalysis of PP_i hydrolysis. [d] Calculated from $K_{p,app}$ (Table III), using a K_D for $Cd^{2+}P_i$ binary complex dissociation of 2 mM (Hietenan et al., 1973). [e] See text.

In contrast to the equilibrium constants, the rate constants compared in Table V, k_3 and k_4, are very much smaller in the presence of Cd^{2+} (by a factor of about 10^5) as compared with Mg^{2+}. This is one example of the general finding that the catalytic activity conferred on PPase by a divalent metal ion correlates to some extent with the ionic radius [these are, using the Goldschmidt values, 0.78 Å for Mg^{2+}, 0.91 Å for Mn^{2+}, and 1.03 Å for Cd^{2+}; this point is discussed in greater detail elsewhere (Welsh et al., 1983, and footnote 5)] and is evidence that catalytic activity is much more dependent on the precise geometry of substrate binding than is overall binding affinity. Such a conclusion is, of course, in accord with prevailing views on the mechanism of enzyme action (Jencks, 1975; Page, 1977).

Our results with Mn^{2+} and Cd^{2+} show an inner-sphere interaction of the metal ion with P_i in site 1 but an outer-sphere interaction with P_i in site 2. On the other hand, Knight et al. (1981) have shown that the P^1,P^2-bidentate complex of Cr^{3+} with PP_i is a substrate for PPase (albeit a relatively poor one, with a high K_m and low V_{max}) and concluded, based on the exchange inertness of the $CrPP_i$ complex, that the natural substrate is a P^1,P^2-bidentate complex of $Mg^{2+}PP_i$. Our results are not necessarily in conflict with this, since it is perfectly plausible that an inner-sphere metal ion bound to one of the phosphoryl groups in PP_i is normally broken (i.e., with metal ions that are exchange labile, such as Mg^{2+}, Mn^{2+}, or Cd^{2+}) during hydrolysis to two P_is on the enzyme surface (eq 10). However, we would point out that the $CrPP_i$ result



(10)

proves only that P^1,P^2-coordination to a metal ion is possible for a substrate, not that it is required, so that the natural substrate for PPase might rather be the monodentate complex

[5] O. A. Moe, S. Pham, B. Selinsky, and T. Dang, unpublished results.

of PP_i with Mg^{2+}, which is the more likely structure suggested by our results. Resolution of this point will require further experimentation.

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Registry No. Cadmium-113, 14336-66-4; phosphorus-31, 7723-14-0; phosphate, 14265-44-2; inorganic pyrophosphatase, 9024-82-2.

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