Yeast Inorganic Pyrophosphatase. A Model for Active-Site Structure Based on $^{113}Cd^{2+}$ and ^{31}P NMR Studies†

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ABSTRACT: Equilibrium dialysis and $^{113}Cd^{2+}$ NMR studies in the presence of inorganic phosphate (P_i) provide clear evidence for the existence of four well-defined Cd^{2+} sites per yeast inorganic pyrophosphatase subunit. Parallel ^{31}P NMR studies demonstrate the existence of two binding sites per subunit for P_i and provide strong confirmatory evidence for a small amount of enzyme-bound inorganic pyrophosphate in equilibrium with enzyme-bound P_i. Such inorganic pyrophosphate formation was demonstrated by chemical analysis earlier [Welsh, K. M., Armitage, I. M., & Cooperman, B. S. (1983) *Biochemistry 22*, 1046–1054]. In this same earlier paper, we provided evidence for inner-sphere contact between enzyme-bound Cd^{2+} and P_i bound in the higher affinity of the two P_i sites. We now present heteronuclear decoupling evidence that one to three different Cd^{2+} ions make such contact. The divalent metal ion cofactor conferring the highest activity on inorganic pyrophosphatase is Mg^{2+}, and we present evidence from competition experiments that such inner-sphere contact is also likely for the Mg^{2+}–enzyme. On the other hand, these experiments also show that some metal ion binding sites on the enzyme bind Mg^{2+} and not Cd^{2+}, and some bind Cd^{2+} and not Mg^{2+}. These results are considered along with others obtained recently in proposing an active-site structure for inorganic pyrophosphatase.

Yeast inorganic pyrophosphatase (PPase),[1] EC 3.6.1.1, is typical of many phosphoryl transfer enzymes in requiring divalent metal ions for activity. In recent years, a considerable effort has been expended to determine both the number of such ions per subunit [PPase is a dimer of identical subunits, 285 amino acid residues long (Heinrikson et al., 1973; Cohen et al., 1978)] required for activity and the relative placement of such ions on the enzyme surface, with respect both to each other and to bound substrate. Although Mg^{2+} confers the highest catalytic activity with inorganic pyrophosphate (PP_i) or inorganic phosphate (P_i) (in H_2O–P_i oxygen exchange) as substrate, Zn^{2+}, Mn^{2+}, and Co^{2+} also confer substantial activity (Kunitz, 1952; Welsh et al., 1983b). Detailed studies of Mg^{2+}, Mn^{2+}, and Co^{2+} binding, both directly, by using equilibrium dialysis or metal ion electrode measurements (Rapoport et al., 1973; Cooperman et al., 1981), and indirectly, by measuring functional properties of PPase as a function of divalent metal ion concentration (Moe & Butler, 1972; Springs et al., 1981; Knight et al., 1984), have led to the conclusion that active enzyme requires three divalent metal ions per subunit and that, in addition, a fourth divalent metal ion, having no clear catalytic function, may also be bound per subunit. EPR studies on the Mn^{2+}–enzyme have provided evidence for the presence of three divalent metal ions in some proximity at the active site (Banerjee & Cooperman, 1983; Knight et al., 1984), and ^{31}P NMR experiments have indicated an inner-sphere interaction between enzyme-bound metal ion and P_i bound in the higher affinity of the two P_i sites but only an outer-sphere interaction between enzyme-bound metal ion and P_i bound in the lower affinity of the two P_i sites (Hamm & Cooperman, 1978; Welsh et al., 1983a).

In this paper, we follow up on our recent demonstration that enzyme-bound Cd^{2+} forms an inner-sphere complex with P_i in the high-affinity site [carried out by comparing the ^{31}P line width in the presence of $^{113}Cd^{2+}$ ($I = {}^1/_2$) to that in the presence of $^{112}Cd^{2+}$ ($I = 0$); Welsh et al., 1983a] by determining (1) the number of well-defined Cd^{2+} sites there are per PPase subunit, (2) the effect of heteronuclear decoupling of enzyme-bound $^{113}Cd^{2+}$ on the line width of P_i bound at the higher affinity site, and (3) the relationship of the enzyme binding sites for Cd^{2+} to those for Mg^{2+}. We conclude by using the results obtained in this and other recent work to present an updated model of the active-site structure of PPase.

Experimental Procedures

Materials

The following materials were obtained from the sources indicated: ^{113}CdO at 96% atom enrichment, Oak Ridge National Laboratory; ^{113}Cd metal at 95% atom enrichment, Prochem; $^{115m}CdCl_2$, New England Nuclear; D_2O (99.8%), Aldrich; ultrapure KOH and HCl and atomic absorption standards for Cd and Mg, Alfa. $^{113}CdCl_2$ was generated by dissolving either ^{113}Cd or ^{113}CdO in ultrapure HCl. (Ethylenedinitrilo)tetramethylenephosphonic acid (ENTMP) was the kind gift of Drs. George Reed and George McDonald.

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). The concentration of protein samples for NMR spectra and the regeneration of metal-free PPase followed standard procedures (Welsh et al., 1983a). Enzyme concentration was determined with A_{280}, by 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).

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[1] Abbreviations: ENTMP, (ethylenedinitrilo)tetramethylenephosphonic acid; FID, free induction decay; Mes, 4-morpholineethanesulfonate; PCHOHP, hydroxymethylenebisphosphonate; PPase, yeast inorganic pyrophosphatase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; EPR, electron paramagnetic resonance. The subscript T indicates the total stoichiometric concentration.

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Methods

Equilibrium Dialysis. Dialysis experiments were performed and analyzed essentially as described previously (Cooperman et al., 1981). The dialysis cell on one side of the membrane contained buffer A (100 mM Mes–100 mM KCl, pH 7.0), PPase, potassium phosphate, and, as indicated, $MgCl_2$. The nonenzyme side contained buffer A, potassium phosphate, and $^{115m}CdCl_2$. Dialysis proceeded for 15–18 h at room temperature, which was sufficient for full equilibration. $^{115m}Cd^{2+}$ concentration was determined by liquid scintillation counting. Protein concentration was determined by A_{280nm}.

^{113}Cd and ^{31}P NMR. ^{113}Cd and ^{31}P spectra were taken on a Bruker CXP 200-MHz spectrometer operating at 44.37 and 80.979 MHz, respectively, and located at the NIH Middle Atlantic NMR Facility at the University of Pennsylvania. Samples were made up in 80% D_2O (100 mM Mes, pH 7.0). Field homogeneity across the sample was optimized on the proton FID of water. Field position was carefully set on the proton FID of water. Proton decoupling was not employed, and all spectra were run without lock. Temperature was maintained at 4 °C.

^{113}Cd NMR spectra were taken with the use of a solenoid coil in a broad-band probe. Operating parameters are given in the text. An exponential line broadening of 30 Hz was utilized to improve the signal to noise ratio. PPase samples were contained in a glass tube (3 × 0.5 cm) which was sealed with parafilm and placed in a horizontal position within the probe. When an external standard was present, it was contained within a sealed glass tube (0.2 × 2.5 cm).

^{31}P NMR spectra were signal enhanced by 5-Hz line broadening, except where noted in the text. Other operating parameters are given in the text. Samples were contained within a 4-mL insert bulb which was placed inside a 20-mm NMR tube. The insert bulb was surrounded on the outside by CCl_4. The sample was held vertically within the ^{31}P high-resolution probe.

^{113}Cd Decoupling of ^{31}P Spectra. The cadmium decoupling frequency was generated by a PTS synthesizer, filtered to remove the ^{31}P frequency, and entered the probe via the deuterium channel. This channel was tuned to the ^{113}Cd frequency of 44.37 MHz. Decoupling power was amplified to 8 W. The ^{31}P frequency came directly from the CXP-200 transmitter after filtration to remove both proton and ^{113}Cd frequencies. It entered the probe via the phosphorus channel. Undecoupled spectra were taken at least once in each decoupling series and served to normalize the line widths of all decoupled spectra.

^{113}Cd and ^{31}P Spin–Lattice Relaxation Times. The 90° pulse for ^{113}Cd and ^{31}P was determined to be 8 μs (solenoid coil) and 26 μs (Helmholtz coil), respectively. Progressive saturation was then utilized to obtain T_1 values for both nuclei in the enzyme sample having a composition of 1:4:3 PPase:Cd:P_i. The progressive saturation pulse sequence, which was reported once per scan, consisted of a 90° pulse followed by a repetition time τ. The value of τ was varied between 1 and 10 s for ^{113}Cd and between 1 and 30 s for ^{31}P. Intensity data were normalized by assigning the intensity measured at the longest repetition time a value of 1 and representing all other intensities as a fraction of this value.

Quantitation of ^{113}Cd Spins. A procedure similar to that of Palmer et al. (1980) was utilized to quantitate the observable resonances of a PPase–Cd–P_i sample. A spectrum was taken of a sample of known concentration of $^{113}CdCl_2$ in 100 mM Mes buffer (pH 7.0) that contained a sealed external 1.5 M $^{113}CdCl_2$ standard. The strong concentration dependence of the ^{113}Cd shift position for chloride complexes allowed the observation of two distinct resonances (Kostelnik & Bothner-By, 1974). A small quantity of $MnCl_2$ was added to both solutions to assure full relaxation under the conditions of the experiment. The calibrated external standard was then added to the enzyme solution and used to calculate the intensities of the peaks in the $ECd_4(P_i)_3$ solution. Integration was accomplished either by cutting out peaks and weighing them or by triangulation.

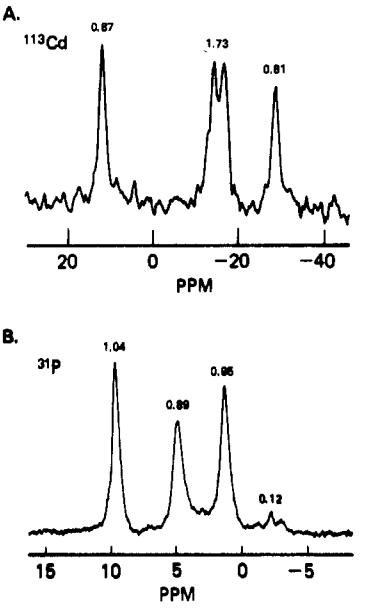

FIGURE 1: ^{31}P and ^{113}Cd NMR spectra of a solution of PPase, $^{113}Cd^{2+}$, and P_i. The following concentrations were employed: PPase, 2.8 mM; $^{113}Cd^{2+}$, 11.2 mM; $[^{31}P]P_i$, 8.4 mM. Spectral parameters for panel A (^{113}Cd) were the following: pulse angle, 90°; repetition time, 10 s; spectral width, 10 000 Hz; number of scans, 7144. Spectral parameters for panel B (^{31}P) were the following: pulse angle, 45°; repetition time, 2.5 s; spectral width, 5000 Hz; number of scans, 16 800. The numbers above the peaks represent mole equivalents of each peak per mole of subunit, after being corrected for incomplete relaxation.

Results

^{113}Cd and ^{31}P NMR Spectra of Solutions Containing PPase, $^{113}Cd^{2+}$, and $[^{31}P]P_i$. Representative ^{113}Cd and ^{31}P NMR spectra of a solution having a composition of 1:4:3 PPase:$^{113}Cd^{2+}$:$[^{31}P]P_i$ are presented in Figure 1. Earlier (Welsh et al., 1983a) we interpreted the $^{113}Cd^{2+}$ spectrum for a similar solution taken at 19.96 MHz as consisting of three defined resonances with the middle resonance having a line width almost double that of the other two. From the current spectrum, taken at 44.37 MHz (Figure 1A), it now seems clear that the center resonance in fact represents two distinct peaks. This interpretation is confirmed by determination of the absolute amount of $^{113}Cd^{2+}$ represented by each of the peaks, using a sealed external standard of $^{113}CdCl_2$ for calibration (see Experimental Procedures). The values obtained per PPase subunit are shown above the peaks in Figure 1A. Thus, under the conditions of the NMR experiment, resonance signals are seen for four bound $^{113}Cd^{2+}$ ions per subunit, with the two middle ones falling quite close to one another. It should be noted that the molar equivalent values shown in Figure 1A have been corrected for incomplete relaxation according to eq 1 where I_0 is the corrected intensity, I_t is the intensity observed

$$I_0 = \frac{I_t}{1 - e^{-t/T_1}} \quad (1)$$

at time t, t is the repetition time during spectrum acquisition,

Table I: Spin–Lattice Relaxation Times and Line Widths for both ^{113}Cd and ^{31}P NMR Resonances of the $ECd_4(P_i)_2$ Complex

nucleus	resonance position (ppm)[a]	T_1 (s)[b]	line width (Hz)[c]	peak designation[d]
^{113}Cd	+12.2	2.5	85	A
	−14.2	2.5	221	B
	−16.5	2.0		C
	−28.4	3.6	102	D
^{31}P	9.6	7.3	48	1
	4.9	5.8	75	2
	1.4	5.4	64	

[a] Resonance position in ppm from 0.1 M $Cd(ClO_4)_2$ for ^{113}Cd and from 0.1 M H_3PO_4 for ^{31}P. The solution used for this experiment is the same as that described in Figure 1. [b] Relaxation times were determined from a semilog plot of the intensity in a fully relaxed spectrum minus the intensity at time t vs. the repetition time. Spectral parameters for ^{113}Cd were the following: pulse angle, 90°; repetition time, 1–5 and 10 s; spectral width, 8200 or 10 000 Hz; number of scans, 7144 or 7208. Spectral width and number of scans were constant within each of two different data sets. Spectral parameters for ^{31}P were the following: pulse angle, 90°; repetition time, 1–30 s; spectral width, 5000 Hz; number of scans, 488. [c] Line widths include contributions from applied line broadening, amounting to 30 Hz for ^{113}Cd and 5 Hz for ^{31}P. [d] See the text.

and T_1 is the spin–lattice relaxation time for the resonance of interest. The measured values of T_1 for each of the enzyme-bound $^{113}Cd^{2+}$ ions are listed in Table I. For the 10-s repetition time employed in obtaining Figure 1A, the maximum correction applied, which is to peak D, is 6%.

The ^{31}P spectrum for the same solution is shown in Figure 1B and is similar to what was presented earlier (Welsh et al., 1983a). The major peaks at 9.6, 4.9, and 1.4 ppm represent P_i bound in the high- and low-affinity sites of PPase and P_i in solution, respectively. The amounts of ^{31}P in each peak, per subunit, are shown above the peaks in Figure 1B. These values have been determined, first, by correcting observed areas for incomplete relaxation according to eq 1 as described above, using the T_1 values listed in Table I, and, second, by making the reasonable assumption that the total area seen for all peaks accounts quantitatively for all of the P_i added to solution. As can be seen, the two P_i sites are virtually fully saturated in the sample solution used for Figure 1.

Previously (Welsh et al., 1983a) we showed that, in the presence of Cd^{2+}, the equilibrium constant for reaction 2 was

$$\text{enzyme·PP}_i \rightleftharpoons \text{enzyme·(P}_i)_2 \qquad (2)$$

19; i.e., at saturating P_i, about 5% of the enzyme is in the form of an enzyme·PP_i complex. We also have shown that the amount of enzyme·PP_i complex formed is higher in D_2O than in H_2O (Welsh et al., 1983b). Noting that the two smaller peaks seen at −2.1 and −2.8 ppm together integrate for 0.12 P/subunit and that the NMR solution is made up in a mixed H_2O–D_2O solvent, we conclude that these peaks represent enzyme-bound PP_i, amounting to 6% of the total enzyme in solution. The observation of two peaks for enzyme-bound PP_i would indicate not only that the two phosphoryl centers in PP_i are in somewhat different environments but also that exchange between the two environments is slow; i.e., there is neither rapid internal rotation of PP_i with respect to the enzyme surface nor rapid dissociation and reassociation of PP_i on and off the enzyme. Slow dissociation of PP_i from enzyme in the presence of Mg^{2+} has previously been demonstrated (Cohn, 1958; Janson et al., 1979; Springs et al., 1981). Both minor peaks are shifted considerably downfield (~7.5 ppm) from the position of PP_i in solution (−10 ppm), paralleling the downfield shifts seen for enzyme-bound P_i (3.5 and 8.2 ppm) with respect to P_i in solution. On this basis, it would appear that enzyme-bound PP_i is in a generally similar environment to enzyme-bound P_i.

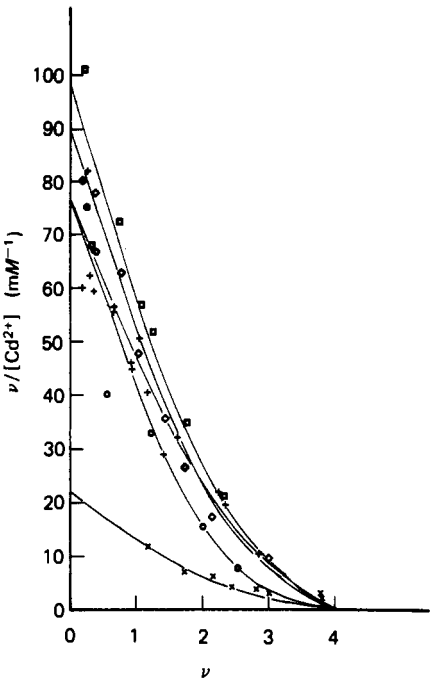

FIGURE 2: Scatchard plots for $^{115m}Cd^{2+}$ binding to PPase as a function of $[P_i]$, as measured by equilibrium dialysis. [PPase], 0.1 mM; $[^{115m}CdCl_2]$, 0.025–1.0 mM. P_i concentrations are (a) 0.5 (×), (b) 1.0 (○), (c) 2.5 (◇), (d) 5.0 (+), and (e) 10 mM (□).

Cd^{2+} Binding to PPase: Equilibrium Dialysis vs. ^{113}Cd NMR. The four peaks we observe in Figure 1A (none of which correspond to $^{113}Cd^{2+}$ in solution which comes at +31 ppm) account for virtually all (~85%) of the $^{113}Cd^{2+}$ in solution. This nearly stoichiometric binding requires dissociation constants for Cd^{2+} binding to be substantially below the free Cd^{2+} levels in solution. In previous work (Welsh et al., 1983a), we obtained data for Cd^{2+} binding to PPase in the absence of P_i, which we interpreted as indicating one tight site (K_D = 0.25 mM) and three weak sites (K_D = 3.0 mM), and for Cd^{2+} binding to PPase in the presence of 0.5 mM P_i, which we interpreted as indicating three similar high-affinity sites (K_D = 0.55 mM). Our data were insufficient to allow estimation of the K_D for a presumed fourth site of lower affinity in the presence of P_i. We also predicted that even tighter binding would be observed as the P_i concentration were raised. In the present work, we have extended our binding studies to higher levels of Cd^{2+} saturation, ν, at a series of P_i levels. Here, ν is defined by eq 3 where n represents the number of equivalent sites of type i and K_i represents the corresponding dissociation constant.

$$\nu = \sum_i \frac{n_i[Cd^{2+}]}{K_i + [Cd^{2+}]} \qquad (3)$$

The two most obvious conclusions from our data, presented in Scatchard form in Figure 2, are, first, that raising the P_i concentrations does indeed result in tighter Cd^{2+} binding and, second, that it is necessary to consider more than one class of Cd^{2+} binding sites, given the evident concavity of the Scatchard plots. Our $^{113}Cd^{2+}$ NMR results indicate the presence of four Cd^{2+} sites per subunit, and the simplest model best fitting our results is for two classes of sites with two roughly equivalent sites in each class. The lines in Figure 2 are theoretical curves for eq 3 using the K_i values listed in Table II. From the K_i values obtained, it appears that the

Table II: Dissociation Constants Used in Fitting Scatchard Binding Curves to Equation 3[a]

expt	[P_i] (mM)	[Mg^{2+}] (mM)	figure (curve)[c]	K_1 (mM)	K_2 (mM)
1	0.5		2 (a)	0.10	1.0
2	1.0		2 (b)	0.027	0.7
3	2.5		2 (c)	0.024	0.3
4	5.0		2 (d)	0.029	0.25
5	10.0		2 (e)	0.022	0.25
6[b]	5.0		5 (a)	0.029	0.25
7	5.0	2.5	5 (b)	0.042	0.46
8	5.0	5.0	5 (c)	0.08	0.50
9	5.0	10.0	5 (d)	0.13	0.52

[a] $n_1 = n_2 = 2$. [b] Same as experiment 4. [c] See legends of Figures 2 and 5.

affinity for the two tighter sites is increased at lower P_i concentration (<1 mM) whereas higher P_i concentration is required to increase affinity for the two weaker sites. It is also important to note that at the free P_i concentration (3 mM) and total PPase concentration (3 mM) present in the solution whose $^{113}Cd^{2+}$ NMR spectrum is shown in Figure 1A, binding to the tighter sites ($K_D \sim 0.025$ mM) would be essentially stoichiometric while binding to the weaker ones ($K_D \sim 0.3$ mM) would be nearly so, in agreement with the NMR results obtained. Furthermore, equilibrium binding data, obtained at higher PPase concentrations more closely approximating the conditions of the ^{113}Cd NMR experiment, give clear evidence for at least four Cd^{2+} sites per subunit. For example, at a PPase concentration of 0.82 mM, and a total initial P_i concentration of 3.0 mM (enzyme side of dialysis cell), 4.4 Cd^{2+} are bound per subunit at a free Cd^{2+} concentration of 1.15 mM.

^{113}Cd Decoupling of ^{31}P NMR Spectra for Cd–ENTMP and for $ECd_4(P_i)_2$ Complexes. The results presented to this point establish the existence of four different Cd^{2+} sites and two different P_i sites per PPase subunit. We next examine the effect of $^{113}Cd^{2+}$ decoupling on the ^{31}P NMR spectrum for enzyme-bound P_i. In the presentation that follows, the $^{113}Cd^{2+}$ peaks in Figure 1A at 12.2, −14.2, −16.5, and −28.4 ppm are designated peaks A, B, C, and D, respectively, and the ^{31}P peaks in Figure 1B at 9.6 and 4.9 ppm are designated peaks 1 and 2, respectively.

Earlier (Welsh et al., 1983a), we interpreted the reduction in the line width of peak 1 when $^{112}Cd^{2+}$ replaces $^{113}Cd^{2+}$ as evidence for inner-sphere coordination of P_i in site 1 to enzyme-bound Cd^{2+}. Such replacement was without effect on the line width of peak 2. We now extend this observation by separately irradiating each of the $^{113}Cd^{2+}$ peaks and measuring the effect of such irradiation on the ^{31}P line width. As expected, narrowing effects were seen on peak 1 but not on peak 2. The results for peak 1 are displayed in Figure 3. The maximal narrowing effects are seen at frequencies corresponding to peaks B and C and clearly do not permit a judgement as to whether they result from irradiation of peak B or peak C, or perhaps of both. An experimentally significant but smaller narrowing is also seen on irradiation of peak D, which is 12 ppm upfield from peak C, and there is essentially no effect on irradiation of peak A.

An important question posed by these results is whether the narrowing seen on peak D irradiation is due to an off-resonance effect of the irradiation of the B/C peak rather than to a direct effect of peak D irradiation. To answer this question, we conducted a model study on the complex formed by $^{113}Cd^{2+}$ with ENTMP. In very good accord with Reed & Bock (1974), whose values are given in parentheses, we determined a $J_{^{113}Cd-O-^{31}P}$ of 47 Hz (46 Hz), a $\delta_{^{113}Cd}$ of 101 (113),[2] and a $\delta_{^{31}P}$

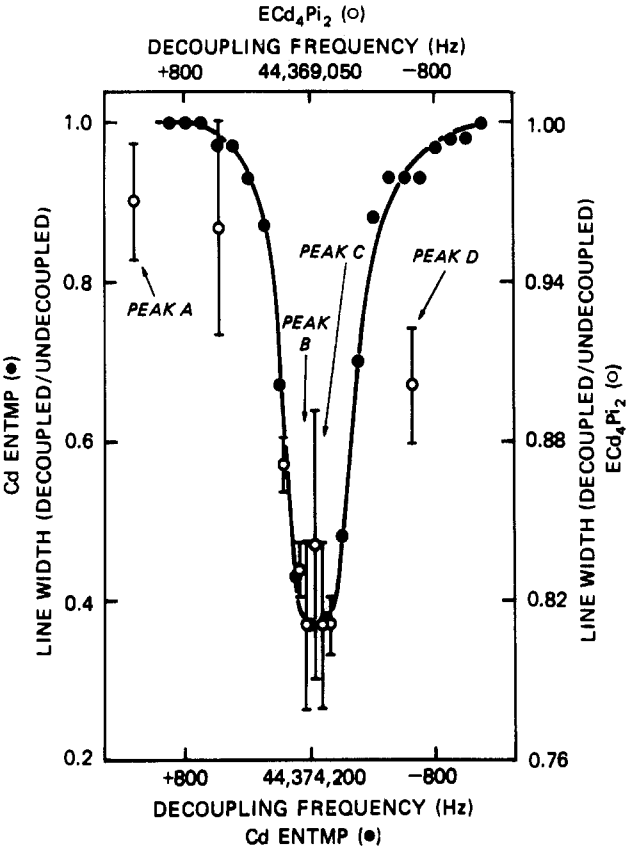

FIGURE 3: ^{31}P NMR peak line widths as a function of ^{113}Cd decoupling frequency: Cd–ENTMP and peak 1 of $ECd_4(P_i)_2$. For Cd–ENTMP (●), the single ^{113}Cd resonance is at 44 374 122 Hz (+101 ppm). For the $ECd_4(P_i)_2$ complex (○), the four $^{113}Cd^{2+}$ resonances are at 44 370 182 (12.2 ppm, peak A), 44 369 011 (−14.2 ppm, peak B), 44 368 909 (−16.5 ppm, peak C), and 44 368 381 Hz (−28.4 ppm, peak D). Error bars for the $ECd_4(P_i)_2$ results represent the average deviations for two independent experiments. Observed line widths, including a small contribution from an applied line broadening (<5 Hz), are normalized to those seen in the absence of decoupling. These were 73 Hz for the total line width of the Cd–ENTMP multiplet and 47 Hz for peak 1 in the $ECd_4(P_i)_2$ complex. Spectral parameters were as in Figure 1B except that the number of scans was 160 for Cd–ENTMP and varied from 4200 to 16 800 for $ECd_4(P_i)_2$.

of 15.4 (15.4) for this complex. Measured ^{31}P line widths for this complex as a function of irradiation frequency are also displayed in Figure 3. The maximal narrowing effect is seen at the frequency corresponding to $\delta_{^{113}Cd}$, half-maximal effects are seen at ±6 ppm (~265 Hz) from $\delta_{^{113}Cd}$, and only a very small effect is seen by ±12 ppm (~530 Hz). From this result, we conclude that the narrowing seen on peak D irradiation in our study of the $ECd_4(P_i)_2$ complex is a direct effect and that irradiation of at least *two* different $^{113}Cd^{2+}$ peaks (B/C and D) causes a narrowing of peak 1.

Competition by Mg^{2+} for Cd^{2+} Binding to PPase: ^{113}Cd NMR and Equilibrium Dialysis Studies. Evidence for the significance of the Cd^{2+} sites observed in Figure 1A was sought by examination of the effects of Mg^{2+}, the divalent metal ion conferring maximal activity on PPase (Kunitz, 1952; Welsh et al., 1983b), on the ^{113}Cd NMR spectrum. Titrations with Mg^{2+} of a solution having a composition of 1:4:3 PPase:$^{113}Cd^{2+}$:P_i were performed in both the absence and presence of KCl. The results are presented in Figure 4 and Table III, and the integrations of the peaks observed are listed

[2] A value of 103 ppm is indicated by more recent data from Reed's laboratory (personal communication).

Table III: Conditions Used for Figure 4 Spectra

spectrum	$[PPase]_T$ (mM)	$[^{113}Cd^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	$[Mg^{2+}]_T$ (mM)	0.2 M KCl	no. of scans
i	2.3	10.6	6.9		-	37 616
ii	2.4	10.2	7.2	2.4	-	37 616
iii	1.9	8.8	5.7	3.8	-	37 616
iv	2.2	9.4	6.5		+	21 040
v	2.0	8.7	6.1	2.4	+	25 896
vi	1.9	7.4	5.6	3.9	+	26 856

Table IV: Effect of Mg^{2+} and KCl on the Integrated Intensities of Enzyme-Bound $^{113}Cd^{2+}$ Resonances

	concn (mM)					integrated peak intensity (mol/mol of subunit)[a]					
expt	PPase	$^{113}Cd^{2+}$	P_i	Mg^{2+}	0.2 M KCl	A (+12 ppm)	B′ (−8 ppm)	B/C (−14 and −16 ppm)	D′ (−23 ppm)	D (−28 ppm)	total
1	2.5	10.5	7.6			0.7	0.1	1.8		0.8	3.4
2	2.7	9.8	8.1	2.7		0.9	0.1	1.6 (5)	1.0	0.4 (5)	4.1
3	2.4	9.5	7.3	5.3		0.2	0.1	0.8	0.5	0.1	1.7
4	2.2	9.4	6.5		+	0.6	0.2	1.7	0.5	0.5	3.5
5	2.0	8.7	6.1	2.4	+	0.4	0.1	1.1	0.3	0.4	2.3
6	1.9	7.4	5.6	3.9	+	0.1		0.7	0.3	0.2	1.3
7	1.5	6.4	4.6	12.5	+			0.4	0.2	0.1	0.7

[a] The integration for sample 1 is calibrated with an external standard and corrected for incomplete relaxation by using the T_1 values in Table I. All other samples were integrated in the same way. This procedure is approximately valid, although some changes in T_1, and therefore in the correction term for incomplete relaxation, would be expected on addition of Mg^{2+} and/or KCl. Spectral parameters were the following: pulse angle, 45°; spectral width, 8065–10 000 Hz; repetition time, 3 s; number of scans, 10 000–27 000.

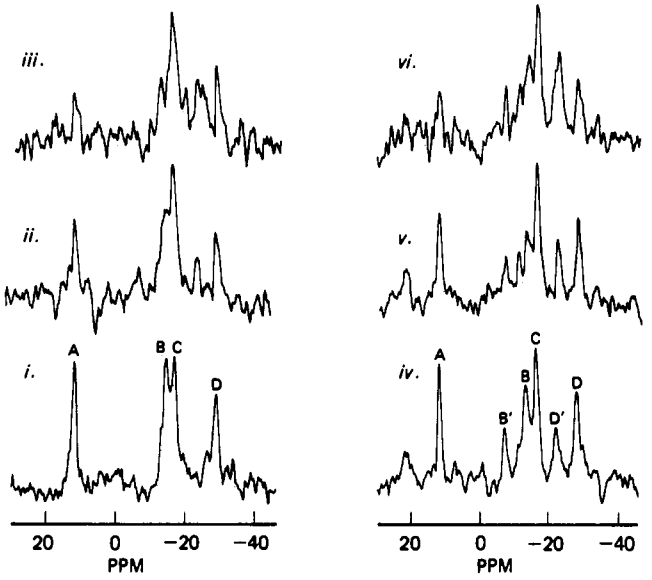

FIGURE 4: Effect of added Mg^{2+} on the ^{113}Cd NMR spectrum of $ECd_4(P_i)_2$. A pulse angle of 45° and a spectral line width of 10 000 Hz were employed for all spectra. Repetition times employed were 1 s for spectra i–iii and 3 s for spectra iv–vi.

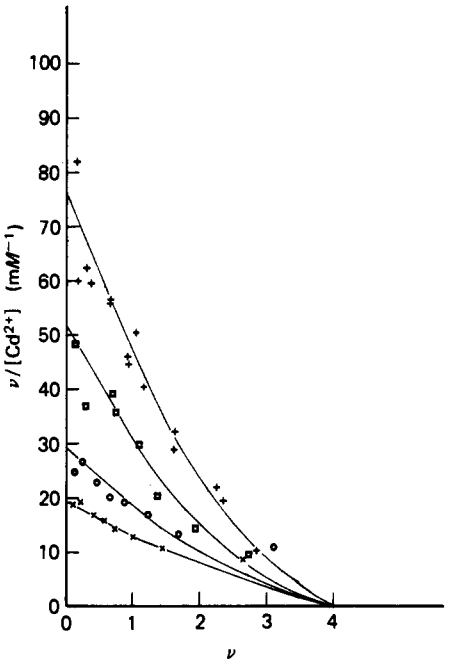

FIGURE 5: Scatchard plots for $^{115m}Cd^{2+}$ binding to PPase as a function of $[Mg^{2+}]$, as measured by equilibrium dialysis. [PPase], 0.1 mM; $[P_i]$, 5.0 mM; $[^{115m}CdCl_2]$, 0.025–1.0 mM. Mg^{2+} concentrations were (a) 0 (+), (b) 2.5 (□), (c) 5.0 (○), and (d) 10.0 mM (×).

in Table IV. The following observations may be made: (a) Two new peaks, D′ at −23 ppm and B′ at −8 ppm, are clearly seen on addition of KCl (Figure 4, spectra i and iv). The appearance of these peaks is accompanied by only minor losses in the integrated intensities of peaks A, B, C, and D. The lack of downfield shifts in peaks A, B, C, and D on addition of KCl is a clear indication that the Cd^{2+} sites these peaks represent are protected from solvent and from the deshielding effects which would be expected from coordination with chloride ion (Ackerman et al., 1979). (b) Peak D′ is also seen on addition of Mg^{2+} (Figure, spectra 4 ii and iii vs. spectrum i). (c) There is a clear decrease in the amount of ^{113}Cd intensity observed in the well-defined peaks A, B′, B, C, D′, and D as the Mg^{2+} concentration is raised. However, this decrease is not uniform. In particular, peaks A and D decrease more rapidly as a function of $[Mg^{2+}]$ than does peak B/C, while peak D′ shows the smallest relative decrease (peak B′ is too small to permit reliable estimation of its amount).

It should be noted that although the integrated intensities listed in Table IV are normalized against an external standard of $^{113}CdCl_2$, they have not been corrected for full relaxation because T_1 values were not remeasured after each addition of Mg^{2+}. Under the conditions of our experiment, failure to make such a correction should not affect the qualitative observations made above.

The effect of Mg^{2+} on Cd^{2+} binding was also examined by equilibrium dialysis experiments. A series of $^{115m}Cd^{2+}$ binding studies were carried out at a constant P_i concentration (5.0 mM) and varying Mg^{2+} concentration (2.5–10 mM). The data, presented in Scatchard form in Figure 5, were again analyzed according to the two class, two sites per class model

presented above. From the K_i values obtained (Table II), it would appear that over the range of Mg^{2+} concentrations investigated, Mg^{2+} competes for high-affinity Cd^{2+} binding but not for low-affinity Cd^{2+} binding.

Discussion

Cd^{2+} Binding to PPase. The $^{113}Cd^{2+}$ NMR spectrum shown in Figure 1A, along with the quantitation of the integrated intensities and the binding data in Figure 2, provides clear evidence for the existence of four well-defined Cd^{2+} sites per PPase subunit of reasonably high affinity ($K_D < 1$ mM). The equilibrium dialysis results in Figure 2 as well as the binding of more than four Cd^{2+} per subunit detected at high PPase concentration also indicate additional weaker binding of Cd^{2+} to other sites on PPase. Such binding may be nonspecific in nature; for example, peripheral carboxylate side chains could serve as ligands for such sites. The presence of weak Cd^{2+} sites would also account for our failure to see free $^{113}Cd^{2+}$ in solution either in Figure 1A, where the observed enzyme-bound $^{113}Cd^{2+}$ ions account for only 85% of added $^{113}Cd^{2+}$, or in the titrations summarized in Table IV, in which decreases in the intensity of enzyme-bound $^{113}Cd^{2+}$ are not matched by the appearance of free $^{113}Cd^{2+}$ in solution. In these cases, signal broadening due to chemical exchange among these sites would result in a lack of detectability of $^{113}Cd^{2+}$. Similar failure to detect $^{113}Cd^{2+}$ in aqueous solutions containing weak ligands has been noted previously (Armitage & Otvos, 1982) as has nonspecific Cd^{2+} binding to enzyme (Coleman et al., 1983).

Cd^{2+} vs. Mg^{2+} Binding. The enzyme activity conferred by Cd^{2+} is much lower (by a factor of 10^{-4}–10^{-5}) than that conferred by Mg^{2+}. From previous work comparing the activities of Mg^{2+}–PPase and Cd^{2+}–PPase (Welsh et al., 1983a), we concluded that the binding of PP_i or both molecules of P_i per PPase subunit was similar in the presence of either divalent metal ion and that the crucial difference between the two ions was in their effects on the microscopic rate constants for PP_i hydrolysis and re-formation on the enzyme surface. In view of these differences and similarities, an important question is whether the Cd^{2+}–P_i(site 1) interaction described above for Cd^{2+}–PPase is a valid model for the Mg^{2+}–P_i(site 1) interaction in Mg^{2+}–PPase. To answer this question, we investigated the effects of added Mg^{2+} both on Cd^{2+} binding to PPase, as measured by equilibrium dialysis, and on the NMR spectrum of PPase-bound $^{113}Cd^{2+}$.

The equilibrium dialysis studies provide evidence for three different kinds of divalent metal ion sites on PPase: those which show a strong, perhaps exclusive preference for binding Mg^{2+} over Cd^{2+}, those which bind both Cd^{2+} and Mg^{2+} competitively, and those which show a strong, perhaps exclusive preference for binding Cd^{2+} over Mg^{2+}. In discussing these conclusions, we assume, for simplicity, two classes of Cd^{2+} binding sites, as we did in calculating theoretical Scatchard binding curves (Figures 2 and 5). However, our qualitative conclusions do not depend on this assumption.

Previously, we proposed a model for Mg^{2+} binding to PPase in which two Mg^{2+} are bound per PPase subunit in the absence of substrate with relatively high affinity and additional Mg^{2+} ions are bound in the presence of substrate with lower affinity (Springs et al., 1981). The presence of the first two sites was demonstrated by Rapoport et al. (1973), who determined dissociation constants of 0.04 and 0.3 mM, under conditions similar to those used in the present studies. The presence of the other sites of considerably lower affinity was inferred by measuring the Mg^{2+} dependence of (a) the half-maximal P_i concentrations for PPase catalysis of water–P_i oxygen exchange and for enzyme-bound PP_i formation (Springs et al., 1981) and (b) the V_{max} for PPase catalysis of PP_i hydrolysis (Moe & Butler, 1972).

Interpreting our present results in light of this model, we conclude, first, that the sites to which Mg^{2+} binds in the absence of substrate have high specificity for Mg^{2+} over Cd^{2+}. This is based on the only modest effects that added Mg^{2+} at 2.5 mM has on the dissociation constants of either class of Cd^{2+} binding sites (Table II, experiment 6 vs. experiment 7). In view of the dissociation constants for the Mg^{2+} sites cited above, were Cd^{2+} binding occurring to these sites, the apparent Cd^{2+} dissociation constants would have increased in the presence of 2.5 mM Mg^{2+} by 8–60-fold. Second, we conclude that the additional sites to which Mg^{2+} binds in the presence of substrate also show strong binding to Cd^{2+}. This is based on the large increases in the dissociation constant for high-affinity Cd^{2+} binding as the Mg^{2+} concentration is increased from 2.5 to 10.0 mM (Table II, experiments 7–9). The presence of sites on PPase having low Mg^{2+} affinity and high Cd^{2+} affinity is in accord with other experiments measuring divalent metal ion binding to PPase, if it is assumed, on the basis of the Goldschmidt ionic radius, that Cd^{2+} (1.03 Å) is a reasonable analogue of Ca^{2+} (1.06 Å)[3] and that Mn^{2+} (0.91 Å) is a reasonable analogue of Mg^{2+} (0.78 Å). Thus, Ridlington & Butler (1972) have shown that although Ca^{2+} binds only weakly to PPase, the $CaPP_i$ complex binds very tightly, without being hydrolyzed. Indeed, it is the tight binding of this complex which accounts for the strong inhibition by Ca^{2+} of yeast PPase activity. In addition, Banerjee & Cooperman (1983) have provided EPR evidence for the binding of Ca^{2+} to one specific site of PPase in the presence of a PP_i analogue, hydroxymethylenebisphosphonate (PCHOHP), which is a competitive inhibitor of PPase, and of 1.5 equiv of Mn^{2+} per PPase subunit. In the latter work, the very strong Mn^{2+}–Mn^{2+} interaction which is induced by addition of PCHOHP to a solution containing enzyme-bound Mn^{2+} is specifically removed on addition of 1 equiv of Ca^{2+}, although no Mn^{2+} is released from PPase. This result has been taken to indicate that Ca^{2+} can displace Mn^{2+} from one specific site on the enzyme, presumably one directly bound to PCHOHP. Third, we conclude that the lower affinity (but still well-defined) sites for Cd^{2+} bind Mg^{2+} very poorly, if at all, on the basis of the insensitivity of low-affinity Cd^{2+} binding to added Mg^{2+} in the range of 2.5–10.0 mM (Table II, experiments 7–9). Although this conclusion is supported by only a limited amount of data, poor Cd^{2+} phosphate solubility posing a technical problem, it is quite straightforward from our results. It is true that very weak Mg^{2+} binding ($K_D > 20$ mM) to these sites would not have been detected by these studies and so cannot be excluded.

Such allosteric sites specific for Cd^{2+} (and, by analogy, Ca^{2+}) binding raise an interesting issue. It has long been a matter of speculation whether the strong inhibition of PPase by $CaPP_i$ is important physiologically as a control element for total flux through PPase. Might activity modulation also be achieved by Ca^{2+} binding to separate, regulatory sites?

Our conclusion that it is the substrate-induced divalent metal ion binding sites that are competitive for Cd^{2+} and Mg^{2+} binding supports the notion that the Cd^{2+}–P_i(site 1) direct interaction we have demonstrated in this work is a valid model for Mg^{2+}–P_i(site 1) interaction. We had hoped that examination of Mg^{2+} effects on the NMR spectrum of enzyme-

[3] The appropriateness of Cd^{2+} as a model for Ca^{2+} has been exploited in several $^{113}Cd^{2+}$ NMR studies of Ca^{2+} binding proteins such as calmodulin and carp parvalbumin, as discussed recently by Armitage & Otvos (1982).

bound $^{113}Cd^{2+}$ would provide additional evidence distinguishing between competitive and noncompetitive sites and, specifically, that the intensity of peaks corresponding to competitive sites would decrease with added Mg^{2+}, while peaks corresponding to noncompetitive sites would be insensitive to such addition. Unfortunately, such straightforward effects are not seen. Instead, new peaks are observed on Mg^{2+} (and even KCl) addition, and intensity is lost in all four of the original peaks (Figure 4 and Table IV). The appearance of new peaks probably reflects an indirect effect of Mg^{2+} (or K^+) binding, given the known sensitivity of $^{113}Cd^{2+}$ chemical shifts to even small changes in environment (Armitage & Otvos, 1982). The decreases in intensity of the peaks can be due to direct competition of Mg^{2+} for Cd^{2+} binding but can also be due to an indirect effect of Mg^{2+} binding that exchange-broadens an enzyme-bound $^{113}Cd^{2+}$ resonance, as has been noted recently in studies on alkaline phosphatase (Gettins & Coleman, 1983a). Such broadening could result, for example, if the chemical shift of an enzyme-bound $^{113}Cd^{2+}$ is altered when Mg^{2+} is bound at another site, and Mg^{2+} exchanges between solvent and enzyme, or if Mg^{2+} binding to one site alters a Cd^{2+} chemical exchange rate at another site. In fact, we attribute to indirect effects the decreases in $^{113}Cd^{2+}$ peak intensity resulting from addition of up to 2 equiv of Mg^{2+} to an $ECd_4(P_i)_3$ solution (Figure 4 and Table IV, experiments 1–3 and 4–6), since our conclusions derived from the equilibrium dialysis results would predict that the first 2 equiv of Mg^{2+} added would bind to sites on PPase different from the Cd^{2+} sites. Although at higher Mg^{2+} concentrations (Table IV, experiment 7) direct competitive effects should also be important, it is clear from the above discussion that Mg^{2+}-induced changes in the NMR spectrum of PPase-bound $^{113}Cd^{2+}$ cannot be used to clearly distinguish competitive from noncompetitive effects.

Cd^{2+}–P_i Contact on PPase. The heteronuclear decoupling experiment presented in Figure 3 confirms our previous finding (Welsh et al., 1983) that some enzyme-bound Cd^{2+} is in inner-sphere contact with P_i bound in site 1 and provides some information on the number of $^{113}Cd^{2+}$ ions making such contact. The simplest interpretation of this experiment is that at least two (but not more than three) Cd^{2+} ions are bound in an inner-sphere manner to P_i in site 1, one (or two) corresponding to peak B and/or peak C and one corresponding to peak D. Such an interpretation, if valid, would, to our knowledge, represent the first demonstration of multiple divalent metal ion coordination to a single enzyme–bound phosphoryl group.[4] However, an alternative interpretation, that a single Cd^{2+}–P_i inner-sphere contact at the Cd^{2+} site corresponding to either peak B or peak C accounts not only for the narrowing of the ^{31}P line width observed on irradiation of peak B/C but also for the lesser narrowing observed on irradiation of peak D, cannot at present be excluded. Such an interpretation would require a chemical exchange between the site for Cd^{2+} binding corresponding to peak D and the site corresponding to either peak B or peak C, with a half-time of about 1 s, comparable to or slightly shorter than the T_1 values (2–3 s, Table I) for $^{113}Cd^{2+}$ relaxation in those sites (if the half-time for exchange were $\ll 1$ s, irradiation at peak D would have the same line-width narrowing effect as irradiation at peak B/C, whereas if the half-time for exchange were $\gg 1$ s, irradiation at peak D would have no effect). It should be noted that such chemical exchange is not inconsistent with the relatively sharp ^{113}Cd peaks seen in Figure 1A, since for the 10–20 ppm (450–900 Hz) separations seen, any exchange time in excess of 1 ms is in the slow exchange limit.

[4] A comparable study of alkaline phosphatase showed only one enzyme-bound Cd^{2+} was bound in an inner-sphere manner to the enzyme-bound phosphoryl group (Gettins & Coleman, 1983b).

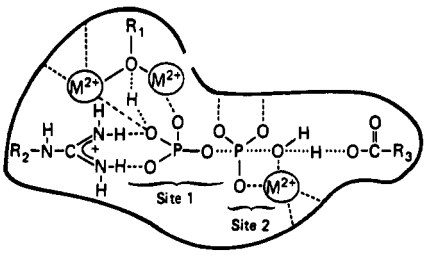

FIGURE 6: Current model for the active-site structure of PPase. See text for discussion.

Future experiments both resolving the effects of peak B from peak C irradiation and measuring the rates of chemical exchange between Cd^{2+} sites [e.g., by saturation or inversion transfer (Jardetsky & Roberts, 1981)] should allow an unambiguous determinations of the number of Cd^{2+} ions bound to P_i in site 1 in an inner-sphere manner. While reserving final judgement until such experiments are performed, we believe it most likely that the correct number is 2. This number would be in good accord with our Mn^{2+} EPR results (Banerjee & Cooperman, 1983), alluded to above, indicating that two enzyme-bound divalent metal ions are brought into close proximity in the presence of a phosphoryl ligand. Moreover, were the number to be 3, at least one Cd^{2+} whose binding does not mimic that of Mg^{2+} would have to be bound at the active site, and such binding would be unexpected.

Active-Site Structure of PPase. The results presented in this paper and other results obtained recently by ourselves and others make this a reasonable time to update the model for the transition state for PP_i hydrolysis or formation on PPase that we put forward 2 years ago (Cooperman, 1982). This model is shown in Figure 6, and its various features are discussed below.

First, the hydrolysis of PP_i is depicted as occurring via direct transfer to water, rather than via a phosphoryl enzyme intermediate. Strong support for this view has recently been provided by stereochemical and single-turnover experiments on PPase (Gonzalez et al., 1984). Second, we identify the electrophilic phosphoryl group as binding to site 2, which has lower affinity, and the leaving (or attacking) phosphoryl group as binding to site 1, which has higher affinity. This assignment was originally made on the basis of our demonstrations that, following hydrolysis, the electrophilic P_i leaves the enzyme first, which is the anticipated (though not mandatory) order for a site of lower affinity, and that P_i binding to site 1 alone protects Arg-77, a residue which we have shown to be essential for activity (Bond et al., 1980), from modification by phenylglyoxal (Cooperman et al., 1981). Recent X-ray crystallographic analysis of $CaPP_i$ binding to PPase also implicates Arg-77 as being present at the active site (Kuranova et al., 1983). The present results show in addition that one to three Cd^{2+} ions are directly coordinated to P_i in site 1 and provide evidence that such coordination should be true of Mg^{2+} as well. Two divalent metal ions are shown coordinated to P_i in site 1 because we believe this number most likely to be correct, as discussed above. We would expect that the simultaneous binding of the leaving group P_i to one to three divalent metal ions as well as to an arginine side chain would greatly increase its conjugate acidity. Such an increase could be a major factor in PPase catalysis given the known high Bronsted leaving group β value (1.2) for phosphoryl transfer in solution (Kirby & Varvoglis, 1967). For example, a pK_a lowering of 4–5 units,

which would not be unreasonable with such a concentration of positive charge, would correspond to a rate acceleration of 10^5–10^6.

Other features of the catalytic mechanism are more open to conjecture, but there is enough information to allow for some intelligent speculation. The abstraction of a water proton by a carboxylate side chain is based on (1) our assumption that some sort of general base activation of the attacking water is necessary, (2) chemical modification experiments implicating an aspartate or glutamate at the active site (Cooperman & Chiu, 1973; Heitmann & Uhlig, 1974; Cooperman et al., 1981) and (3) pH–rate profile studies implicating a basic group of $pK_a = 6$ as being essential for activity (Knight et al., 1981). Kuranova et al. (1983) have, on the basis of their X-ray results, proposed that Glu-58 is the general base depicted in Figure 6. Our proposed catalytic mechanism also includes a general acid, R_1OH. The presence of such a group is inferred from pH–rate profile studies implicating an acidic group of $pK_a = 8$ as being essential for enzyme activity (Knight et al., 1981) and from the need for each of the P_i's formed on PP_i hydrolysis to pick up a proton prior to release from PPase. This need arises because the true substrate for PPase is a Mg^{2+} complex of $P_2O_7^{4-}$. We should point out that since the microscopic steps for PP_i hydrolysis and the release of each of the P_i's are all partially rate determining (Springs et al., 1981), proton donation from R_1OH to the phosphoryl group in site 1 need not be simultaneous with water attack on the phosphoryl group in site 2. On the basis of the available evidence from chemical modification studies (Heitmann et al., 1972; Cooperman & Chiu, 1973; Chiu, 1974; Heitmann & Uhlig, 1974) which exclude essential lysine, cysteine, or histidine residues, a tyrosine residue or a bound water molecule is the most likely candidate for R_1OH and several tyrosine residues (numbers 88, 92, and 191 in the sequence) have been placed at or near the active site by Kuranova et al. (1983). Complexation of R_1OH to either or both of the metal ions bound to P_i in site 1, indicated as a possibility in Figure 7, could lower the pK_a of R_1OH to 8.

In addition to the divalent metal ion(s) directly coordinated to the leaving (or attacking) phosphoryl group in site 1, our model for the catalytic mechanism also depicts an additional divalent metal ion complexed to the electrophilic phosphoryl center. This arrangement of divalent metal ions, two very close to one another and one farther away, is also in accord with the Mn^{2+} EPR results alluded to earlier (Banerjee & Cooperman, 1983). Divalent metal ion coordination to the phosphoryl group in site 2 would be consistent with results showing that such metal ion complexation allows anionic nucleophiles to attack an anionic phosphoryl center, a reaction that is otherwise very difficult to demonstrate, presumably because of charge repulsion (Cooperman, 1976; Hsu & Cooperman, 1976). However, our results showing, first, no apparent $^{113}Cd^{2+}$–^{31}P inner-sphere interaction with P_i in site 2 (Welsh et al., 1983a) and, second, only outer-sphere interaction (estimated distance, 6.2 Å) between enzyme-bound Mn^{2+} and P_i in site 2 (Hamm & Cooperman, 1978) are strong evidence that such interaction does not persist into the product state. If we posit that the divalent metal ion in question is bound directly to enzyme in the absence of P_i, then we might imagine that the bonds to enzyme could constrain its motion and cause it to separate from P_i as the reaction proceeds from the transition to the product state. From our interpretation of Mg^{2+} effects on Cd^{2+} binding, such an assignment would have as a consequence that the divalent metal ion site in question has strong, perhaps exclusive preference for Mg^{2+} over Cd^{2+}. The inability of Cd^{2+} to occupy the site would then provide a rationale for the poor catalytic activity of the Cd^{2+}–enzyme. Finally, it is worth noting that our model for the active site ascribes function to three different metal ions. This is consistent with recent model studies on metal ion catalysis of PP_i hydrolysis in solution (Hubner & Milburn, 1980) showing that the active species has a stoichiometry of three metal ions to one PP_i and with X-ray diffraction studies of PPase indicating the presence of a site on the enzyme containing a cluster of three metal ions (Kuranova et al., 1981, 1983).

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Registry No. PPase, 9024-82-2; P_i, 14265-44-2; Cd, 7440-43-9; Mg, 7439-95-4; ^{113}Cd, 14336-66-4.

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X-ray Analysis of the Kinetics of *Escherichia coli* Lipid and Membrane Structural Transitions†

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ABSTRACT: Synchrotron radiation was used to follow the time course of the transitions, induced by temperature jump, in *Escherichia coli* membranes and their lipid extracts isolated from a fatty acid auxotroph grown with different fatty acids. We measured the relaxation times associated with the phase transitions as well as with the conformational transition of the hydrocarbon chains and observed different behavior as a function of chemical composition. Relaxation times of about 1–2 s were found at a hexagonal to lamellar phase transition and within a lamellar phase whose parameters display important variations with temperature when the conformational transition takes place. On the other hand, no delay was observed for a phase transition where large lipid or water diffusion was not needed. We have shown that phase transitions and conformational transitions are, to a large extent, uncoupled and that the relaxation times corresponding to the latter transition could be related to the size of the ordered domains. In all cases, the order to disorder conformational transition is more rapid than the disorder to order transition. Finally, the relaxation times of the disorder to order transition observed with the membranes and with their lipid extracts were found to be strongly correlated, indicating that the proteins do not play a role in this transition.

Lipid–water systems are known to exhibit a remarkable polymorphism as a function of temperature and water content (Luzzati & Tardieu, 1974). In addition, the hydrocarbon chains of the lipids undergo reversible, temperature-dependent transitions between disordered (type α) and partially ordered (type β, β') conformations (Tardieu et al., 1973; Ranck, 1983). Such systems have been extensively studied by using a variety of physical techniques (X-ray diffraction, calorimetry, spectroscopy, etc.) and a number of phases, and a variety of hydrocarbon chain conformations are now fairly well characterized [for recent reviews, see Zannoni et al. (1983), Kimmich et al. (1983), Hemminga (1983), and Ranck (1983)].

In the past, we have used high-angle X-ray diffraction to investigate the behavior as a function of temperature of various cytoplasmic membranes and lipid extracts isolated from an *Escherichia coli* unsaturated fatty acid auxotroph. This enabled us to establish correlations between structural, morphological, and physiological properties of these systems (Shechter et al., 1974; Letellier et al., 1977). In the present study, the earlier work is extended to an analysis of the time course of the transition phenomena induced by temperature jumps.

First, we recorded both high- and low-angle diffraction patterns for the lipid extracts in the presence of water. The structure of the phases observed as a function of temperature

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