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Catalytic Specificity of Yeast Inorganic Pyrophosphatase for Magnesium Ion as Cofactor. An Analysis of Divalent Metal Ion and Solvent Isotope Effects on Enzyme Function†

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ABSTRACT: This work extends our functional studies of yeast inorganic pyrophosphatase, previously performed in the presence of Mg^{2+} in H_2O [Springs, B., Welsh, K. M., & Cooperman, B. S. (1981) *Biochemistry 20*, 6384–6391], to studies in the presence of Zn^{2+}, Mn^{2+}, or Co^{2+} in H_2O and of Mg^{2+} in D_2O. Measurements of equilibrium formation of enzyme-bound pyrophosphate as a function of added inorganic phosphate and of the rates of enzyme catalysis of inorganic pyrophosphate hydrolysis and H_2O–inorganic phosphate oxygen exchange are used to calculate microscopic rate constants for (a) pyrophosphate hydrolysis and formation on the enzyme surface and (b) release of inorganic phosphate from enzyme. These rate constants allow the following conclusions to be drawn: (1) The solvent isotope effect on overall enzyme-catalyzed pyrophosphate hydrolysis that we previously measured [Konsowitz, L., & Cooperman, B. S. (1976) *J. Am. Chem. Soc. 98*, 1993–1995] derived primarily from an effect on the rate of inorganic phosphate release from enzyme. Only a modest effect is found on the rate of pyrophosphate hydrolysis on the enzyme surface. (2) The lower effectiveness, compared with Mg^{2+}, of Zn^{2+}, Mn^{2+}, and Co^{2+} as cofactors for inorganic pyrophosphate hydrolysis is due mainly or entirely to the slower rates of phosphate release from enzyme in the presence of each of these ions. Put another way, the specificity of inorganic pyrophosphatase for Mg^{2+} as a cofactor derives in large measure from the rapid rates of phosphate release achievable in its presence. Similar considerations might explain the specificity of other phosphoryl enzymes for Mg^{2+} as cofactor. In a second series of experiments, solvent isotope effects on overall enzyme-catalyzed PP_i hydrolysis are determined in the presence of Zn^{2+}, Co^{2+}, and Mn^{2+} and compared with that found in the presence of Mg^{2+}. The magnitudes of the effect are found to decrease in the order $Mn^{2+} > Zn^{2+} > Co^{2+} > Mg^{2+}$. This result supports conclusions 1 and 2 reached above, since the rate of inorganic phosphate release is uniquely rate determining in the presence of Zn^{2+} or Mn^{2+} and only partly rate determining in the presence of Co^{2+} or Mg^{2+}, and, as already mentioned, inorganic phosphate release displays a higher solvent isotope effect than does pyrophosphate hydrolysis on the enzyme surface.

Yeast inorganic pyrophosphatase (EC 3.6.1.1) (PPase),[1] a dimeric enzyme composed of identical subunits, has long been known to require divalent metal ions for activity [Kunitz, 1952; for a recent review of the properties of this enzyme, see Cooperman (1982)]. With PP_i as a substrate, the relative activity conferred by divalent metal ions falls in the order $Mg^{2+} > Zn^{2+} > Co^{2+}$, $Mn^{2+} \gg Cd^{2+}$ (Butler & Sperow, 1977; Janson et al., 1979; Hackney, 1980; O. A. Moe, Jr., S. Pham, B. Selinsky, and T. Dang, unpublished experiments; Welsh et al., 1983). PPase catalysis of PP_i hydrolysis in the presence of Mg^{2+} displays a solvent isotope effect of just under 2 (Konsowitz & Cooperman, 1976), measured at the pH and pD maxima.

In recent work (Springs et al., 1981), we presented a unified scheme for PPase action and demonstrated the following.

(1) Equation 1, in which three Mg^{2+} are bound per PPase subunit in the presence of P_i or PP_i, quantitatively accounts

$$MgPP_i + Mg_2E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mg_2EMgPP_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MgE(MgP_i)_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} MgEMgP_i + MgP_i \underset{k_8}{\overset{k_7}{\rightleftharpoons}} Mg_2E + P_i \quad (1)$$

for PPase catalysis of PP_i hydrolysis, of H_2O–P_i oxygen exchange, and of P_i–PP_i exchange at Mg^{2+} concentrations below 10 mM. At higher Mg^{2+} concentrations, it is also necessary to consider eq 2, in which four Mg^{2+} are bound per subunit in the presence of P_i or PP_i. In these equations, we are con-

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; PPase, yeast inorganic pyrophosphatase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; the subscript T indicates the total stoichiometric concentration of a species added to a solution.

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$$Mg_2PP + Mg_2E \underset{k_{2'}}{\overset{k_{1'}}{\rightleftharpoons}} Mg_2EMg_2PP_i \underset{k_{4'}}{\overset{k_{3'}}{\rightleftharpoons}} Mg_2E(MgP_i)_2 \underset{k_{6'}}{\overset{k_{5'}}{\rightleftharpoons}} Mg_2EMgP_i + MgP_i \underset{k_{8'}}{\overset{k_{7'}}{\rightleftharpoons}} Mg_2E + MgP_i \quad (2)$$

cerned only with binding stoichiometries per enzyme subunit and not with relative physical arrangements of Mg^{2+}, P_i, and PP_i on the enzyme. Thus, for example, $MgEMgP_i$ refers to an enzyme subunit binding two Mg^{2+} and one P_i and does not signify that there is necessarily an enzyme-bound Mg^{2+}–P_i complex.

(2) There is little difference in the kinetic or thermodynamic parameters for eq 1 vs. eq 2. That is, equilibrium formation of enzyme-bound PP_i from P_i in solution and rates of PPase catalysis are little changed by the binding of an additional Mg^{2+} to each of the important enzyme forms.

(3) The first P_i released from enzyme following PP_i hydrolysis (via step 5) is the one which exchanges oxygen with water.

(4) All eight microscopic rate constants involved in overall PPase catalysis (k_1–k_8, eq 1) can be evaluated by determination of the equilibrium constant for enzyme-bound PP_i formation ($1/K_3 = k_4/k_3$) and of steady-state rate parameters for PP_i hydrolysis and H_2O–P_i oxygen exchange.

In the present work, we apply our previously developed methodology to evaluate microscopic rate constants when either Zn^{2+}, Co^{2+}, or Mn^{2+} replaces Mg^{2+} as the required divalent metal ion cofactor or when D_2O replaces H_2O in the presence of Mg^{2+}. We also measure the kinetic solvent isotope effect in the presence of Zn^{2+}, Co^{2+}, or Mn^{2+}. These determinations allow us to define which steps in catalysis are most affected by changes either in divalent metal ion or in solvent and provide a basis for understanding the catalytic specificity of PPase for Mg^{2+}.

Experimental Procedures

Materials. PPase was prepared as described previously (Cooperman et al., 1973) with modifications (Bond, 1979) and ranged in specific activity from 480 to 680 μmol of PP_i min^{-1} mg^{-1} as determined by standard titrimetric assay (Cooperman et al., 1973). The following materials were obtained from the sources indicated: D_2O (99.8%, Aldrich or Stohler); carrier-free [^{32}P]PP_i (New England Nuclear); carrier-free [^{32}P]P_i (ICN); ^{18}O-enriched water (≥99%, Norsk-Hydro). All other chemicals were reagent grade and used without further purification.

Methods. Initial rates of PP_i hydrolysis were performed by using either [^{32}P]PP_i, as described in Springs et al. (1981), or colorimetric determination of P_i, as described in Cooperman et al. (1973). Determination of enzyme-bound PP_i formation was carried out by the selective extraction method described previously (Springs et al., 1981). Rates of H_2O–P_i oxygen exchange were determined by measuring ^{18}O release from ^{18}O-labeled P_i [prepared according to Hackney et al. (1980) as modified in Springs et al. (1981)], using ^{31}P NMR analyses (Cohn & Hu, 1978), essentially as reported previously (Springs et al., 1981). Some modifications were introduced in order to completely remove paramagnetic metal ions such as Mn^{2+} and Co^{2+}. In typical analyses, reaction aliquots were quenched with a final concentration of 0.17 N HCl and 0.17% sodium dodecyl sulfate. Quenched samples (3.0 mL) were incubated for at least 3 h at room temperature before application to a Dowex 50 column (0.8 cm × 7 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, and adjusted to pH 8.2 ± 0.2 at a final P_i concentration of ~20 mM. ^{31}P NMR spectra were taken of these final solutions.

With the exception of the solvent isotope effect studies, all rate and equilibrium measurements were performed in buffer A [50 mM Tris (pH 7.0 or pD 7.4) and 200 mM KCl] at 25 °C. For all rate or equilibrium measurements performed in D_2O, the pD was set equal to the read pH plus 0.40 (Glasoe & Long, 1960).

Table I: Parameters for EPP_i Formation

M^{2+}	$[M^{2+}]_T$ (mM)	$[PPase]_T$ (mM)	K_3	$K_{p,app}$ (mM)
Mn^{2+}	0.7	0.09–0.11	47	2.2
Co^{2+}	2.0	0.11–0.125	3.2	1.25
Zn^{2+}	2.0	0.25	139	2.8
		0.50	165	2.0
Mg^{2+} (D_2O)	30[a]	0.11	3.0	18
Mg^{2+} (H_2O)	30[a]	0.11–0.14	4.8	12
Mg^{2+} (H_2O)	10[a]	0.11–0.14	4.9	27

[a] Calculated free $[Mg^{2+}]$. All other values are total concentrations.

Results

Functional Studies of PPase at pH 7.0 or pD 7.4. We previously evaluated microscopic rate constants for overall PPase catalysis of PP_i–P_i equilibration in the presence of Mg^{2+} and H_2O at pH 7.0 and 25 °C (Springs et al., 1981). We here present measurements of several enzyme functions in the presence of Zn^{2+}, Co^{2+}, and Mn^{2+} (all in H_2O) and Mg^{2+} (in D_2O), under the same conditions, which allow calculation of rate constants for comparison with those obtained in the presence of Mg^{2+} and H_2O. The quantities measured are (1) equilibrium formation of enzyme-bound PP_i, (2) steady-state rates of PP_i hydrolysis, and (3) steady-state rates of H_2O–P_i oxygen exchange.

PPase has both high- and low-affinity sites for P_i (Hamm & Cooperman, 1978; Cooperman et al., 1981; Springs et al., 1981; Welsh et al., 1983). Thus, at relatively high P_i concentrations, the total enzyme concentration is given by eq 3,

$$[E]_T = [EP_i] + [E(P_i)_2] + [EPP_i] \quad (3)$$

$$EP_i + P_i \rightleftharpoons E(P_i)_2 \rightleftharpoons EPP_i \quad (4)$$

and the equilibria in eq 4 obtain, it being understood 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 the bound divalent metal ion or the protonation state. The equilibrium formation of EPP_i as a function of $[P_i]$ is then given by

$$\frac{[E]_T}{[EPP_i]} = 1 + K_3 + \frac{K_3 K_{p,app}}{[P_i]} \quad (5)$$

where $K_3 = [E(P_i)_2]/[EPP_i]$ and $K_{p,app} = [EP_i][P_i]/[E(P_i)_2]$. Plots of $[E]_T/[EPP_i]$ vs. $1/[P_i]$ are shown in Figure 1, yielding the values for K_3 and $K_{p,app}$ presented in Table I. The extent of formation of EPP_i from P_i is not very sensitive to free divalent metal ion concentration at the divalent metal ion concentrations used in this study. Thus, K_3 is insensitive to free $[Mg^{2+}]$ in the range of 10–30 mM (Table I), and in experiments not shown, similar results have been demonstrated for Co^{2+} (1–3 mM), Zn^{2+} (1–2 mM), and Mn^{2+} (1–2 mM).

One interesting result which is apparent from Table I is that, in the presence of Mg^{2+}, D_2O has the effect of increasing the amount of EPP_i formed at saturating P_i concentrations. That

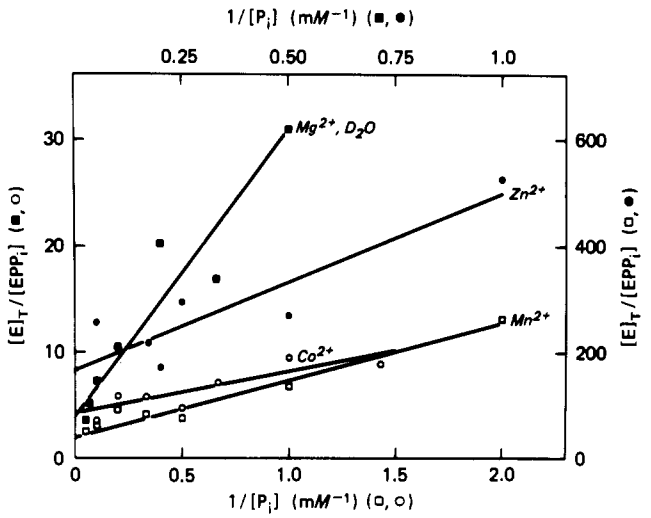

FIGURE 1: Dependence of $[E]_T/[EPP_i]$ on $1/[P_i]_T$. All reactions are in buffer A at 25 °C. Equilibrium mixtures contained (■) 30–54 mM $MgCl_2$ and 1–54 mM P_i in D_2O, (●) 2 mM $ZnSO_4$ and 1–10 mM P_i, (○) 2 mM $CoCl_2$ and 0.5–5 mM P_i, and (□) 0.7 mM $MnCl_2$ and 0.5–20 mM P_i.

Table II: Solvent Isotope Effect on EPP_i Formation

M^{2+}	$[M^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	$([EPP_i]/[E]_T, D_2O)/([EPP_i]/[E]_T, H_2O)$
Mg^{2+}	43	30	1.55
Co^{2+}	2	10	1.29
Mn^{2+}	0.7	20	1.68
Cd^{2+}	1.5	10	1.73

Table III: Rate Parameters for PP_i Hydrolysis

M^{2+}	concn (mM)	$[PP_i]_T$ (mM)	$k_{cat,hyd}$ (s^{-1})	$K_m^{PP_i}$ (μM)
Zn^{2+}	0.25	0.005–0.030	60	12
Zn^{2+}	0.5	0.005–0.050	37	10
Zn^{2+}	1.0	0.005–0.050	19.1	7.1
Co^{2+}	0.1	0.005–0.100	8.9	23
Co^{2+}	0.25	0.005–0.100	10.3	23
Co^{2+}	0.5	0.002–0.200	9.7	20
Co^{2+}	1.0	0.002–0.200	8.8	26
Co^{2+}	2.0	0.002–0.200	7.4	24
Mn^{2+}	0.1	0.020–0.200	34	19
Mn^{2+}	0.25	0.015–0.200	29	18
Mn^{2+}	0.5	0.010–0.200	22	20
Mn^{2+}	0.75	0.010–0.200	16.7	21
Mn^{2+}	1.0	0.010–0.200	16.3	25

this solvent isotope effect is general for all of the ions tested is shown by the data presented in Table II.

Values for $k_{cat,hyd}$ and $K_m^{PP_i}$ as a function of divalent metal ion concentration, determined by Eadie–Hofstee plots of initial velocity/$[PP_i]_T$ vs. initial velocity, are presented in Table III. These results show a clear reduction in k_{cat} at higher M^{2+} concentrations, in agreement with earlier reports of others (Moe & Butler, 1972; Moe et al., 1979; Knight et al., 1981; Volk et al., 1981). By contrast, $K_m^{PP_i}$ values vary much less as a function of divalent metal ion concentration.

Rate constants for H_2O–P_i oxygen exchange were determined by ^{31}P NMR analysis (Cohn & Hu, 1978). Sample spectra for a single kinetic run are shown in Figure 2. Hackney & Boyer (1978) have defined a partition coefficient, P_c, for enzyme-catalyzed H_2O–P_i oxygen exchange, which for PPase is equal to the rate at which enzyme-bound P_i loses oxygen in the exchange step divided by the sum of this rate and the rate of release of P_i to the medium. They have also shown that P_c can be calculated from the ratio (R) of the rate

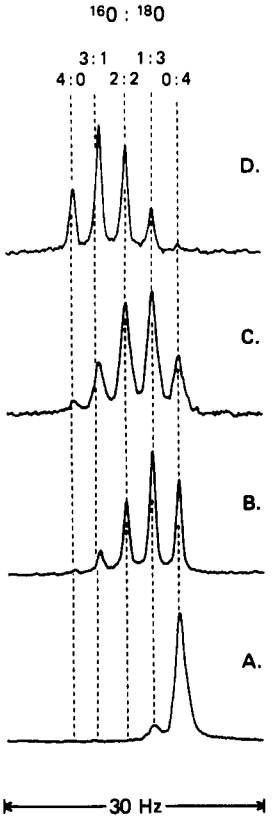

FIGURE 2: ^{31}P NMR spectra showing PPase catalysis of H_2O–P_i oxygen exchange at 25 °C. The initial reaction mixture contained 0.1 mM $MnCl_2$, 10 mM $P^{18}O_4$, and 0.8 μM PPase in buffer A. The times of incubation were (A) 0, (B) 2.5, (C) 4, and (D) 10 h. Spectral parameters were the following: frequency, 145.7 MHz; spectral width, 1000 Hz; acquisition time, 8.2 s; and number of scans, ≃64. In suitable control experiments, no exchange was seen in the absence of either PPase or Mn^{2+}.

Table IV: Rate Parameters for H_2O–P_i Oxygen Exchange

no.	M^{2+}	$[M^{2+}]_T$ (mM)	$[P_i]_T$ (mM)	k_{ex} (s^{-1})	R[b]	P_c
1	Zn^{2+}	0.1	10	3.6	3.99 ± 0.20 (10)	0.00–0.07
2	Zn^{2+}	0.2	10	4.4	3.95 ± 0.16 (10)	0.00–0.07
3	Zn^{2+}	0.3	10	5.2	3.87 ± 0.18 (9)	0.00–0.10
4	Zn^{2+}	0.5	10	6.0	3.60 ± 0.26 (3)	0.05–0.22
5	Co^{2+}	0.1	7.5	0.79	3.59 ± 0.25 (2)	0.05–0.22
6	Co^{2+}	0.25	7.5	2.1	3.01 ± 0.25 (2)	0.25–0.41
7	Co^{2+}	0.5	7.5	3.6	2.46 ± 0.20 (8)	0.45–0.58
8	Co^{2+}	1.0	7.5	4.5	2.55 ± 0.25 (2)	0.40–0.57
9	Co^{2+}	2.0	7.5	4.7	2.38 ± 0.25 (2)	0.46–0.62
10	Mn^{2+}	0.1	10	1.9	3.92 ± 0.15 (11)	0.00–0.06
11	Mn^{2+}	0.25	10	2.6	3.64 ± 0.12 (11)	0.08–0.16
12	Mn^{2+}	0.50	10	2.8	3.58 ± 0.20 (12)	0.07–0.21
13	Mg^{2+}	10[a]	20	154	3.21 ± 0.14 (8)	0.22–0.31
14	Mg^{2+}	10[a]	50	175	3.34 ± 0.19 (9)	0.16–0.28
15	Mg^{2+}	10[a]	100	168	3.32 ± 0.19 (9)	0.16–0.29

[a] Calculated free metal ion. All other values are total concentrations. [b] Values in parentheses are number of determinations.

constant for $P^{18}O_4$ disappearance to the rate constant for overall oxygen exchange via eq 6:

$$P_c = \frac{4 - R}{3} \quad (6)$$

Values for k_{ex} and P_c are listed in Table IV. Since we earlier showed that EPP_i formation and PPase catalysis of H_2O–P_i oxygen exchange have the same concentration dependence on P_i (Springs et al., 1981), the observed $K_{p,app}$ values for Mn^{2+}, Co^{2+}, Zn^{2+}, and Mg^{2+} (D_2O) (Table I) should also apply as K_m values for the exchange process. Thus, the k_{ex}

Table V: Observed and Calculated Equilibrium and Rate Parameters

		Zn^{2+}, H_2O[a]	Co^{2+}, H_2O[a]	Mn^{2+}, H_2O[a]	Mg^{2+}, D_2O[a]	Mg^{2+}, H_2O[b]	Cd^{2+}, H_2O[c]
observed	$k_{cat,hyd}$ (s^{-1})	37	7.4	22	121	212	0.015
	$k_{cat,ex}$ (s^{-1})	7.2	5.3	3.4	191	171	8×10^{-4}
	K_3	165	3.2	47	3.0	4.8	19
	P_c	0.05-0.22	0.46-0.62	0.07-0.21	0.16-0.29	0.16-0.30	<0.05
calculated	k_3^* (s^{-1})	1210-1280	27-31	166-174	800-840	1040-1100	0.015
	k_4^* (s^{-1})	7.3-7.8	8.4-9.8	3.5-3.7	270-280	220-230	8×10^{-4}
	k_5^* (s^{-1})	139-28[d]	9.9-6.0	47-14	1400-690	1140-530	ind
	k_7^* (s^{-1})	53-ind[d]	ind	56-ind	165-195	370-650	ind

[a] This work. For $k_{cat,hyd}$ and $k_{cat,ex}$ measurements, $[Zn^{2+}]_T = 0.5$ mM, $[Co^{2+}]_T = 2.0$ mM, and $[Mn^{2+}]_T = 0.5$ mM. [b] Springs et al. (1981); $[Mg^{2+}]_{free} = 10$ mM. [c] Welsh et al. (1983); $[Cd^{2+}]_T = 1.5$ mM. [d] ind, indeterminate. Underlined lower limit values of k_5^* are impossibly low, since they fall below $k_{cat,hyd}$.

Table VI: Kinetic Solvent Isotope Effect on PP_i Hydrolysis

M^{2+}	peak rate pH	peak rate pD	peak solvent isotope effect	rel peak PPase activities
Mg^{2+}	6.75	7.25	1.75 ± 0.05	1.00
Zn^{2+}	6.25	6.50	2.56 ± 0.21	0.26
Co^{2+}	6.50	7.00	2.22 ± 0.22	0.08
Mn^{2+}	7.00	7.50	2.86 ± 0.11	0.09

values in Table IV are close to those expected at saturating P_i concentration. Estimates of k_{ex} at saturating P_i concentration (equal to $k_{cat,ex}$) were calculated from eq 7 and are listed in Table V. Values of $k_{cat,ex}$ differ from the values of k_{ex} in Table IV by 10–20%.

$$k_{cat,ex} = k_{ex}\left\{1 + \frac{K_3 K_{p,app}}{(K_3 + 1)[P_i]}\right\} \quad (7)$$

Over the range of M^{2+} concentrations studied, k_{ex} values clearly increase with increasing $[M^{2+}]$, in marked contrast to what was noted above for $k_{cat,hyd}$ values. It is also clear for Co^{2+} that R decreases and P_c increases with increasing $[M^{2+}]$. The same trends, albeit less marked, also seem to apply for both Zn^{2+} and Mn^{2+}, although the error range in P_c is large enough with these ions that a definitive judgement cannot be made.

pH/pD Dependence. The pH– and pD–rate profiles for PPase catalysis of PP_i hydrolysis in the presence of Mg^{2+}, Zn^{2+}, Co^{2+}, or Mn^{2+} are shown in Figure 3. The PP_i concentrations used in these studies are substantially in excess of apparent $K_m^{PP_i}$ values [see Table III and Springs et al. (1981)]. As described above, the rate of PP_i hydrolysis passes through a maximum as a function of divalent metal ion concentration. The metal ion concentrations chosen were those giving maximal PPase activities.

Each of the four divalent metal ions examined shows similar bell-shaped curves for activity vs. pH (or pD) (Figure 3). The most salient features of these results are summarized in Table VI, leading to the following observations: (1) The pD optima are 0.25–0.50 log unit higher than the pH optima, which is typical for studies of this kind. (2) The pH optima show a small variation (0.75 log unit) as a function of the added divalent metal ion. (3) The magnitude of the solvent isotope effect varies greatly with divalent metal ion, with the effects conferred falling in the order $Mn^{2+} > Zn^{2+} > Co^{2+} > Mg^{2+}$.

Discussion

In our previous study (Springs et al., 1981), we found that steps 3, 5, and 7, as defined in eq 1, or steps 3′, 5′, and 7′, as defined in eq 2, were each partially rate determining for PPase catalysis of PP_i hydrolysis, while step 4 or 4′ was essentially uniquely rate determining for PPase catalysis of P_i–water oxygen exchange. Assuming that the same scheme applies for

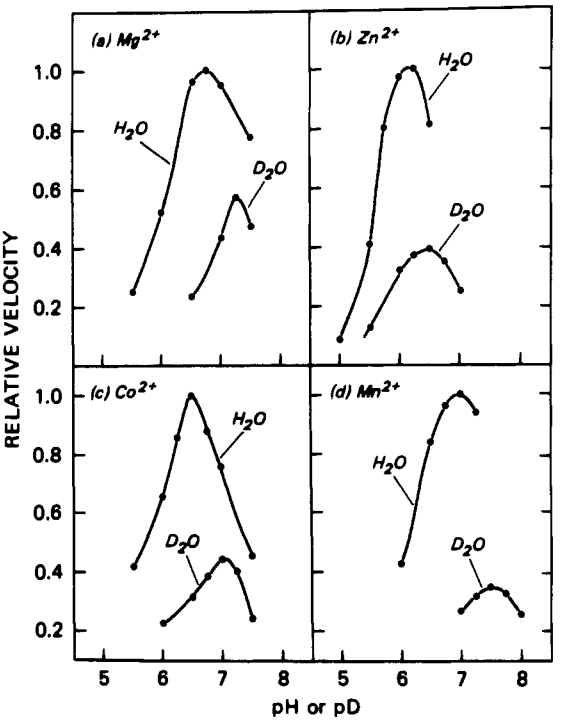

FIGURE 3: pH/pD rate profiles of PP_i hydrolysis by PPase in H_2O–D_2O at 30 °C. All solutions were 200 mM KCl and 100 mM Mes. Reaction solutions also contained (a) 5 mM $MgCl_2$ and 1 mM PP_i, (b) 0.5 mM $CoCl_2$ and 0.3 mM PP_i, (c) 0.5 mM $ZnSO_4$ and 0.3 mM PP_i, and (d) 0.1 mM $MnSO_4$ and 0.1 mM PP_i. Data in each panel are normalized to the highest rate for each divalent metal ion in water.

the four additional conditions studied in this work (Mg^{2+}–D_2O, Zn^{2+}–H_2O, Co^{2+}–H_2O, Mn^{2+}–H_2O) allows calculation of the four rate constants of interest, k_3^*, k_4^*, k_5^*, and k_7^* (here, the numbers with asterisks indicate that the calculated rate constants may be unprimed and pertain to eq 1 or primed and pertain to eq 2, vide infra), for each of these conditions from eq 8 to 11 (Springs et al., 1981) and the measured values of

$$k_4^* = k_{cat,ex}\frac{K_3 + 1}{K_3}\frac{4 - 3P_c}{4(1 - P_c)} \quad (8)$$

$$k_3^* = K_3 k_4^* \quad (9)$$

$$k_5^* = \frac{k_4^*(1 - P_c)}{P_c} = k_{cat,ex}\frac{K_3 + 1}{K_3}\frac{4 - 3P_c}{4P_c} \quad (10)$$

$$k_7^* = \left(\frac{1}{k_{cat,hyd}} - \frac{k_3^* + k_4^* + k_5^*}{k_3^* k_5^*}\right)^{-1} \quad (11)$$

$k_{cat,hyd}$, $k_{cat,ex}$, K_3, and P_c. The measured and calculated values are listed in Table V, along with values for rate constants previously determined for Mg^{2+}–H_2O (Springs et al., 1981) and for Cd^{2+}–H_2O (Welsh et al., 1983). Because $k_{cat,hyd}$ (Table

III) and $k_{cat,ex}$ and P_c (Table IV) values depend on M^{2+} concentration, it is important that these parameters be determined at comparable M^{2+} concentration. In these experiments, the concentration of enzyme employed is much less than that of divalent metal ion, and it suffices to use the same $[M^{2+}]_T$. The same is true for K_3 measurements in the presence of Co^{2+} or Mg^{2+}, but in the presence of Zn^{2+} or Mn^{2+}, the concentration of enzyme employed is significant compared to the total divalent metal ion concentration. As a result, sufficient divalent metal ion was added to give non-enzyme-bound metal ion concentrations reasonably close to those used in the exchange and hydrolysis experiments. Given the relative insensitivity of K_3 values to free divalent metal ion concentration (see Results), this procedure need only be approximately correct to be valid.

Of the four experimental parameters in Table V, the experimental error in P_c is by far the largest. The ranges of P_c indicated are taken from Table IV, and values of the microscopic constants are calculated in Table V according to eq 8–11 for both the high and low extremes of P_c. These calculations demonstrate that both k_3* and k_4* are almost totally insensitive to P_c, rising only very slightly with marked increases in P_c, so that firm conclusions about these rate constants can be reached from our data. By contrast, k_5* decreases sharply, and k_7* increases with increasing values of P_c, making conclusions about these constants less certain. It should also be noted that some lower limit values for k_5* fall below $k_{cat,hyd}$ and are thus impossible and that k_5* values which approach $k_{cat,hyd}$ lead, via eq 11, to calculated values of k_7* which are negative and indicated as indeterminate. Within these limitations, the calculated rate constants presented in Table V, in combination with other properties of PPase determined in this work and elsewhere, lead to the following conclusions:

(1) The solvent isotope effect found in the presence of Mg^{2+} derives primarily from an effect on P_i dissociation, certainly via step 7 (k_{H_2O}/k_{D_2O} = 1.9–3.9) and perhaps also via step 5. For this step, the calculated range of k_{H_2O}/k_{D_2O} is 0.38–1.65, but we think the more probable range is 1.00–1.65, given the rarity of inverse solvent isotope effects. The effect on actual hydrolysis, step 3 (k_{H_2O}/k_{D_2O} = 1.24–1.38), is more modest. In previous work (Konsowitz & Cooperman, 1976), we had carefully measured the dependence of the rate of PPase catalysis of PP_i hydrolysis on the H_2O:D_2O ratio [the so-called "proton inventory" (Schowen & Schowen 1982)] and rationalized the results obtained in terms of possible proton movements during PP_i hydrolysis on the enzyme surface (step 3). Our current work shows that this analysis not only was too simplistic but also focused attention on the wrong step. Rather, it is most likely changes in the energetics of hydrogen bonds between P_i and enzyme, and thus in the activation energy for their disruption, which are most responsible for the observed solvent isotope effect in the presence of Mg^{2+}.

(2) For all divalent metal ions tested, step 4 is uniquely rate determining, or nearly so, for PPase catalysis of H_2O–P_i oxygen exchange. In the presence of Mg^{2+}, steps 3, 5, and 7 are all partially rate determining for PPase catalysis of PP_i hydrolysis, while for Zn^{2+}, Mn^{2+}, and, to a lesser extent, Co^{2+}, k_3 is much more rapid than $k_{cat,hyd}$, and P_i release, via either (or both) of steps 5 and 7, is exclusively rate determining. The lower effectiveness of these latter ions as cofactors for PP_i hydrolysis is thus due mainly or entirely to the slower rates of P_i dissociation which are found in their presence. In fact, k_3*, the rate constant for PP_i hydrolysis on the enzyme, is larger in the presence of Zn^{2+} than of Mg^{2+}, while the ratio $k_3^*(Mn^{2+})/[k_3^*(Mg^{2+})]$, equal to 0.21, is much larger than the ratio $k_{cat,hyd}(Mn^{2+})/[k_{cat,hyd}(Mg^{2+})]$, equal to 0.08.

That different steps are rate determining for PP_i hydrolysis on the one hand and H_2O–P_i oxygen exchange on the other suggests a straightforward rationale for the observation (see Results) that these two processes depend in opposite ways on divalent metal ion concentration. Specifically, this observation can be accounted for with the assumptions, first, that over the range of divalent metal ion concentrations studied in Tables III and IV the dominant enzyme species in solution changes from those included in eq 1 to those included in eq 2 and, second, that $k_{4'} > k_4$ and k_5 and/or $k_7 > k_5'$ and/or k_7'. At least for Co^{2+} and Mn^{2+}, the fact that the previously determined (Cooperman et al., 1981) dissociation constants for the binding of a third divalent metal ion per subunit in the presence of saturating P_i concentration (0.026 mM for Mn^{2+}, 0.045 mM for Co^{2+}; the dissociation constant for Zn^{2+} was not determined) are clearly too low to account for the changes in k_{ex} between 0.1 and 0.5 mM Mn^{2+} or 0.1–1.0 mM Co^{2+} (Table IV) provides strong evidence for the first assumption. Evidence consistent with the second assumption is that P_c, equal to $k_4^*/(k_4^* + k_5^*)$, increases with increasing divalent metal ion concentration (Table IV).

(3) Changes in the magnitude of the solvent isotope effect for PP_i hydrolysis as a function of divalent metal ion (Table VI) are explainable as reflecting changes in the rate-determining step. Since the solvent isotope effect is larger for P_i dissociation than for PP_i hydrolysis, PPase activity in the presence of those divalent metal ions (Zn^{2+} and Mn^{2+}) for which P_i dissociation is mainly or entirely rate determining should and does display higher solvent isotope effects than does PPase activity in the presence of Mg^{2+}, for which both PP_i hydrolysis and P_i dissociation are partly rate determining. Furthermore, PPase activity in the presence of Co^{2+}, which is intermediate between Mg^{2+} on the one hand and Zn^{2+} and Mn^{2+} on the other with respect to the relative contribution of k_3* to $k_{cat,hyd}$, is also intermediate with respect to the magnitude of the solvent isotope effect it displays.

Rapidly growing cells, such as yeast cells, need high levels of PPase activity in order to avoid PP_i accumulation from a large variety of biosynthetic reactions which result in PP_i formation. Yeast PPase not only is a plentiful enzyme, constituting about 1% of the yeast proteins released following toluene plasmolysis, but it also has a relatively high turnover number (212 s^{-1}, see Table IV), which is about 10^{10} times faster than that for uncatalyzed PP_i hydrolysis in water. What our analysis shows is that the yeast enzyme has such a highly evolved catalytic active site [in the sense discussed by Knowles & Albery (1977)] that enzyme-bound PP_i hydrolysis is itself not uniquely rate determining and the rate of release of product must also be considered. From the data in Table V, it is clear that the major factor in the catalytic specificity of PPase for Mg^{2+} is the much faster rate of product release found in the presence of this ion than in the presence of any of the other ions considered in this work.

We propose that it is the high lability of Mg^{2+}–oxygen bonds which accounts for the rapid product release found in the presence of Mg^{2+}. That M^{2+}–oxygen bonds are broken during product release is suggested by the following observations: first, one or two divalent metal ions per subunit are taken up and released during a complete turnover (eq 1 and 2); second, NMR chemical shifts of $^{113}Cd^{2+}$ bound to PPase are characteristic of predominant, if not exclusive, oxygen ligand environments (Welsh et al., 1983); third, the effects of Mn^{2+} (Hamm & Cooperman, 1978) and $^{113}Cd^{2+}$ (Welsh et al., 1983) on enzyme-bound [^{32}P]P_i NMR show that there is inner-sphere

Table VII: Association Constants (log Values)[a]

divalent metal ion	ligand: oxalate dianion	ADP trianion	citric acid trianion	3,4-dihydroxybenzoic acid trianion
Mg^{2+}	2.76	3.17	3.29	5.67
Zn^{2+}	4.9	4.28	4.85	8.91
Co^{2+}	4.7	4.20	4.83	7.96
Mn^{2+}	3.9	4.16	3.67	7.22

[a] Sillén & Martell (1964). $\mu = 0.05$–0.15, 20–30 °C.

M^{2+} coordination to P_i bound in the high-affinity site and outer-sphere coordination to the second P_i bound in the low-affinity site. That Mg^{2+}–O ligand bonds should be particularly labile is suggested by the distinctly weaker complexes that Mg^{2+}, as compared with Zn^{2+}, Co^{2+}, and Mn^{2+}, forms with oxygen ligands (Table VII). If a dissociative mechanism for ligand substitution at the divalent metal ion center is assumed, lower formation constants would correlate with higher rates of dissociation (Langford, 1965).

Since an active PPase subunit requires at least three bound divalent metal ions, it is to be expected that factors in addition to M^{2+}–O bond lability will be important in determining divalent metal ion specificity. In recent work, O. A. Moe, Jr., et al. (unpublished experiments) have emphasized the importance for PPase activity of the M^{2+} ionic radius, and it is clear that this factor can be used, in approximate fashion, to rationalize the trend observed in k_3*. Using Goldschmidt ionic radii, we determined that the two smallest ions, Zn^{2+} (0.69 Å) and Mg^{2+} (0.78 Å), have the highest k_3* values, the larger ions, Co^{2+} (0.82 Å) and Mn^{2+} (0.91 Å), have lower k_3* values, and the largest ion, Cd^{2+} (1.03 Å), has the lowest k_3* value.

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Registry No. PPase, 9024-82-2; P_i, 14265-44-2; PP_i, 14000-31-8; Mg, 7439-95-4; Zn, 7440-66-6; Co, 7440-48-4; Mn, 7439-96-5.

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