Catalysis by *Escherichia coli* Inorganic Pyrophosphatase: pH and Mg^{2+} Dependence†

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ABSTRACT: Steady-state rates of PP_i hydrolysis by *Escherichia coli* inorganic pyrophosphatase (E-PPase) were measured as a function of magnesium pyrophosphate (substrate) and free Mg^{2+} ion (activator) in the pH range 6.0−10.0. Computer fitting of hydrolysis data in combination with direct measurements of Mg^{2+} binding to enzyme has resulted in a model that quantitatively accounts for our results. The major features of this model are the following: (a) E-PPase catalysis proceeds both with three and with four (and possibly with five) Mg^{2+} ions per active site; (b) catalysis requires both an essential base and an essential acid, and the pK_as of these groups are modulated by the stoichiometry of bound Mg^{2+}; and (c) the four-metal route predominates for concentrations of free Mg^{2+} >0.2 mM. The model straightforwardly accounts for the apparent linkage between increased pK_a of an essential base and activity requirements for higher Mg^{2+} concentration observed for several active site variants. Microscopic rate constants for overall catalysis of PP_i−P_i equilibration were determined at pH 6.5−9.3 by combined analysis of enzyme-bound PP_i formation and rates of PP_i hydrolysis, PP_i synthesis, and P_i−H_2O oxygen exchange. The catalytic activity of E-PPase at saturating substrate increases toward PP_i hydrolysis and decreases toward PP_i synthesis and P_i−H_2O oxygen exchange with increasing pH. These changes are mainly due to an increased rate of dissociation of the second released P_i and a decreased rate of enzyme-bound PP_i synthesis from enzyme-bound P_i, respectively, as the pH is raised.

Escherichia coli inorganic pyrophosphatase (E-PPase)[1] catalysis of PP_i−P_i equilibration is a complex function of pH and Mg^{2+} and substrate (Mg_2PP_i) concentrations. The current model of E-PPase catalysis, shown in Scheme 1, was developed from measurements of steady-state rates of PP_i hydrolysis, net PP_i synthesis, and P_i−H_2O oxygen exchange, as well as equilibrium measurements of enzyme-bound PP_i formation and Mg^{2+} binding to enzyme (Baykov et al., 1990; Käpylä et al., 1995). These earlier studies were limited in scope. Thus, the pH−rate profile for steady-state PP_i hydrolysis was determined at a single Mg^{2+} concentration (20 mM), Mg^{2+} binding was determined at a single pH value (7.2), and the pH dependencies of the individual rate constants (k_1−k_8) were determined over only a narrow pH range (6.5−8.0).

Although Scheme 1 provided an adequate description of E-PPase catalysis for the wild-type enzyme under the conditions tested, its limitations as a general scheme became manifest in seeking to apply it to data obtained with E-PPase variants generated by site-specific mutagenesis (Käpylä et al., 1995; Salminen et al., 1995). Thus, catalysis by both the D97E and E20D variants at pH 7.2 clearly requires the involvement of a fifth Mg^{2+} (Käpylä et al., 1995; Volk et al., 1996); similar results have been obtained for other active site variants as well (Käpylä et al., in preparation). Further, Scheme 1 provided no cogent explanation for the observation that virtually all E-PPase active site variants examined show an increase in the pK_a of the essential base required for catalysis (Salminen et al., 1995). In addition, Scheme 1 requires the involvement of four Mg^{2+} ions per active site, whereas *Saccharomyces cerevisiae* PPase (Y-PPase), which has an active site structure quite similar to that of E-PPase (Kankare et al., 1994; Oganessyan et al., 1994), is active with either three or four Mg^{2+} ions (Baykov & Shestakov, 1992).

In the work presented below we present a much more comprehensive examination of E-PPase leading to the formulation of a general scheme (Scheme 2) that quantitatively accounts for its catalytic properties as a function of both Mg^{2+} concentration and pH. This scheme also provides

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[1] Abbreviations: E-PPase, *Escherichia coli* inorganic pyrophosphatase; P_i, inorganic phosphate; PPase, inorganic pyrophosphatase; PP_i, inorganic pyrophosphate.

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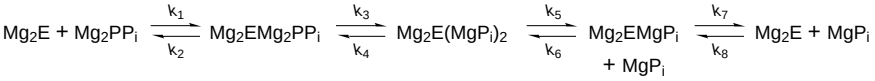
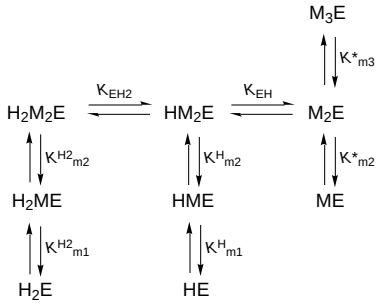
Scheme 1: Minimal Scheme of E-PPase Catalysis

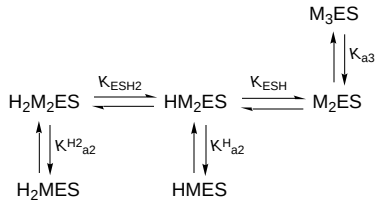


Scheme 2: pH and Mg^{2+} Dependence of E-PPase Catalysis of PP_i Hydrolysis[a]

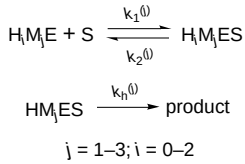
(A) Equilibria Linking Stoichiometrically Significant Species within $[E]_t$



(B) Equilibria Linking Stoichiometrically Significant Species within $[ES]_t$



(C) Rate Processes Defining the Steady State



[a] Definitions: $K_{m1} = [Mg^{2+}][E]_t/[ME]_t$; $K_{m2} = [Mg^{2+}][ME]_t/[M_2E]_t$; $K_{m3} = [Mg^{2+}][M_2E]_t/[M_3E]_t$. $K^H_{m1} = [Mg^{2+}][HE]/[HME]$; $K^H_{m2} = [Mg^{2+}][HME]/[HM_2E]$. $K^{H2}_{m1} = [Mg^{2+}][H_2E]/[H_2ME]$; $K^{H2}_{m2} = [Mg^{2+}][H_2ME]/[H_2M_2E]$. $K^*_{m2} = [Mg^{2+}][ME]/[M_2E]$; $K^*_{m3} = [Mg^{2+}][M_2E]/[M_3E]$. $K_{a2} = [Mg^{2+}][MES]/[M_2ES]$; $K_{a3} = [Mg^{2+}][M_2ES]/[M_3ES]$. $K^H_{a2} = [Mg^{2+}][HMES]/[HM_2ES]$; $K^H_{a3} = [Mg^{2+}][HM_2ES]/[HM_3ES]$. $K^{H2}_{a2} = [Mg^{2+}][H_2MES]/[H_2M_2ES]$. $K_{EH2} = [H^+][HM_2E]/[H_2M_2E]$; $K_{EH} = [H^+][M_2E]/[HM_2E]$. $K_{ESH2} = [H^+][HM_2ES]/[H_2M_2ES]$; $K_{ESH} = [H^+][M_2ES]/[HM_2ES]$. The letter t refers to all protonated forms of E or M_jE.

a straightforward rationale for the properties of active site variants noted above.

EXPERIMENTAL PROCEDURES

Enzyme. E-PPase used in most studies was isolated from *E. coli* strain MRE-600 according to Wong et al. (1970). The enzyme used in equilibrium dialysis measurements was isolated from an overproducing *E. coli* HB101 strain transformed with a suitable plasmid derived from PUC19 as described by Lahti et al. (1990). Both types of preparations were at least 95% pure, as determined by electrophoresis in nondenaturing polyacrylamide gel. Activity measurements carried out with both preparations gave essentially identical results.

Methods. Initial rates of PP_i hydrolysis were estimated from continuous recordings of P_i liberation obtained with an automatic P_i analyzer (Baykov & Avaeva, 1981). Initial rates of PP_i synthesis were measured luminometrically, with ATP-sulfurylase and luciferase as coupling enzymes (Nyrén & Lundin, 1985; Baykov & Shestakov, 1992). Since perchlorate was found to suppress final luminescence, trifluoroacetic acid was used for E-PPase inactivation prior to PP_i measurements (Smirnova et al., 1995). Rates of oxygen exchange between $[^{18}O]P_i$ and H_2O were measured by mass spectrometry (Baykov et al., 1990). All rate data were normalized to the highest specific activity of the enzyme (205 s^{-1} at pH 9, 5 mM Mg^{2+}). Enzyme-bound PP_i was determined according to Springs et al. (1981). Parallel measurements carried out at pH 7.2 indicated that this procedure determines the same amounts of enzyme-bound PP_i as the luminometric procedure used previously (Baykov et al., 1990). Equilibrium dialysis measurements of Mg^{2+} binding to E-PPase were performed as described previously (Käpylä et al., 1995). All experiments were carried out at 25 °C.

The following buffers were used (μ = 0.1 M) for measurement of enzyme-bound PP_i formation and of steady-state rates of PP_i hydrolysis, net PP_i synthesis, and P_i-H_2O oxygen exchange: 0.11 M imidazole/HCl (pH 6.0); 0.05 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid/0.05 M Tris (pH 6.5, adjusted with HCl); 0.16 M Tris-HCl (pH 7.9); 0.25 M Tris-HCl (pH 8.5); 0.25 M 2-amino-2-methyl-1,3-propanediol/HCl (pH 9.3); 0.41 M 2-ethanolamine/HCl (pH 10.0). Equilibrium dialysis measurements of Mg^{2+} binding to enzyme employed the following buffers: 0.10 M imidazole/HCl (pH 6.0); 0.13 M imidazole/HCl (pH 6.6); 0.10 M Tris-HCl (pH 7.2); 0.13 M Tris-HCl, (pH 7.9); 0.25 M Tris-HCl (pH 8.5); 0.25 M 2-amino-2-methyl-1,3-propanediol/HCl (pH 9.3). All buffers contained EGTA: 50−100 μM (pH 6.0−7.9); 10 μM (pH 8.5−9.3); 1 μM (10.0).

CALCULATIONS AND DATA ANALYSIS

Fitting of various equations used in this work to data was performed using a program for nonlinear regression analysis (Duggleby, 1984).

Solution Equilibrium Constants. Values of apparent equilibrium constants used to calculate the concentrations of free Mg^{2+}, Mg_2PP_i, and MgP_i at different pH values are presented in Table 1.

pH and Mg^{2+} Dependence of Initial Rates of PP_i Hydrolysis. Scheme 2 was found to account quantitatively for the dependence of k_h and k_h/K_m values on pH and $[Mg^{2+}]$. This scheme assumes that the substrate S is Mg_2PP_i (Käpylä et al., 1995). However, it is clear that our results only suffice to specify overall Mg^{2+} stoichiometry within an enzyme−substrate complex and that forms such as $HMg_2EMg_2PP_i$ and HMg_3EMgPP_i are kinetically indistinguishable. Other assumptions of the model are (a) enzyme species lacking substrate, which sum to $[E]_t$, are in equilibrium with one another, as are all enzyme−substrate species, which sum to $[ES]_t$; (b) only monoprotonated forms within $[ES]_t$ can react to give product P_i; (c) $[ES]_t$ is in the steady state; (d) over the range of pH and Mg^{2+} concentration values examined, the nine species E, H_2M_3E, HM_3E, H_2M_3ES, HM_3ES, MES, H_2ES, HES, and ES may be ignored as not contributing significantly to $[E]_t$, $[ES]_t$, or the rate term; i.e., eliminating these species did not significantly affect the quality of the fits obtained in Figures 2 and 4. On the other hand,

Table 1: pH-Dependent Values of Dissociation Constants for Mg^{2+} Complexes of PP_i and P_i[a]

pH	K_A[b] (μM)	K_{A2}[b] (mM)	K_B[c] (mM)
6.0	696	16.2	
6.5	294	6.28	18.7
7.2	85.9	2.83	8.5
7.9	21.1	2.17	
8.5	6.44	2.04	6.1
9.3	2.12	2.01	6.0
10.2	1.40	2.00	

[a] Definitions (the subscript t refers to total concentration, i.e., all species having the stoichiometry shown without regard to protonation state): $K_A = [Mg][PP_i]/[MgPP_i]$; $K_{A2} = [Mg][MgPP_i]/[Mg_2PP_i]$; $K_B = [Mg][P_i]/[MgP_i]$. Values reported were determined by us earlier (see footnotes *b* and *c*) at 25 °C and $\mu = 0.1$. [b] Volk et al., 1982. [c] Values at pH 6.5, 8.5, and 9.3 are calculated on the basis of the value at pH 7.2 (Smirnova et al., 1989; Smith & Alberty, 1956), assuming that $MgHPO_4$ is the only form of magnesium phosphate present (Childs, 1970).

eliminating any of the other 15 species presented in Scheme 2 did lead to poorer fits.

Mg^{2+} Binding to Enzyme. Values of the dissociation constants for Mg^{2+} binding to up to three sites on E-PPase at fixed pH were estimated by fitting equilibrium dialysis data to eq 1, where n measures the number of Mg^{2+} bound per subunit and the parameters K_{mj} ($j = 1-3$) are apparent dissociation constants at fixed pH.

$$n/[Mg^{2+}] = \frac{K_{m2}K_{m3} + 2K_{m3}[Mg^{2+}] + 3[Mg^{2+}]^2}{K_{m1}K_{m2}K_{m3} + K_{m2}K_{m3}[Mg^{2+}] + K_{m3}[Mg^{2+}]^2 + [Mg^{2+}]^3} \quad (1)$$

Fitting n as a function of both $[Mg^{2+}]$ and pH, according to Scheme 2A, was accomplished by making the following substitutions:

$$K_{m1} = K^{H}_{m1}(b/c) \quad (1A)$$

$$K_{m2} = K^{H}_{m2}(c/d) \quad (1B)$$

$$K_{m3} = K^{*}_{m3}(e) \quad (1C)$$

$$b = 1 + [H^+]K^{H2}_{m2}K^{H2}_{m1}/K_{EH2}K^{H}_{m2}K^{H}_{m1}$$

$$c = 1 + [H^+]K^{H2}_{m2}/K_{EH2}K^{H}_{m2} + K_{EH}K^{*}_{m2}/[H^+]K^{H}_{m2}$$

$$d = 1 + [H^+]/K_{EH2} + K_{EH}/[H^+]$$

$$e = 1 + [H^+]/K_{EH} + [H^+]^2/K_{EH}K_{EH2}$$

k_h. Values of k_h as a function of varying pH and $[Mg^{2+}]$ were fit to eq 2, derived from Scheme 2B.

$$k_h = k_h^{(2)}W/C \quad (2)$$

where $W = 1 + k_h^{(1)}K^{H}_{a2}/k_h^{(2)}[Mg^{2+}] + k_h^{(3)}[Mg^{2+}]/k_h^{(2)}K^{H}_{a3}$ and $C = 1 + [Mg^{2+}]/K^{H}_{a3} + K^{H}_{a2}/[Mg^{2+}] + (1 + K^{H2}_{a2}/[Mg^{2+}])[H^+]/K_{ESH2} + (1 + [Mg^{2+}]/K_{a3})K_{ESH}/[H^+]$.

k_h/K_m. Values of k_h/K_m at fixed pH and varying Mg^{2+} were fit to eq 3, derived from Scheme 2 assuming $k_h^j \gg k_2^j$ for all values of $j = 1-3$ (see below).

$$k_h/K_m = \frac{k_1^{(1)}K_{m2}/[Mg^{2+}] + k_1^{(2)} + k_1^{(3)}[Mg^{2+}]/K_{m3}}{1 + K_{m1}K_{m2}/[Mg^{2+}]^2 + K_{m2}/[Mg^{2+}] + [Mg^{2+}]/K_{m2}} \quad (3)$$

Rate and Equilibrium Constants at Fixed Mg^{2+} Concentration and pH. Values for the catalytic constant (k_h) and Michaelis constant (K_m) for PP_i hydrolysis were determined by computer fitting of hydrolysis rate values to the Michaelis−Menten equation. The value of k_s, the catalytic constant for net PP_i synthesis, by extrapolating v_s to infinite $[MgP_i]$, using the phenomenological eq 4, where $[E]_T$ refers to the total enzyme concentration in solution is

$$k_s = (v_s/[E]_T)(1 + A/[MgP_i] + B/[MgP_i]^2) \quad (4)$$

Because of poor precision in the measurement of v_s at low $[MgP_i]$, parameters A and B were found to be highly correlated; i.e., many pairs of A and B values gave equally good fits to the data. As a result, these values did not afford reliable estimates of K_5 and K_7, as previously (Springs et al., 1981; Käpylä et al., 1995), although they did permit reliable estimates of k_s to be made.

Values for k_{ex}, the catalytic constant for oxygen exchange, and K_3 ($=k_3/k_4$), the equilibrium constant for enzyme-bound PP_i formation, were obtained directly from eqs 5 and 6, using the values of A and B determined from the v_s data. $[EPP_i]$ refers to the total concentration of all forms of enzyme-bound PP_i.

$$k_{ex} = (v_{ex}/[E]_T)(1 + A/[MgP_i] + B/[MgP_i]^2) \quad (5)$$

$$K_3 = \{[E]_T/\{[EPP_i](1 + A/[MgP_i] + B/[MgP_i]^2)\}\} - 1 \quad (6)$$

The partition coefficient, P_c, which defines the probability of enzyme-bound P_i undergoing oxygen exchange with water vs being released into solution (Hackney & Boyer, 1978), was calculated from the distribution of all ^{18}O-labeled forms of P_i according to Hackney (1980).

Values for six rate constants, k_1-k_5 and k_7, were calculated from eqs 7−12 (Springs et al., 1981; Baykov et al., 1990).

$$k_5 = k_{ex}(1 - 0.75P_c)(K_3 + 1)/P_cK_3 \quad (7)$$

$$k_4 = k_5P_c/(1 - P_c) \quad (8)$$

$$k_3 = k_4K_3 \quad (9)$$

$$k_2 = (K_3 + 1)/(1/k_s - 1/k_4) \quad (10)$$

$$k_1 = (1 + k_2/k_3 + k_2k_4/k_3k_5)k_h/K_m \quad (11)$$

$$k_7 = 1/(1/k_h - 1/k_3 - 1/k_5 - k_4/k_3k_5) \quad (12)$$

RESULTS

Equilibrium Dialysis Measurements of Mg^{2+} Binding at pH 6.0−9.3. Mg^{2+} binding to E-PPase in the absence of substrate was determined using equilibrium dialysis in combination with atomic absorption to determine Mg^{2+} concentration (Käpylä et al., 1995). The resulting Scatchard plots (Figure 1) allow the estimation of dissociation constants

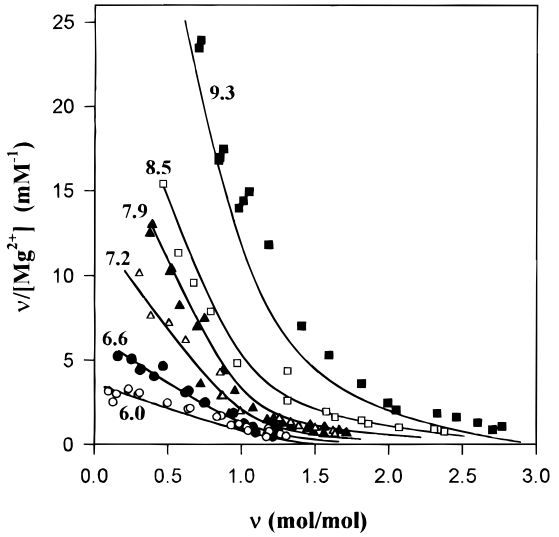


FIGURE 1: Scatchard plots of Mg^{2+} binding to E-PPase. Values of pH are indicated on the curves. Lines are fit to eq 1, using parameter values found in Table 3 (Scheme 2A). Experimental conditions as in Table 2 legend.

Table 2: Dissociation Constants for Mg^{2+} Binding to E-PPase[a]

pH	K_{m1}	K_{m2}	K_{m3}
6.0	0.28 ± 0.01 (0.28)	3.1 ± 0.4 (3.3)	nd (649)
6.6	0.153 ± 0.004 (0.155)	4.3 ± 0.4 (3.2)	nd (90)
7.2	0.080 ± 0.003(0.079)	1.74 ± 0.17 (2.9)	nd (19.5)
7.9	0.054 ± 0.003 (0.049)	2.1 ± 0.3 (2.0)	nd (5.4)
8.5	0.041 ± 0.003 (0.037)	1.1 ± 0.2 (1.1)	2.5 ± 0.7 (3.0)
9.3	0.016 ± 0.002 (0.019)	0.34 ± 0.05 (0.41)	1.3 ± 0.3 (2.3)
10.0	(0.0056)	(0.28)	(2.2)

[a] Expressed in units of mM. Determined by equilibrium dialysis. Values with deviations were derived by separately fitting data at each pH to eq 1. Values in parentheses are calculated from eqs 1A−C and the parameter values in Table 3. Experimental conditions: [PPase], 0.4−1.0 mM; [Mg^{2+}], 0.03−3.0 mM.

Table 3: Equilibrium Constants for Scheme 2[a]

constant	value	constant	value
K^*_{m2}	0.25 ± 0.10	K_{a3}	8 ± 3
K^*_{m3}	2.2 ± 0.7	K^H_{a2}	0.017 ± 0.012
K^H_{m1}	0.044 ± 0.004	K^{H2}_{a2}	1.7 ± 0.4
K^H_{m2}	3.2 ± 0.5	pK_{ESH2}	6.70 ± 0.07
K^{H2}_{m1}	0.42 ± 0.04	pK_{ESH}	10.04 ± 0.08
K^{H2}_{m2}	3.0 ± 0.9	$k_h^{(1)} = k_h^{(2)}$	199 ± 8 s^{-1}
pK_{EH2}	6.2 ± 0.2		
pK_{EH}	8.06 ± 0.14		

[a] In units of mM, except for p*K*s and rate constants.

(eq 1) for binding to two sites per monomer from pH 6.0 to pH 7.9 and to three sites at pH 8.5 and 9.3 (Table 2). Fitting all of the data to eqs 1 and 1A−C allowed evaluation of eight equilibrium parameters in Scheme 2A (Table 3) that quantitatively account for the observed pH dependence of Mg^{2+} binding to E-PPase. From these results it is clear that (a) two deprotonation steps are pertinent; (b) tight binding of the first Mg^{2+} depends on loss of the more acidic proton; (c) tight binding of the second Mg^{2+} depends on loss of the less acidic proton; (d) binding of the third Mg^{2+} is only stoichiometrically significant when both protons are lost.

Steady-State Rates of PP_i Hydrolysis as a Function of pH and Mg^{2+} Concentration. k_h and K_m values for E-PPase catalysis of PP_i hydrolysis were determined at seven pH values over a wide range of Mg^{2+} concentration.

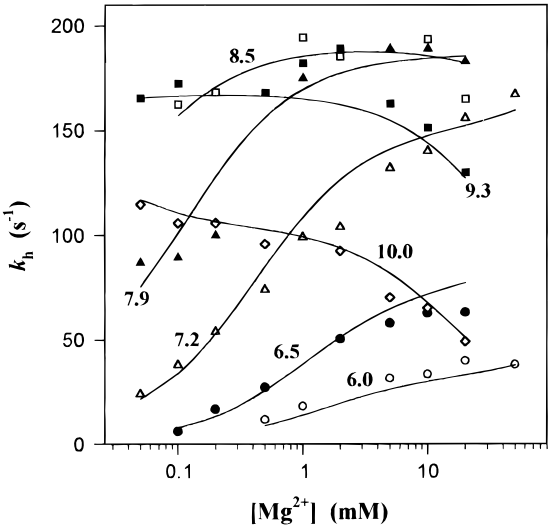


FIGURE 2: Dependence of k_h on [Mg^{2+}] at fixed pH values. The lines are drawn to eq 2, using parameter values found in Table 3 (Scheme 2B). Curves: pH 6.0 (○); pH 6.5 (●); pH 7.2 (△); pH 7.9 (▲); pH 8.5 (□); pH 9.3 (■); pH 10.0 (◇).

At pH 6.0−7.9, k_h increases to a saturating value with increasing Mg^{2+} concentration (Figure 2). By contrast, at higher pH (9.3 and 10.0) optimal activity is achieved at quite low levels of Mg^{2+} concentration, and raising Mg^{2+} concentration leads to dramatic decreases in k_h. Intermediate behavior is observed at pH 8.5, with high k_h values observed at low Mg^{2+} concentration and little dropoff observed as Mg^{2+} concentration is increased.

A large number of models were considered in attempting to account quantitatively for this rather complex behavior. The minimal model providing an acceptable fit to all the data obtained (Figure 2) is depicted in Scheme 2B,C. It results in satisfactorily low values of mean relative deviation of calculated vs measured rates compared to experimental error and the absence of systematic deviation. The model requires that catalytically active forms of the enzyme−substate complex have both an essential acid group and an essential base group, consistent with pH−profile analyses showing a clear pH optimum for k_h at fixed Mg^{2+} concentration (Salminen et al., 1995; Käpylä et al., 1995).

Fitting k_h values to eq 2 allowed evaluation of the rate and equilibrium constants, as displayed in Table 3. These values demonstrate that the dominant route for hydrolysis is via HMg_2ES over the range of pH and Mg^{2+} concentration investigated. By contrast, for at least two active site variants, D20E-PPase and D97E-PPase, HMg_3ES is the dominant reactive species (Volk et al., 1996). However, because Mg_3-ES accounts for virtually all enzyme containing five bound Mg^{2+} ions, we cannot even approximate a catalytic activity for HMg_3ES for the wild-type enzyme. On the other hand, the downward deviation from linearity at low [Mg^{2+}] of a plot of $1/k_h$ vs $1/[Mg^{2+}]$ (Figure 3) provides clear qualitative evidence that HMgES has significant catalytic activity without, however, permitting precise estimation of $k_h^{(1)}$, since HMg_2ES is always stoichiometrically dominant over HMgES under our conditions. Assuming that $k_h^{(1)}$ and $k_h^{(2)}$ are equal, in order to minimize the number of fitted parameters, gives a best fit value of 199 ± 8 s^{-1}. Alternatively, allowing nonequal values had little effect on $k_h^{(2)}$ (186 ± 8 s^{-1}) and gave a poorly determined value of $k_h^{(1)}$ (302 ± 253 s^{-1}) without markedly improving the quality of the fit.

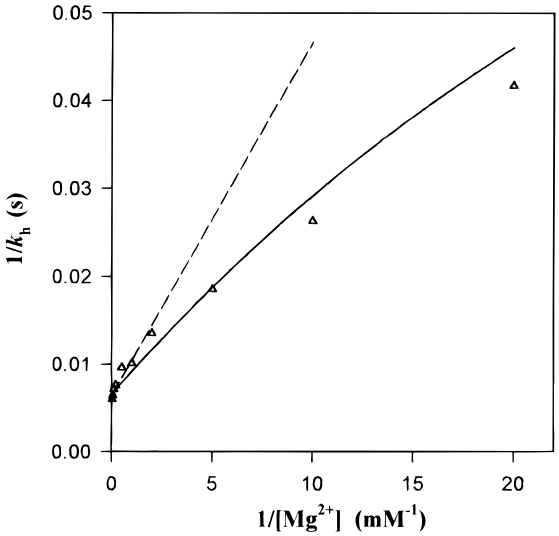


FIGURE 3: $1/k_h$ vs $1/[Mg^{2+}]$, pH 7.2. The solid line is drawn to eq 2, using parameter values found in Table 3 (Scheme 2B); the dashed line shows the best linear fit for the data from 0.5−20 mM.

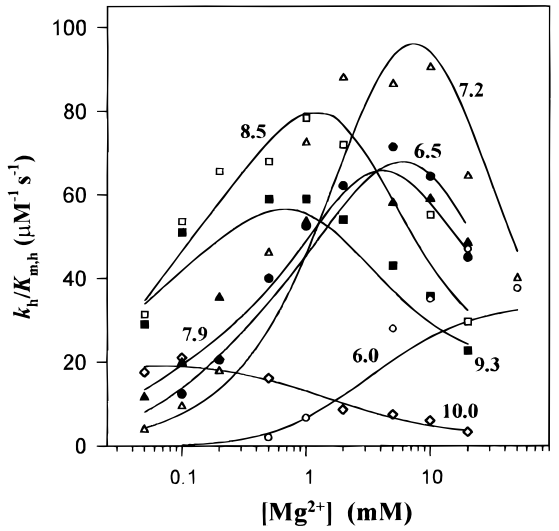


FIGURE 4: Dependence of k_h/K_m on $[Mg^{2+}]$ at fixed pH values. Lines are fitted to eq 3, using the parameter values in Table 4. Deviations observed are principally due to uncertainties in the value of K_{m3} (Table 2). Curves: pH 6.0 (○); pH 6.5 (●); pH 7.2 (△); pH 7.9 (▲); pH 8.5 (□); pH 9.3 (■); pH 10.0 (◇).

The parameter values in Table 3 allow straightforward rationalization of the results presented in Figure 2. At low pH values, relatively high Mg^{2+} concentrations, corresponding to the binding of the fourth Mg^{2+} ion, are required to deprotonate the essential basic group, while the essential acidic group retains its proton at even relatively high Mg^{2+} concentrations, which are insufficient to bind a fifth Mg^{2+} (only a lower limit, >50 mM, can be estimated for K^H_{a3}). In contrast, at high pH values, the essential basic group loses its proton at much lower Mg^{2+} concentration, with either three or four Mg^{2+} bound, and the binding of a fifth Mg^{2+} at high Mg^{2+} concentrations is accompanied by deprotonation of the essential acidic group. The model also accounts for the shift to a lower pH optimum as Mg^{2+} concentration is raised from 1.3 mM (pH 9.1; Josse, 1966) to 20 mM (pH 8; Käpylä et al., 1995).

As demonstrated below (see Table 6), $k_h \gg k_2$, so that k_h/K_m values (Figure 4) depend only on k_1 values. Even so, it proved difficult to fit k_h/K_m values to a general scheme

Table 4: k_1 Values[a]

pH	$k_1^{(1)}$	$k_1^{(2)}$	$k_1^{(3)}$
6.0	nd[b]	35 ± 5	nd
6.5	35 ± 3	107 ± 8	nd
7.2	28 ± 9	167 ± 16	nd
7.9	26 ± 3	113 ± 24	23 ± 16
8.5	71 ± 30	159 ± 24	23 ± 7
9.3	41 ± 7	78 ± 12	18 ± 4
10.0	21 ± 3	16 ± 3	2.2 ± 0.6

[a] Fitted to eq 3. Units are μM^{-1} s^{-1}. [b] Not determined.

explicitly accounting simultaneously for pH and Mg^{2+} concentration dependence, as was possible for k_h. This was in part due to the complexity of the equation for this parameter if more than one binding pathway of Mg_2PP_i to enzyme was permitted (i.e., in principle, substrate could bind to H_iMg_jE, where $i = 0-2$ and $j = 1-3$) and in part due to the sensitivity of K_m to buffer effects, which vary over the pH range studied (Baykov et al., in preparation). As a consequence, k_h/K_m values at fixed pH were fit to eq 3, giving the pH-dependent values of $k_1^{(1)}$, $k_1^{(2)}$, and $k_1^{(3)}$ listed in Table 4. These values indicate that substrate Mg_2PP_i binds most rapidly to HMg_2E (1.6×10^8 M^{-1} s^{-1}), somewhat more slowly to Mg_2E ($\sim 1 \times 10^8$ M^{-1} s^{-1}), and slower yet to HMgE ($\sim 0.3 \times 10^8$ M^{-1} s^{-1}) and Mg_3E (0.2×10^8 M^{-1} s^{-1}).

Rate Constants for E-PPase Catalysis of PP_i−P_i Equilibration at pH 6.5−9.3. We previously determined all rate constants in Scheme 1 for E-PPase at pH 6.5, 7.2, and 8.0 by measuring steady-state rates of PP_i hydrolysis and P_i−H_2O oxygen exchange and equilibrium EPP_i formation (Käpylä et al., 1995). Here we extend these studies by covering the pH range of 6.5−9.3 and performing direct measurements of net PP_i synthesis. All measurements were made at fixed free Mg^{2+} concentrations corresponding approximately to maximal values of k_h (Figure 2) and k_h/K_m (Table 5): i.e., 20, 20, 5, and 1 mM at pH 6.5, 7.2 (taken earlier; Baykov et al., 1990), 8.5, and 9.3, respectively.

The dependence of the rate of net PP_i synthesis on MgP_i concentration at three pH values is shown in Figure 5, and data on oxygen exchange and EPP_i formation at a fixed total P_i concentration are presented in Table 5, permitting calculation of k_s, k_{ex}, P_c, and K_3 (Table 6; see Calculations and Data Analysis). At all pHs these values, in combination with those determined for k_h and k_h/K_m, permitted evaluation of the rate constants k_1-k_5 and k_7. The results, summarized in Table 6, are in general agreement with those reported previously for the pH range 6.5−8.0 (Kapyla et al., 1995). The only significant difference is in the value of k_2 at pH 6.5, which is considerably lower (10.6 ± 2.5) than that previously estimated (106 ± 37). We believe the current value to be more trustworthy, as it is based on direct measurement of k_s. The earlier method was based on a calculated value of K_1, with the potential for propagating several accumulated errors. The most significant observations are that increasing pH over the measured range increases k_h, decreases k_s and k_{ex}, and destabilizes the central EPP_i complex (i.e., increases K_3) and that such effects stem largely from a decrease in k_4 and an increase in k_7 with increased pH.

An attractive rationale for the decrease in k_4 at increasing pH is that it is due to loss of a proton from one of the two

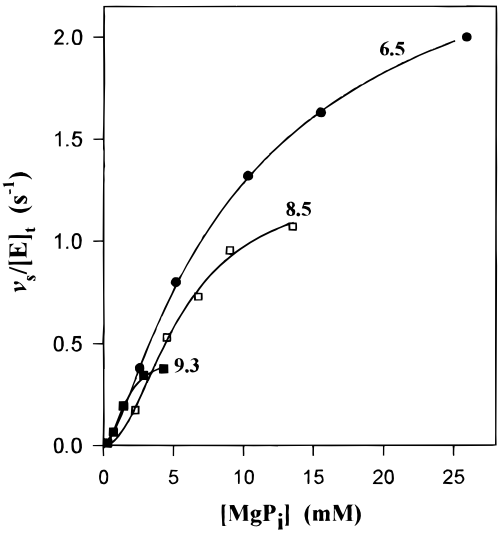


FIGURE 5: Rate of enzyme-catalyzed PP_i synthesis in solution as a function of $[MgP_i]$ and pH. The lines show the best fits to eq 4, using the following values of A (mM) and B (mM^2), respectively: pH 6.5, 10.9 and 14.7; pH 8.5, 2.7 and 25; pH 9.3, 2.3 and 1.7. Free Mg^{2+} concentrations were as follows: pH 6.5, 20 mM; pH 8.5, 5 mM; pH 9.3, 1 mM.

Table 5: Rates of Oxygen Exchange and Amounts of Enzyme-Bound PP_i[a]

pH	$[Mg^{2+}]$ (mM)	v_{ex} (s^{-1})	$[EPP_i]/[E]_T$ (mol/mol)
6.5	20	36 ± 2	0.122 ± 0.024
8.5	5	39 ± 3	0.114 ± 0.020
9.3	1	9.5 ± 0.7	0.040 ± 0.011

[a] Measured at 20 mM P_i.

Table 6: Values of Kinetic and Binding Parameters and of Calculated Rate Constants for E-PPase Catalysis of PP_i-P_i Equilibration[a]

	pH 6.5	pH 7.2[b]	pH 8.5	pH 9.3
k_h (s^{-1})	64 ± 2	139 ± 4	207 ± 5	206 ± 8
k_h/K_m (μM^{-1} s^{-1})	45 ± 5	65 ± 6	74 ± 7	59 ± 2
k_s (s^{-1})	2.9 ± 0.2	2.0 ± 0.2	1.9 ± 0.1	0.68 ± 0.02
k_{ex} (s^{-1})	79 ± 6	63 ± 5	71 ± 7	19 ± 2
P_c	0.14 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.04 ± 0.01
K_3	3.2 ± 0.7	5.0 ± 0.4	3.2 ± 0.6	13 ± 4
k_1 (μM^{-1} s^{-1})	47 ± 7	67 ± 11	76 ± 8	60 ± 3
k_2 (s^{-1})	10.6 ± 2.5	12 ± 3	10 ± 2	10 ± 3
k_3 (s^{-1})	350 ± 70	400 ± 80	290 ± 60	250 ± 100
k_4 (s^{-1})	110 ± 15	81 ± 8	100 ± 20	20 ± 2
k_5 (s^{-1})	670 ± 130	390 ± 80	450 ± 80	500 ± 190
k_7 (s^{-1})	92 ± 15	600 ± 80	>1000	>1000

[a] Measured at a free $[Mg^{2+}]$ of 20 mM (pH 6.5 and 7.2), 5 mM (pH 8.5), or 1 mM (pH 9.3). [b] Values from Baykov et al. (1990).

phosphates bound to the enzyme, i.e., that both MgP_is have to be protonated in order to be able to synthesize Mg_2PP_i but that both $MgHPO_4$ and $MgPO_4^-$ can bind to enzyme. An approximate pK_a value of 8.8 can be estimated from the k_4 values presented in Table 6. This is about 1 unit lower than the pK_a for $MgHPO_4$ in solution (9.7; Childs, 1970), which is not unreasonable considering the possible interactions of enzyme-bound $MgHPO_4$ with other enzyme-bound Mg^{2+} ions as well as Arg and Lys residues present at the active site (Kankare et al., 1994; Salminen et al., 1995). The increase in k_7 with increasing pH would then reflect an increased rate of dissociation of the second released MgP_i as a result of this proton loss.

DISCUSSION

Catalysis by E-PPase is clearly a complex function of both Mg^{2+} concentration and pH. Our studies, leading to the formulation of Scheme 2, provide the first reasonably complete, self-consistent models accounting for the pH dependence of Mg^{2+} binding to three sites on the enzyme in the absence of substrate and for the pH and Mg^{2+} concentration dependencies of k_h. From a mechanistic point of view it is especially interesting that the value of $k_h^{(1)}$, though estimated only imprecisely in this work, is of the same order of magnitude as that of $k_h^{(2)}$ (0.25−2.5 in relative terms). Thus, for the protonation state of the enzyme−substrate complex that reacts to give product, $HMg_jEMg_2PP_i$, the fourth Mg^{2+} (i.e., $j = 2$) may perhaps modulate catalytic activity but is not essential for it. Of course, at pH values <8 achieving the catalytically active protonation state depends on the binding of the fourth Mg^{2+}. It will be interesting to determine whether the observed differences in catalytic activity with different numbers of bound Mg^{2+}, observed for PPases from yeast, rat liver cytosol, and rat liver mitochondria (Baykov & Shestakov, 1992; Unguryte et al., 1989), may also be rationalized according to Scheme 2.

Two of the three Mg^{2+} sites per monomer that we observe in the absence of substrate can be placed unequivocally within the active site, based on crystal structures of the Mg^{2+} complex of E-PPase (Kankare et al., 1996) and the Mn^{2+} complex of Y-PPase (Chirgadze et al., 1991; Heikinheimo et al., in preparation). While the remaining site might also be within the active site, there is no direct evidence for such placement. An intriguing alternative is that the remaining site corresponds to the Mg^{2+} binding site at the subunit interface, described in the accompanying paper (Kankare et al., 1996). This possibility presents the difficulty that the interface site only has a stoichiometry of 0.5/monomer, whereas the results presented in Figure 1 (especially for the data at pH 8.5 and 9.3) indicate an apparent stoichiometry of three full sites. However, we cannot presently exclude interpretations of the binding stoichiometry of three as reflecting four sites having monomer stoichiometries of 1.0:1.0:0.5:0.5, or of 2.5 specific sites/monomer with some relatively weak, nonspecific binding accounting for the additional 0.5 site/monomer.

The similarity in the values of pK_{EH2} and pK_{ESH2} (Table 3) prompts the speculation that both constants refer to deprotonation of the same group. Elsewhere we have presented arguments that this group is an active site water molecule, with binding to 1−2 Mg^{2+} ions, as well as possible hydrogen bonding to acidic residues at the active site (protonated Lys and Arg residues) accounting for its low pK_a value, and that the bound hydroxide ion is the essential base, possibly involved as the nucleophilic species attacking PP_i at the active site (Salminen et al., 1995). As we have seen, our results on the pH dependence of k_4 may be interpreted as indicating that enzyme-bound PP_i synthesis (or, in the reverse direction, PP_i hydrolysis) occurs as $Mg_2PP_i \rightleftharpoons 2MgHP_i$, necessitating proton transfer to each of the two phosphate groups. Hydroxide ion or water attack on the electrophilic phosphoryl group accounts for the protonation of this group. It would make sense mechanistically for proton transfer to the leaving group P_i to occur during the course of hydrolysis (Cooperman et al., 1992), thus

providing a rationale for the essential acidic group. Detailed pH−rate profile analyses of variants for each of the potential essential polar amino acid residues at the active site of E-PPase (Salminen et al., 1995) were inconclusive on this point.

The elaboration of Scheme 2 allows more cogent rationalization of the catalytic efficiencies of E-PPase active site variants than was possible heretofore. Almost all of our previous results with variants have involved determination of k_h and k_h/K_m values either as a function of Mg^{2+} concentration at one fixed pH (7.2) (Käpylä et al., 1995; Volk et al., 1996; Käpylä et al., in preparation) or as a function of pH at one fixed Mg^{2+} concentration (20 mM) (Salminen et al., 1995). In virtually all cases thus far studied, such variants have catalytic efficiencies that, at pH 7.2, are clearly dependent on the involvement of a fifth Mg^{2+} and, at 20 mM Mg^{2+}, have essential basic groups with pK_a values substantially higher than that found for wild-type enzyme. Scheme 2 provides a rationale linking these observations: i.e., as mutations at the active site raise the pK_a of the essential base by distorting an extensive hydrogen bond network (Kankare et al., 1996), a higher Mg^{2+} stoichiometry is required to achieve the deprotonation conferring activity. This requires that the species HMg_3ES (which is not accessible in wild-type enzyme) has catalytic activity, a point explored in greater detail in Volk et al. (1996). More generally, the availability of Scheme 2 to account for the pH and Mg^{2+} concentration dependence of E-PPase activity should allow the effects of active site residue mutation to be understood in terms of changes in discrete equilibrium or rate constants.

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