# Effect of D97E Substitution on the Kinetic and Thermodynamic Properties of *Escherichia coli* Inorganic Pyrophosphatase†

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ABSTRACT: Aspartic acid 97 in the inorganic pyrophosphatase of *Escherichia coli* (E-PPase) has been identified as an evolutionarily conserved residue forming part of the active site [Cooperman *et al.* (1992) *Trends Biochem. Sci. 17*, 262−266]. Here we determine the effect of D97E substitution on several kinetic and thermodynamic properties of E-PPase, including rate and equilibrium constants for enzyme-catalyzed $PP_i$•$P_i$ equilibration at pH 7.2 and 8.0, $Mg^{2+}$ affinity in the presence and absence of substrate, and the $Mg^{2+}$ and pH dependence of $k_{cat}$ and $K_m$. We find the major effects of D97E substitution are to (a) decrease markedly the pH-independence rates of both $PP_i$ hydrolysis and, especially, $PP_i$ resynthesis on the enzyme, (b) selectively destabilize both the $EMg_4PP_i$ complex and the transition state between this complex and the $EMg_2(MgP_i)_2$ complex, (c) raise the $pK_a$ of the basic group "essential" for $PP_i$ hydrolysis and for productive $PP_i$ binding by 1.5 and >2.2 log units, respectively, (d) distort a site to which $Mg^{2+}$ binds in the absence of substrate such that occupancy of the site by $Mg^{2+}$ no longer confers enzymatic activity, and (e) decrease the affinity of one of the two $Mg^{2+}$ ions that binds to enzyme in the presence of substrate. That this multiplicity of effects arises from a single Asp to Glu substitution suggests, in the absence of any evidence for a generalized structural change, a tightly integrated active site in which the perturbation induced by conservative substitution at a single location can have widespread functional effects.

Phosphoryl-transfer enzymes form one of the largest classes of enzymes in nature (Knowles, 1980), yet their mechanisms of action remain incompletely understood (Herschlag & Jencks, 1990). This class includes soluble inorganic pyrophosphatase (PPase),[1] a ubiquitous enzyme known to be essential for cell growth (Chen *et al.*, 1990; Lundin *et al.*, 1991), presumably because of its role in controlling the concentration balance between the central metabolites, inorganic pyrophosphate ($PP_i$) and inorganic phosphate ($P_i$). Recently, we reviewed the compelling evidence supporting the suggestion that the catalytic mechanism and active site structure of soluble PPases are, with minor variation, evolutionarily highly conserved (Cooperman *et al.*, 1992). Four sets of results support this suggestion. First, a group of 15 putative active site residues originally placed at the active site of *Saccharomyces cerevisiae* PPase by X-ray crystallographic analysis and chemical modification have been found to be fully conserved in all 8 known sequences of soluble PPases, despite there being only modest homology between the entire protein sequences. Second, virtually all of the variant *Escherichia coli* PPases (E-PPases) in which each of these putative residues has been mutated have much lower enzymatic activity than does wildtype (Salminen *et al.*, 1995). Third, the two best studied soluble PPases, those from *E. coli* and *S. cerevisiae*, apparently not only share a common mechanism but, in addition, have rate constants for each of eight microscopic steps that differ by less than a factor of 3 from each other. Fourth, these same two enzymes have the distinctive property that enzyme activity requires several divalent metal ions to be bound per active site, three or four for *S. cerevisiae* PPase and four for E-PPase. In each case, $Mg^{2+}$ confers the highest enzymatic activity. More recent studies have shown that the overall fold of the E-PPase subunit is the same as the *S. cerevisiae* subunit, except that the latter is longer at both the N- and C-termini (Kankare *et al.*, 1994).

We seek to understand the basis for PPase catalysis of $PP_i$ hydrolysis, which affords a $10^{10}$ rate acceleration as compared with the hydrolysis of $PP_i$ in solution (Cooperman, 1982). Knowledge of the functions of essential groups at the active site, how they act singly or in combination to affect specific aspects of overall catalysis, is important for such understanding. Site-specific mutagenesis provides a direct approach to the acquisition of such knowledge. In the accompanying paper (Salminen *et al.*, 1995) we summarize the results of preliminary structural and functional studies of E-PPase variants in which each of the putative active site residues has been mutated. Here we explore in detail the functional consequences of conservatively mutating one of these residues, Asp97, to Glu.

Scheme 1 is a slightly simplified version of the minimal scheme we put forward, fully accounting for overall catalysis

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[1] Abbreviations: BSA, bovine serum albumin; E-PPase, *Escherichia coli* inorganic pyrophosphatase; $P_c$, partition coefficient; $P_i$, inorganic phosphate; PPase, inorganic pyrophosphatase; $PP_i$, inorganic pyrophosphate; WT-PPase, wild-type E-PPase.

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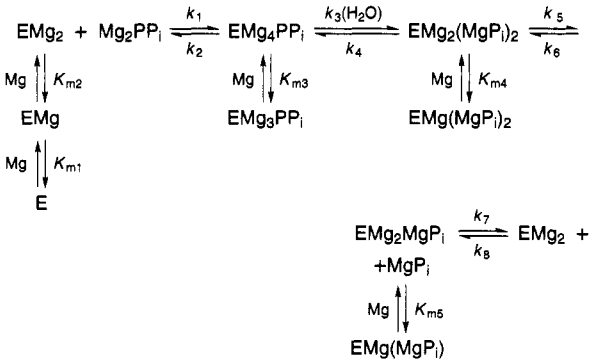
Scheme 1: Minimal Scheme of E-PPase Catalysis[a]




[a] Definitions: $K_{m1}$ = [E][Mg]/[EMg]; $K_{m2}$ = [EMg][Mg]/[EMg$_2$]; $K_{m3}$ = [EMg$_3$PP$_i$][Mg]/[EMg$_4$PP$_i$]; $K_{m4}$ = [EMg(MgP$_{i2}$][Mg]/[EMg$_2$-(MgP$_i$)$_2$]; $K_{m5}$ = [EMg(MgP$_i$)][Mg]/[EMg$_2$MgP$_i$]; $K_1 = k_1/k_2$; $K_3 = k_3/k_4$; $K_5 = k_5/k_6$; $K_7 = k_7/k_8$.

of PP$_i$·P$_i$ equilibrium by E-PPase at a single pH (7.2) (Baykov *et al.*, 1990), following procedures that had been developed earlier in studies on *S. cerevisiae* PPase catalysis of the same reaction (Springs *et al.*, 1981; Welsh *et al.*, 1983; Baykov & Shestakov, 1992). In the current work we extend our analysis to measure the pH dependence of the equilibrium and rate constants in the range 6.5−8.0 for wild-type E-PPase (WT-PPase) catalysis and compare the results obtained with similar experiments carried out on D97E-PPase. In addition, we compare WT-PPase and D97E-PPase with respect to their $Mg^{2+}$ affinities and to the pH dependencies of their $k_{cat,hyd}$ and $k_{cat,hyd}/K_m$ values. Our results show that the single conservative D97E substitution has several important effects on PPase function and leads to a suggestion that a tightly organized network of bound water molecules and metal ions at the active site may be crucial for enzymatic activity.

## EXPERIMENTAL PROCEDURES

*Materials.* The expression and purification of wild-type PPase (WT-PPase) was carried out using an overproducing *E. coli* HB101 strain transformed with a suitable plasmid derived from pUC19 as described by Lahti *et al.* (1990). Two samples of D97E-PPase were utilized in these studies. The first was obtained in a manner similar to that of WT-PPase, using the appropriate mutant plasmid. As described in Results, it was contaminated with 0.5 ± 0.1% (by weight) endogenous, chromosomal-encoded, WT-PPase derived from pUC19. The second was obtained free of WT-PPase by using an *E. coli* MC1061 (Sambrook *et al.*, 1989) derivative in which chromosomal E-PPase was replaced by the *S. cerevisiae* PPA1 gene (Kolakowski *et al.*, 1988), using the method of Hamilton *et al.* (1989), as a host strain for the amplification of D97E-PPase. The chromosome-encoded *S. cerevisiae* PPase was readily separated from D97E-PPase by ion-exchange chromatography (Salminen *et al.*, 1995). Enzyme concentration was estimated on the basis of a subunit molecular weight of 20 000 (Josse, 1966) and an $A^{1\%}_{280}$ equal to 11.8 (Wong *et al.*, 1970; Lahti *et al.*, 1988). Protein determination was by Bradford (1976) assay.

Carrier-free [$^{32}$P]P$_i$ was from NEN-Du Pont. Contaminating [$^{32}$P]PP$_i$ was removed according to Cohn (1958) using a Dowex-1 column. $^{18}$O-enriched water (97−98%), $D_2O$ (99.8%), Dowex-1 (1-X2, 100 mesh), and Dowex-50 (50-X8, 200 mesh) were from Aldrich. Chelex-100 was from Bio-Rad and BSA was from Sigma. Scintillation cocktail EcoLite-(+) was purchased from ICN. All other chemicals were reagent grade and were used without further purification.

Table 1: pH-Dependent Values of Equilibrium Constants Involving PP$_i$, P$_i$, and $Mg^{2+}$ [a]

| pH | $K_B$[b] (mM) | $K_A$[c] (mM) | $K_{A2}$[c] (mM) | $K_{PP}$[d] (M) |
|---|---|---|---|---|
| 6.5 | 18.7 | 0.294 | 6.28 | $1.29 \times 10^4$ |
| 7.2 | 8.5 | 0.086 | 2.83 | $4.45 \times 10^4$ |
| 8.0 | 6.36 | 0.017 | 2.13 | $22.2 \times 10^4$ |

[a] Definitions (the subscript t refers to total concentration, i.e., all species having the stoichiometry shown without regard to protonation state): $K_B$ = [Mg][P$_i$]$_f$/[MgP$_i$]$_t$; $K_A$ = [Mg][PP$_i$]$_f$/[MgPP$_i$]$_t$; $K_{A2}$ = [Mg][MgPP$_i$]$_f$/[Mg$_2$PP$_i$]$_t$; $K_{PP}$ = [P$_i$]$_f^2$/[PP$_i$]$_t$. [b] Values at pH 6.5 and 8.0 are calculated on the basis of the value at pH 7.2 (Smirnova *et al.*, 1989). [c] Volk *et al.*, 1982. [d] Values at pH 6.5 and 8.0 are calculated on the basis of the value at pH 7.2 (Baykov *et al.*, 1990).

*Methods.* Unless otherwise noted, experiments were conducted at an ionic strength of 0.16−0.24. Suitable control experiments showed negligible changes in the properties measured over this range of ionic strength.

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). Reactions were started by addition of enzyme and were carried out at 25 °C. All rates were normalized to a standard rate of WT-PPase-catalyzed PP$_i$ hydrolysis, measured with a reaction mixture containing 40 μM PP$_i$ and 20 mM $Mg^{2+}$ at pH 7.2, in order to correct for minor differences observed for data taken over extended periods of time with different preparations of enzyme. The total variation in this standard assay was ±20% for all samples determined.

$Mg^{2+}$ binding to PPase was measured by equilibrium dialysis in a Tris (0.1 M)−EGTA (20 μM) buffer at 23 ± 2 °C, using a microapparatus similar to that described by Overall (1987) equipped with a dialysis membrane having a cutoff of 12 000 (Spectrum Medical Industries, Los Angeles, CA). PPase samples used in such experiments were treated to remove small amounts of contaminating DNA by adsorbing protein on a DEAE-cellulose column equilibrated with 0.05 M Tris·HCl (pH 7.2) and eluting protein with 0.3 N NaCl in the same buffer. Following equilibration, $Mg^{2+}$ concentrations were determined by atomic absorption spectrometry (Perkin-Elmer 5100 PC, 285.2 nM).

Enzyme-bound PP$_i$ formation (EPP$_i$ formation) at equilibrium was measured at 25 °C by using the selective extraction method described by Springs *et al.* (1981). $^{32}$P radioactivity was determined with a Beckman LS1801 scintillation counter.

Rates of $H_2O$·P$_i$−oxygen exchange were measured at 25 °C as described by Springs *et al.* (1981) with the slight modification that a larger Dowex-50 column was used (1 × 15 cm) in order to remove $Mg^{2+}$ more completely. Chelex-100 columns were used to remove paramagnetic metal ions, as described by Welsh *et al.* (1983). $^{18}$O-labeled P$_i$ typically contained 90% [$^{18}O_4$]P$_i$ and 10% [$^{18}O_3$]P$_i$. $^{31}$P NMR spectra were taken on a Bruker AM-500 at 202.46 MHz.

*Calculations and Data Analysis.* Values of equilibrium constants used to calculate the concentrations of free $Mg^{2+}$, Mg$_2$PP$_i$, and MgP$_i$ are presented in Table 1. Parameter values giving best fits of the data to eqs 1−7 and other equations fitted in this work were determined using either a

program for nonlinear regression previously described (Duggleby, 1984) or the Igor program available from Wavemetrics.

Values for $k_{cat,hyd}$ and for $K_{m,hyd}$ were determined by computer fitting of $k_{hyd}$ values as a function of $Mg_2PP_i$ concentration to the Michaelis–Menten equation. Values for $K_3$, $K_5$, and $K_7$ were determined by fitting the observed dependence of $EPP_i$ formation on $[MgP_i]$ (Figure 2) to eq 1, in which $[E]_t$ refers to the total enzyme concentration in solution and $[EPP_i]_t$ refers to the total concentration of all forms of enzyme-bound $PP_i$, as described (Springs *et al.*, 1981). Values for $K_1$ were calculated from eq 2.

$$[E]_t/[EPP_i]_t = K_3K_5K_7/[MgP_i]^2 + K_3K_5/[MgP_i] + K_3 + 1 \quad (1)$$

$$K_1 = K_AK_{A2}K_{PP}/K_3K_5K_7K_B^2 \quad (2)$$

Values of $k_{cat,ex}$ were calculated either by fitting the results of measuring $k_{ex}$ as a function of $[MgP_i]$ to the Michaelis–Menten equation (data taken at pH 7.2) or, when less extensive data were taken (at pH values of 6.5 and 8.0), directly from eq 3 (Springs *et al.*, 1981), using values for

$$k_{cat,ex} = k_{ex}\{1 + K_3K_5/((K_3 + 1)[MgP_i]) + K_3K_5K_7/((K_3 + 1)[MgP_i]^2)\} \quad (3)$$

$K_3$ and $K_5$ determined from $EPP_i$ formation data. First approximations to the partition coefficient, $P_c$, defined as the rate at which enzyme-bound $P_i$ loses water in the exchange step divided by the sum of this rate and the rate of release of $P_i$ to the medium (Hackney & Boyer, 1978), were determined from the ratio of the rate of $P^{18}O_4$ disappearance to the rate of overall exchange, as previously described (Springs *et al.*, 1981). Using these values as starting points, the computer program developed by Hackney (1980), which considers the distribution of all $^{18}O$-labeled forms of $P_i$ as a function of time, was employed to obtain more precise estimates of $P_c$.

Calculations of values for the eight rate constants, $k_1$–$k_8$, from the four equilibrium constants, $K_1$, $K_3$, $K_5$, and $K_7$, and the values of $k_{cat,hyd}$, $K_{m,hyd}$, $k_{cat,ex}$, and $P_c$ were carried out as described earlier (Springs *et al.*, 1981), setting $P_c$ equal to $k_4/(k_4 + k_5)$. For the D97E variant, $k_{cat}/K_m$ is well approximated by $k_1k_3/(k_3 + k_2)$, in principle permitting estimation of $k_1$ and $k_2$ knowing $k_3$ and $K_1$. However, accumulated errors in calculated parameter values did not permit reliable estimations of $k_1$ and $k_2$ by this approach.

Values of $K_{m1}$ and $K_{m2}$ were estimated by fitting equilibrium dialysis data to eq 4, where $n$ measures the number of $Mg^{2+}$ bound per subunit and $n_1$ and $n_2$ (each set equal to 1.0) and $K_{m1}$ and $K_{m2}$ are the stoichiometries and dissociation constants of the higher affinity and lower affinity sites, respectively.

$$n/[Mg^{2+}] = (n_1/K_{m1})/(1 + [Mg^{2+}]/K_{m1}) + (n_2/K_{m2})/(1 + [Mg^{2+}]/K_{m2}) \quad (4)$$

Values of $K_{m3}$, as well as an alternative values of $K_{m2}$, were estimated by fitting $k_{cat,app}$ and $K_{m,app}$ values for PPase catalysis of $PP_i$ hydrolysis as a function of $Mg^{2+}$ concentration to eq 5, which may be derived from Scheme 1 and equations presented in Springs *et al.* (1981), making the simplifying assumption that $K_{m4}$ and $K_{m5}$ are both equal to $K_{m3}$. $k_{cat,app}$ is equal to $k_{cat}'/B$ and $K_{m,app}$ is equal to $AK_m'/B$. Primed constants refer to values at saturating $[Mg^{2+}]$.

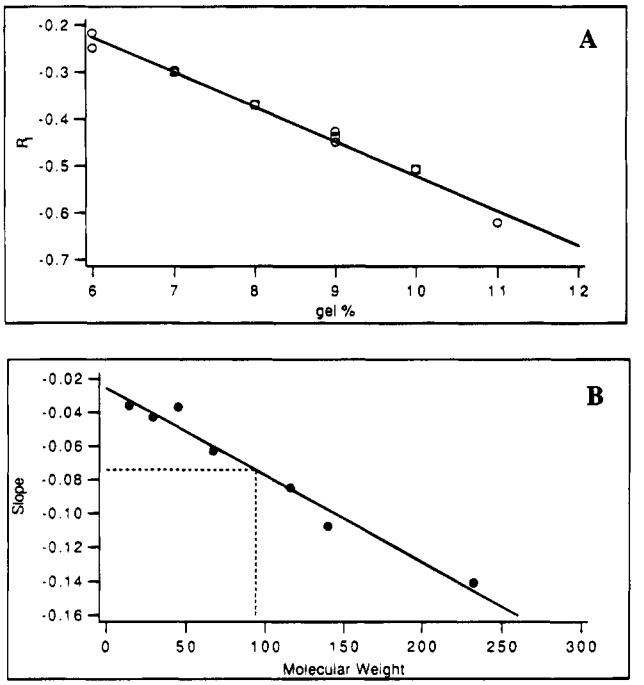



FIGURE 1: (A) Electrophoretic mobility of WT-PPase (○) and D97E-PPase (□) in nondenaturing polyacrylamide gels as a function of polyacrylamide content. The identity in migration of the two PPases at several polyacrylamide concentrations is evidence that both enzymes have the same molecular weight. (B) Molecular weight estimation by the method of Hedrick and Smith (1968). The slope determined in part A was compared with slopes determined for standard proteins, leading to an estimated molecular weight for both PPases of 95 000 ± 5000. The standard proteins (weights) were α-lactalbumin (14 200), carbonic anhydrase (29 000), chicken egg albumin (45 000), bovine serum albumin (67 000), β-galactosidase (116 000), lactate dehydrogenase (140 000), and catalase (232 000).

$$v/[E]_t = (k_{cat}'/B)/\{1 + (AK_m'/B)/[Mg_2PP_i]\} \quad (5)$$

where

$$A = 1 + K_{m1}K_{m2}/[Mg]^2 + K_{m2}/[Mg] \quad (5a)$$

$$B = 1 + K_{m3}/[Mg] \quad (5b)$$

$$k_{cat}' = k_3'k_5'k_7'/[k_3'k_5' + k_7'(k_3' + k_4' + k_5')] \quad (5c)$$

$$K_m' = k_7'[k_3'k_5' + k_2'(k_4' + k_5')]/k_1'[k_3'k_5' + k_7'(k_3' + k_4' + k_5')] \quad (5d)$$

## RESULTS

*Structural Characterization of D97E-PPase.* Previously (Lahti *et al.*, 1990) we showed that D97E-PPase was as thermostable as WT-PPase and had approximately the same surface hydrophobicity, as measured by Nile red binding. In this work we show further that D97E-PPase has the same molecular weight as does WT-PPase as measured by the dependence of electrophoretic mobility on polyacrylamide concentration during nondenaturing polyacrylamide gel elec-

Table 2: Rate and Equilibrium Constants for WT-PPase and D97E-PPase

| | WT,[a] pH 7.2 | WT, pH 6.5 | WT, pH 7.2 | WT, pH 8.0 | D97E, pH 7.2 | D97E, pH 8.0 |
|---|---|---|---|---|---|---|
| $K_{m,hyd}$ ($\mu$M) | | 1.6 ± 0.1 | 3.5 ± 0.5 | 3.4 ± 0.2 | 1.8 ± 0.2 | 2.4 ± 0.1 |
| $k_{cat,hyd}$ ($s^{-1}$) | | 86 ± 4 | 155 ± 8 | 187 ± 5 | 12 ± 1 | 55 ± 2 |
| $k_{cat,ex}$ ($s^{-1}$) | | 104 ± 4 | 116 ± 21 | 114 ± 13 | 0.34 ± 0.03[b] | 0.67 ± 0.18[b] |
| | | | | | 0.30 ± 0.09[c] | 0.6 ± 0.2[c] |
| $P_c$ | 0.19 | 0.18 ± 0.04 | 0.24 ± 0.02 | 0.21 ± 0.03 | <0.03[d] | <0.03[d] |
| | | | | | <0.01[e] | <0.01[e] |
| $K_1 \times 10^6$ ($M^{-1}$) | 11 ± 4 | 0.6 ± 0.2 | 2.3 ± 0.6 | 5.2 ± 3.9 | 0.19 ± 0.08 | 0.68 ± 0.43 |
| $K_3$ | 5.0 ± 0.4 | 4.1 ± 0.5 | 5.8 ± 0.5 | 6.0 ± 2.8 | 56 ± 6 | 74 ± 22 |
| | | | | | | 82[e] |
| $K_5$ (mM) | 3.1 ± 0.6 | 10.5 ± 2.3 | 7.4 ± 1.2 | 13.2 ± 6.7 | 5.7 ± 1.6 | 8.8 ± 3.3 |
| $K_7$ (mM) | 0.85 ± 0.15 | 2.9 ± 0.7 | 1.55 ± 0.24 | 0.49 ± 0.14 | 2.5 ± 0.7 | 0.45 ± 0.19 |
| $k_1 \times 10^7$ ($M^{-1}$ $s^{-1}$) | 13 ± 2 | 6.4 ± 0.6 | 4.6 ± 0.5 | 5.6 ± 0.4 | | |
| $k_2$ ($s^{-1}$) | 12 ± 3 | 106 ± 37 | 20 ± 5 | 11 ± 9 | | |
| $k_3$ ($s^{-1}$) | 400 ± 80 | 550 ± 200 | 800 ± 180 | 800 ± 400 | 19 ± 2 | 50 ± 20 |
| $k_4$ ($s^{-1}$) | 81 ± 8 | 134 ± 47 | 140 ± 30 | 130 ± 20 | 0.34 ± 0.09 | 0.67 ± 0.18 |
| $k_5$ ($s^{-1}$) | 390 ± 80 | 610 ± 165 | 440 ± 110 | 500 ± 140 | >45 | >150 |
| $k_6 \times 10^4$ ($M^{-1}$ $s^{-1}$) | 13 ± 4 | 5.9 ± 2.1 | 5.9 ± 1.7 | 3.8 ± 2.2 | | |
| $k_7$ ($s^{-1}$) | 600 ± 80 | 129 ± 27 | 400 ± 100 | 560 ± 310 | >45 | >150 |
| $k_8 \times 10^5$ ($M^{-1}$ $s^{-1}$) | 7 ± 2 | 0.47 ± 0.15 | 2.6 ± 0.7 | 11 ± 7 | | |

[a] Baykov *et al.*, 1990. [b] Measured for D97E-PPase free of endogenous WT-PPase. [c] Corrected value obtained for D97E-PPase contaminated with endogenous E-PPase after correction for contribution from endogenous WT-PPase. [d] Observed. [e] Calculated; see text.

trophoresis (Figure 1). That molecular weight is the same for D97E-PPase as for WT-Ppase shows that mutation does not lead to change in oligomerization and demonstrates that the observed similarity in surface hydrophobicity does not mask a change in conformation that is offset by a change in oligomerization state.

The slope determined in Figure 1A for both proteins was compared with slopes determined for standard proteins in Figure 1B, leading to an estimated molecular mass for both PPases of 95 ± 5 kDa. Wakagi *et al.* (1992) have recently reported an apparent molecular mass for E-PPase of 105 kDa, as measured by gel filtration. Both of these values are lower than the values of 121 ± 2 kDa for E-PPase determined by equilibrium sedimentation (Wong *et al.*, 1970) and of 117 kDa calculated from the primary structure (Lahti *et al.*, 1988) for a hexamer, suggesting that E-PPase may have a compact structure. In data not presented, we have also shown that WT-PPase and D97E-PPase have superimposable CD spectra. As measured by these four criteria, then, there is no gross structural change in PPase on D97E substitution, although smaller changes cannot be excluded.

*Determination of Rate and Equilibrium Constants for WT-PPase at High $Mg^{2+}$ and pH 6.5, 7.2, and 8.0.* We previously demonstrated that equilibrium and rate constants for PPase catalysis of $PP_iP_i$ equilibration can be evaluated on the basis of measurements of steady-state rates of $PP_i$ hydrolysis ($k_{hyd}$) and $H_2O$-$P_i$ oxygen exchange ($k_{ex}$) and of equilibrium $EPP_i$ formation as a function of [$MgP_i$] (Springs *et al.*, 1981; Welsh *et al.*, 1983). Here we investigate the pH dependence of these processes for WT-PPase by performing measurements at pH 6.5, 7.2, and 8.0. These studies, the results of which are compared directly in Table 2 with parallel results obtained for D97E-PPase (see below), were performed at a relatively high concentration of free $Mg^{2+}$ (20 mM). This was done in order to minimize the effects of changes in $Mg^{2+}$ affinity resulting from D97E substitution on the derived rate and equilibrium constants (see below).

Results measuring the dependence of $EPP_i$ formation and of $k_{ex}$ on [$MgP_i$] are presented in Figures 2 and 3 and Table 3. The limited solubility of $MgP_i$ at alkaline pH prevented

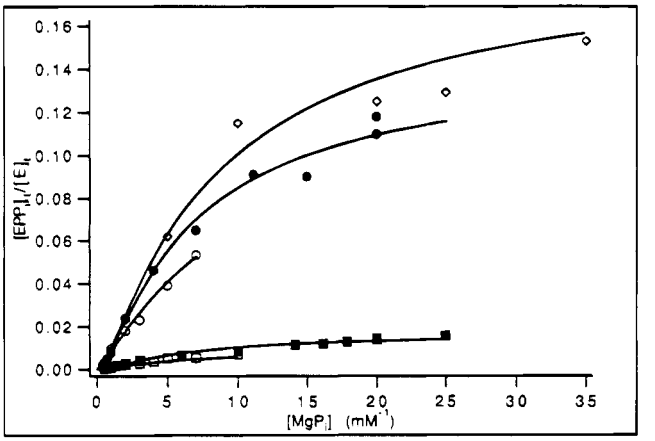



FIGURE 2: Dependence of $[EPP_i]_t/[E]_t$ on [$MgP_i$] for WT-PPase and D97E-PPase. Symbols: (◇) WT-PPase, pH 6.5; (●) WT-PPase, pH 7.2; (○) WT-PPase, pH 8.0; (■) D97E-PPase, pH 7.2; (□) D97E-PPase, pH 8.0. Lines are drawn to eq 1, using parameter values found in Table 2 (Springs *et al.*, 1981). Experimental conditions: $[Mg]_{free}$, 20 mM. For WT-PPase, [$MgP_i$] was 0.5−35 mM at pH 6.5, 0.4−20 mM at pH 7.2, and 0.5−7 mM at pH 8.0; [enzyme] was 140−360 $\mu$M; buffers were 150 mM Tris at pH 7.2 and 8.0 and 150 mM MES at pH 6.5. For D97E-PPase, [$MgP_i$] was 0.5−25 mM at pH 7.2 and 0.5−10 mM at pH 8.0; [enzyme] was 525−675 $\mu$M; buffer was 150 mM Tris at both pH values.

us from approaching saturation levels at pH 8.0, leading to considerable uncertainty in some of the parameters in Table 2, and prevented extension of our studies to higher pH. These same limitations apply as well to measurements carried out on D97E-PPase (see below).

The values of the equilibrium and rate constants derived for WT-PPase at pH 7.2 are similar to those determined earlier by Baykov *et al.* (1990), demonstrating that WT-PPases prepared from an ordinary strain of *E. coli* and from the transformed, overproducing strain are essentially identical, and confirm that E-PPase shares with *S. cerevisiae* PPase the properties that (a) for $PP_i$ hydrolysis all three steps following $PP_i$ binding are partially rate determining, (b) for $P_i$-$H_2O$ oxygen exchange, $PP_i$ synthesis (step 4) is almost uniquely rate determining, and (c) $K_3$, the equilibrium constant for the hydrolysis of enzyme-bound $PP_i$ to two enzyme-bound $P_i$s, only slightly favors hydrolysis, quite

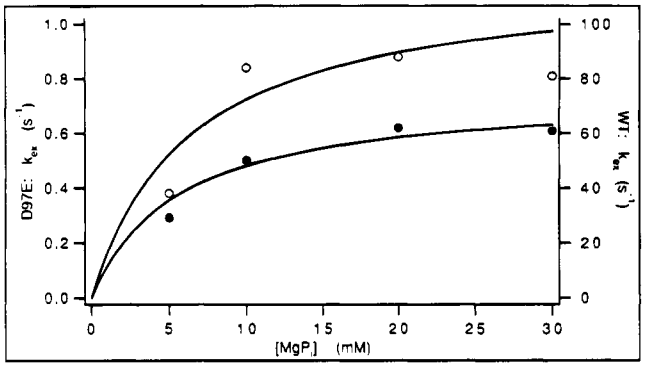



FIGURE 3: Dependence of $k_{ex}$ on [MgP$_i$] for WT-PPase (○) and D97E-PPase (●) at pH 7.2. Lines are drawn to eq 3, using parameter values found in Table 2 (Springs *et al.*, 1981). Experimental conditions: $[Mg]_{free}$, 20 mM; [PPase], 0.5−10 μM; buffer, Tris (150 mM).

Table 3: Values of $k_{ex}$

| | [MgP$_i$] (mM) | $k_{ex}$ ($s^{-1}$) | $k_{cat,ex}$ ($s^{-1}$)[a] | $k_{cat,ex}$ ($s^{-1}$)[b] |
|---|---|---|---|---|
| WT, pH 6.5 | 10.4 | 45 | 104 | 104 |
| WT, pH 7.2 | 5 | 38 | 101 | 116 ± 16 |
| | 10 | 84 | 145 | |
| | 14 | 73 | 116 | |
| | 20 | 88 | 118 | |
| | 30 | 81 | 99 | |
| WT, pH 8.0 | 4 | 28 | 117 | 114 ± 13 |
| | 7 | 38 | 104 | |
| | 8.5 | 54 | 130 | |
| | 10 | 47 | 103 | |
| D97E, pH 7.2 | 20 | 0.26 ± 0.01 | 0.34 ± 0.01 | 0.34 ± 0.01 |
| D97E, pH 7.2[c] | 5 | 0.29 | 0.78 | 0.80 ± 0.05[c] |
| | 10 | 0.50 | 0.85 | |
| | 20 | 0.62 | 0.82 | |
| | 30 | 0.61 | 0.73 | |
| D97E, pH 8.0 | 4 | 0.22 | 0.76 ± 0.26 | 0.67 ± 0.18 |
| | 8 | 0.27 | 0.58 ± 0.11 | |
| D97E, pH 8.0[c] | 2 | 0.28 | 1.77 | 1.25 ± 0.52[c] |
| | 5 | 0.37 | 1.07 | |
| | 8 | 0.43 | 0.92 | |

[a] Calculated according to eq 3. [b] Average value ± standard deviation. [c] Measured on samples contaminated with endogenous WT-PPase.

different than the large equilibrium constant found for PP$_i$ hydrolysis in solution.

*Determination of Rate and Equilibrium Constants for D97E-PPase at High $Mg^{2+}$ and pH 7.2 and 8.0.* Measurements identical to those described above for WT-PPase were also performed on D97E-PPase, giving results presented in Tables 2 and 3 and Figures 2 and 3. Compared at pH 7.2, D97E-PPase has substantially lower values than WT-PPase for $k_{cat,hyd}$ (13-fold), $k_{cat,ex}$ (340-fold), and $K_1$ (12-fold), a substantially (10-fold) higher value for $K_3$, and similar values for $K_m$, $K_5$, and $K_7$. These differences and similarities persist at pH 8.0, with the only major changes being that the ratio of $k_{cat,hyd}$ values drops to 3.4 and the ratio of $k_{cat,ex}$ values drops to 170.

Only a lower limit for $P_c$ of <0.03 at pH 7.2 and 8.0 can be estimated from the $^{31}P$ NMR data. However, these estimates can be reduced to <0.01 on the basis of eq 6, which is derivable from eq 11 in Springs *et al.* (1981), taking into account that $K_3 \gg 1$ for D97E-PPase.

$$P_c = k_{cat,ex}/(k_5 + 0.75\, k_{cat,ex}) \quad (6)$$

This low and imprecise value makes it impossible (Springs *et al.*, 1981) to estimate values for $k_5$ and $k_7$ (and therefore of $k_6$ and $k_8$). However, we can estimate values for $k_3$ and $k_4$ for D97E-PPase at both pH 7.2 and pH 8.0, since when $P_c \ll 1$ and $K_3 \gg 1$, $k_4$ equals $k_{cat,ex}$ (Welsh *et al.*, 1983) and $k_3$ may be calculated from the product of $k_4$ and $K_3$. Such calculation shows $k_3$ to be very nearly equal to $k_{cat,hyd}$ at both pH values. Thus, for D97E-PPase, steps 3 and 4 are essentially uniquely rate determining in the forward (hydrolysis) and backward (exchange) directions, respectively, of PPase catalysis. Finally, we note that whereas the values of $k_3$ and $k_4$ for WT-PPase are essentially unchanged between pH values 7.2 and 8.0 (see also A. A. Baykov, S. E. Volk, V. N. Kasho, A. V. Vener, T. Hyytiä, R. Lahti, and B. S. Cooperman, manuscript in preparation), both rate constants increase between these pH values for D97E-PPase. As a result, while D97E substitution decreases $k_4$ by 400-fold and $k_3$ by 40-fold at pH 7.2, these values fall to 200-fold and 16-fold, respectively, at pH 8.0 (Table 2).

Table 4: Measures of $Mg^{2+}$ Binding[a]

| | WT | D97E |
|---|---|---|
| $k_{cat}'$ ($s^{-1}$) | 154 ± 3 | 13.7 ± 0.4 |
| $K_m'$ (mM) | 3.5 ± 0.2 | 0.66 ± 0.17 |
| $K_{m1}$ (mM) dialysis | 0.083 ± 0.004 | 0.076 ± 0.02 |
| $K_{m2}$ (mM) dialysis | 1.67 ± 0.17 | 1.86 ± 0.14 |
| $K_{m2}$ (mM) kinetics | 1.0 ± 0.1 | 85 ± 35 |
| $K_{m3}$ (mM) kinetics | 0.57 ± 0.08 | 3.7 ± 0.3 |

Many of the earlier measurements reported in this paper were carried out with D97E-PPase that was contaminated with a minor amount of endogenous WT-PPase. While the low level of contamination had negligible effects on the values determined for $K_1$, $K_3$, $K_5$, $K_7$, $k_{cat,hyd}$, and $K_{m,hyd}$, it did result in a measured value for $k_{cat,ex}$ that was markedly higher than the correct value and did not permit direct determination of $P_c$. Fitting the observed $^{18}O$-labeled P$_i$ distributions (a total of 39 at pH 7.2 and 16 at pH 8.0) obtained with this contaminated sample to those calculated assuming two catalytic processes occurring simultaneously, one via WT-PPase with a $P_c$ value of either 0.24 (pH 7.2) or 0.21 (pH 8.0) (Table 2) and one via D97E-PPase with a $P_c$ value ≤0.01, yielded corrected $k_{ex}$ values for D97E-PPase that agree very well with those measured directly using D97E-PPase free of WT-PPase contamination (Table 2), as well as an estimate of the level of endogenous WT-PPase contamination of the D97E-PPase. The latter, given by the ratio of the contribution WT-PPase makes to the measured value of $k_{cat,ex}$ for contaminated D97E-PPase to the value of $k_{cat,ex}$ for WT-PPase, was 0.5 ± 0.1%.

*Effects of D97E Substitution on $Mg^{2+}$ Affinity.* E-PPase is known to require four $Mg^{2+}$ per active site for activity. Two of these $Mg^{2+}$ ions are bound directly to PPase and two additional $Mg^{2+}$ are bound in the presence of substrate (Baykov *et al.*, 1990). The effects of D97E substitution on $Mg^{2+}$ affinity were measured in two ways: first, by equilibrium dialysis measurements of direct $Mg^{2+}$ binding to both WT-PPase and D97E-PPase in the absence of substrate (Figure 4) and, second, by determination of the $Mg^{2+}$ dependence of $k_{cat,hyd}$ and $K_{m,hyd}$.

Measured at pH 7.2 in the absence of substrate, equilibrium dialysis measurement of the binding of $Mg^{2+}$ to both WT-PPase and D97E-PPase is well described by eq 4, giving values for $K_{m1}$ and $K_{m2}$ displayed in Table 4. These values make clear that D97E substitution has little effect on $Mg^{2+}$ binding to either the higher or lower affinity site. We found

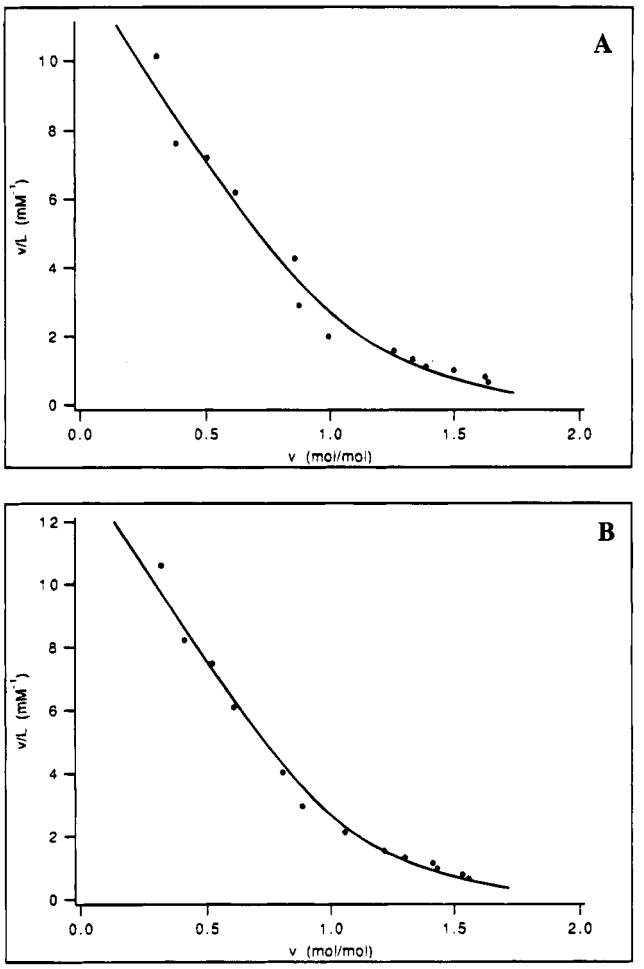



FIGURE 4: Scatchard plot of $Mg^{2+}$ binding to WT-PPase (A) and to D97E-PPase (B). Solid lines are drawn to eq 4, using parameter values found in Table 4. Experimental conditions: $[PPase]_t$, 0.4–0.5 mM; $[Mg^{2+}]_t$, 0.03–2.5 mM.

no evidence for a third binding site for $Mg^{2+}$ in the absence of substrate; i.e., if it is present, it is too weak at pH 7.2 to detect in our experiment.

$k_{cat,app}$ and $K_{m,app}$ values for $PP_i$ hydrolysis catalyzed by both WT-PPase and D97E-PPase were determined as a function of $Mg^{2+}$ concentration (0.2–50 mM) at a fixed pH (7.2). As Figure 5 makes clear, 20 mM corresponds to a fully or nearly saturating $Mg^{2+}$ concentration for $k_{cat,hyd}$ for both WT-PPase and D97E-PPase and for $K_m$ for WT-PPase but not for $K_m$ for D97E-PPase. The data obtained (88 points for WT-PPase, 101 points for D97E-PPase) were fit to eq 5, yielding best fit values for $K_{m2}$, $K_{m3}$, $K_m'$, and $k_{cat}'$. $K_{m1}$ for each PPase was fixed at the value determined by equilibrium dialysis. These values are collected in Table 4. They show that D97E substitution substantially increased the kinetically determined values of $K_{m2}$ (85-fold) and $K_{m3}$ (6.5-fold).

For WT-PPase, the kinetically determined $K_{m2}$ value differs by less than a factor of 2 from that determined by equilibrium dialysis. Given the precision of each measurement and the differences in experimental conditions for the two measurements, the two values may be considered to agree within experimental error. However, the two $K_{m2}$ values for D97E-PPase differ markedly from one another, with the kinetically determined $K_{m2}$ value being 46-fold larger than that determined by equilibrium dialysis. We return to this point in the Discussion.

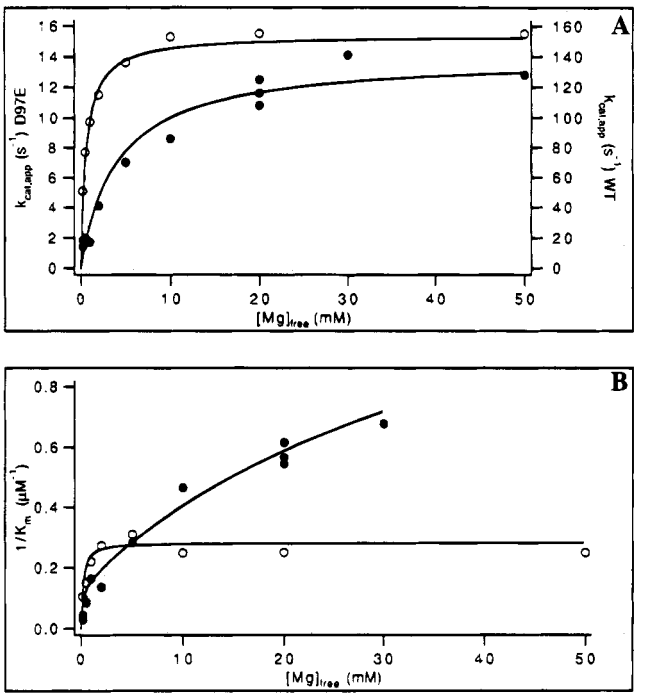



FIGURE 5: Dependence of $k_{cat,hyd,app}$ (A) and $K_{m,hyd,app}$ (B) on $[Mg]_{free}$ at pH 7.2 for WT-PPase (○) and D97E-PPase (●). Ionic strength varied from 0.16 at low $[Mg^{2+}]$ to 0.19 at high $[Mg^{2+}]$. Substrate concentrations were calculated for $Mg_2PP_i$. Solutions contained $MgCl_2$ (0.2–20 mM with the wild type; 0.2–50 mM with the variant D97E) and $NaPP_i$ (0.8–375 mM). Lines are drawn to eq 5, using parameters listed in Table 4.

*Effect of D97E Substitution on the pH–Rate Profile.* $k_{cat}$ and $K_m$ values for $PP_i$ hydrolysis catalyzed by both WT-PPase and D97E-PPase were determined as a function of pH at fixed, high $[Mg^{2+}]$ (20 mM), giving the results displayed in Figure 6. The pH dependencies of $k_{cat,app}$ and $k_{cat,app}/K_{m,app}$ allow calculation of apparent ionization constants for enzyme with substrate bound, indicated as ESH, and for enzyme lacking substrate, indicated as EH, as well as of pH-independent values for $k_{cat}$ and $k_{cat}/K_m$, according to eqs 7a and 7b. The values of these parameters, collected in Table

$$k_{cat,app}/K_{m,app} = (k_{cat}/K_m)/(1 + [H^+]/K_{EH_2} + K_{EH}/[H^+]) \quad (7a)$$

$$k_{cat,app} = k_{cat}/(1 + [H^+]/K_{ESH_2} + K_{ESH}/[H^+]) \quad (7b)$$

5, clearly demonstrate that, measured at 20 mM $Mg^{2+}$, the major effect accompanying D97E substitution is an increase in the apparent $pK_a$ (from 6.6 to 8.1) of a basic group needed for catalytic turnover in the $EMg_4PP_i$ complex and a similar increase in the apparent $pK_a$ (from <6 to 8.2) of a basic group in EH presumably needed for productive $Mg_2PP_i$ binding. Particularly striking are the comparatively minor effects of D97E substitution on the pH-independent values of both $k_{cat}$ (a 1.6-fold decrease) and $k_{cat}/K_m$ (a 1.4-fold increase).

The pH optimum of $k_{cat}$ for WT-PPase, 8.15, is considerably lower than the value of 9.1 reported earlier by Josse (1966), but this latter value was determined at a much lower $[Mg^{2+}]$, 1.3 mM, than that used in these studies.

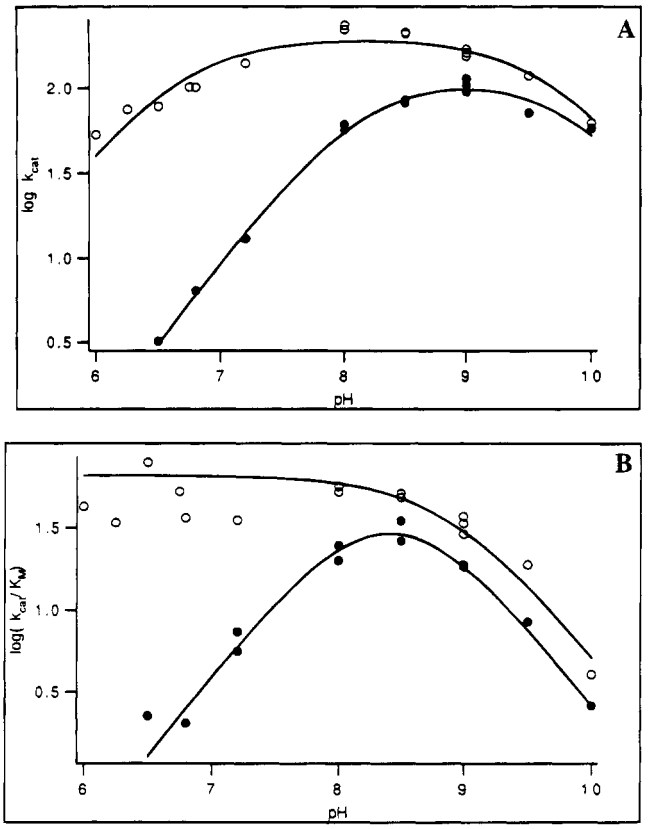



FIGURE 6: Dependence of $k_{cat,app}$ (A) and $k_{cat,app}/K_{m,app}$ (B) on pH for WT-PPase (○) and for D97E-PPase (●). Experimental conditions: 20 mM $[Mg]_{free}$ and $NaPP_i$ (1.5−80 $\mu$M) in 120 mM buffer (MES, HEPES, Tris, or 2-amino-2-methyl-1,3-propanediol) at desired pH. Substrate concentrations were calculated for $Mg_2PP_i$. Lines are drawn to eqs 7a and 7b, using parameters listed in Table 5.

Table 5: pH−Rate Profile Parameters

| | $pK_{EH_2}$ | $pK_{EH}$ | $k_{cat}/K_m \times 10^6$ ($M^{-1}$ $s^{-1}$) | $pK_{ESH_2}$ | $pK_{ESH}$ | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|---|---|---|
| WT | <6 | 9.1 ± 0.1 | 46 ± 4 | 6.6 ± 0.2 | 9.7 ± 0.1 | 200 ± 14 |
| D97E | 8.2 ± 0.2 | 8.6 ± 0.2 | 65 ± 19 | 8.1 ± 0.1 | 9.9 ± 0.1 | 123 ± 8 |

## DISCUSSION

An advantage in choosing the D97E variant of E-PPase to study the effects of conservative substitution of a potentially essential residue is its significant partial activity, which allows the effects of substitution to be measured on a variety of parameters that characterize enzymatic activity. In addition, we detect no major conformational changes arising from such substitution, thus increasing the likelihood that the changes we observe result from a direct effect of the substitution on the active site, rather than from some more generalized change in PPase structure. More detailed examination of this point must await the results of X-ray crystallography studies currently underway comparing the structures of both WT-PPase and D97E-PPase. A 2.7 Å structure of WT-PPase has just become available (Kankare *et al.*, 1994).

We have chosen to conduct most of our studies at a constant $[Mg^{2+}]$ of 20 mM. This concentration represents a saturating value for $k_{cat,hyd}$ for both WT-PPase and D97E-PPase, permitting an evaluation of the effects of D97E substitution on constants that depend on $k_{cat,hyd}$ ($k_3$, $pK_{ESH}$, $pK_{ESH_2}$), which are independent of effects on $Mg^{2+}$ affinity for any of its various sites. We assume that D97E substitution effects on $k_{cat,ex}$ and $k_4$ will be similarly independent of $Mg^{2+}$ affinity at this concentration.

The parameter values collected in Table 2 permit the construction of a diagram (Figure 7) illustrating the effects of D97E substitution on the energetics of PPase catalysis. From this figure it is clear that the largest effects of D97E substitution are in increasing the free energies of both the $EMg_4PP_i$ complex and the transition state between $EMg_4PP_i$ and $EMg_2(MgP_i)_2$ (labeled $EM4PP_i$*), with smaller increases seen for the $EMg_2(MgP_i)_2$ and $EMg_2MgP_i$ complexes and for $EMg_2$.

D97E substitution changes the mechanism of PPase catalysis of $PP_i$ hydrolysis, from one in which three steps, 3, 5, and 7, are all partially rate determining to one in which step 3, the only one involving bond cleavage and formation, is uniquely rate determining. While our results are not informative regarding the effects of D97E substitution on steps 5 and 7, they do clearly show two major effects on step 3, which is the most important step from a mechanistic point of view. The first is that the apparent $pK_a$ of the essential group for step 3 is raised, from a value of approximately 6.5, which can be estimated from the decline in $k_3$ for WT-PPase between pH 7.2 and pH 6.5 (Table 2), to 8.1, which is the value for the $pK_a$ of the essential basic group for $k_{cat}$ of D97E-PPase (Table 5; recall that, for D97E-PPase, $k_3$ is approximately equal to $k_{cat,hyd}$). The second is that the pH-independent value of $k_3$ is decreased by approximately 6.5-fold, from a value of 800 $s^{-1}$ for WT-PPase (Table 2) to a value of 123 $s^{-1}$ for D97E-PPase (Table 5).

Step 4 is almost exclusively rate determining for WT-PPase catalysis of $H_2O$•$P_i$ oxygen exchange and becomes exclusively so for D97E-PPase. Although the limited solubility of $MgP_i$ at alkaline pH did not permit us to determine a complete pH profile for $k_{cat,ex}$, the values of $k_4$ presented in Table 2 nevertheless clearly indicate that D97E substitution decreases the pH-independent value of $k_4$ by some 2 orders of magnitude, from a value of approximately 140 $s^{-1}$ for WT-PPase to a value that can be estimated as approximately 1 $s^{-1}$ for D97E-Ppase. The greater decrease in $k_4$ as compared with $k_3$ on D97E substitution results in a large increase in $K_3$ (Table 2).

In considering the effects of D97E substitution on $Mg^{2+}$ binding, we designate the two sites observed by equilibrium dialysis in the absence of substrate as sites A and B, corresponding to the higher and lower affinity sites, respectively, and the sites corresponding to the two $Mg^{2+}$ bound as the $Mg_2PP_i$ complex as sites C1 and C2. The values of $K_{m2}$ for WT-PPase determined both by equilibrium dialysis and by the $Mg^{2+}$ dependence of $K_{m,hyd}$ (see eq 5) agree reasonably well with one another (Table 4), consistent with the notion that $Mg^{2+}$ binding to site B, as measured by equilibrium dialysis, fulfills a catalytically essential role in Scheme 1. The value of $K_{m3}$ is only determined from kinetic studies, from the dependence of $k_{cat,hyd}$ on $Mg^{2+}$ (eq 5). The agreement between the data and the calculated lines in Figure 5 demonstrates that our results can be adequately fit with the assumption that $K_{m4}$ and $K_{m5}$ are equal to $K_{m3}$ (Scheme 1).

The value of $K_{m2}$ measured by equilibrium dialysis (1.86 mM) is about the same for D97E-PPase as for WT-PPase,

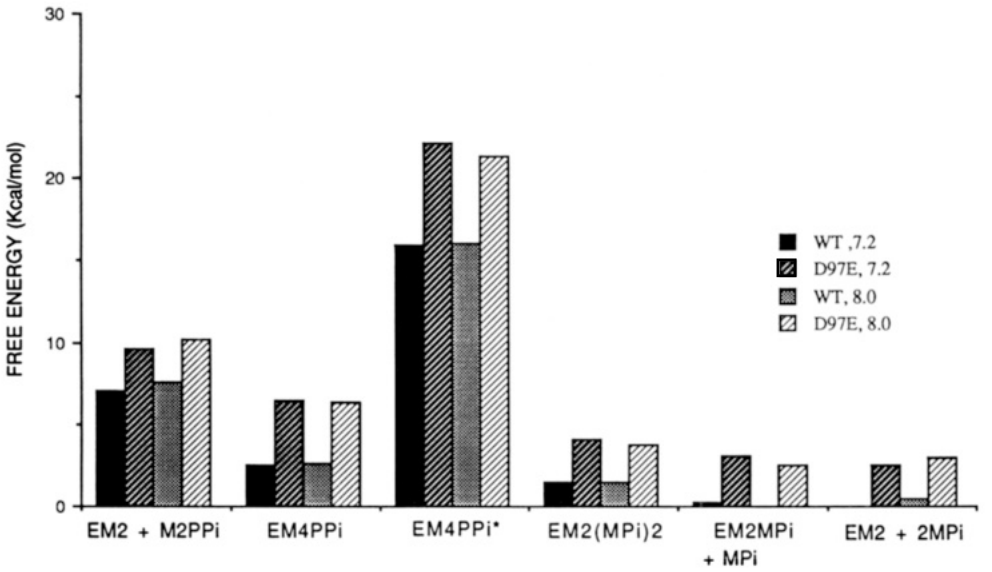



FIGURE 7: Free energy profile for catalysis of $PP_i$ to $2P_i$ equilibration by WT-PPase and by D97E-PPase at pH 7.2 and pH 8.0, at [$Mg^{2+}$] equal to 20 mM. Only one energy barrier is shown (marked with an asterisk) corresponding to the transition state for $PP_i$ hydrolysis on the enzyme. The concentrations of $Mg_2PP_i$ and $MgP_i$ were taken as 1 mM. The free energies of $EM_2 + 2MP_i$ (pH 7.2) and of $EM_2MP_i + MP_i$ (pH 8.0), both for WT-PPase, were set equal to 0. Free energies for all D97E-PPase species relative to WT-PPase species were calculated using $K_{m2}$ values reflecting the kinetically important $Mg^{2+}$ binding site, 1.0 mM for WT-PPase and 85 mM for D97E-PPase (Table 4).

but by contrast, the fitted value of $K_{m2}$ from kinetic studies for D97E-PPase is much higher (85 ± 35 mM). Any kinetic scheme that rationalizes these results must also take into account that $k_{cat,hyd}$ saturates as a function of $Mg^{2+}$ with an apparent $K_{m3}$ of 3.7 mM and does not decrease at higher $Mg^{2+}$ values. A plausible model and our current working hypothesis is that for WT-PPase the binding of $Mg^{2+}$ to sites A and B is catalytically essential, whereas for D97E-PPase, although such binding is retained, one or both of these sites has (have) been distorted such that $Mg^{2+}$ binding to it is no longer sufficient to confer catalytic activity. Rather, activity depends upon the binding of an additional (*i.e.*, fifth) $Mg^{2+}$ to the active site, which, as it is not part of the normal mechanism, binds with very low affinity.

Scheme 1 would thus be altered by binding $Mg_2PP_i$ and $MgP_i$ to $EMg_3$ rather than to $EMg_2$. A larger number of alternative kinetic schemes were considered which preserved a $Mg^{2+}$ stoichiometry of 4, including models in which $Mg^{2+}$ binding to PPase in the absence of $Mg_2PP_i$ was in steady state, rather than in equilibrium, and in which $Mg_2PP_i$ to enzyme preceded $Mg^{2+}$ binding. None was found that adequately fit the data presented in Figure 5.

In the X-ray crystallographic structure of the $Mn_3P_{i2}$•*S. cerevisiae* PPase complex (Chirgadze *et al.*, 1991), D147, aligning with D97 in E-PPase, does not bind directly to any of the three bound $Mn^{2+}$. It is, however, quite close to two aspartate residues, D115 and D152 (corresponding to residues D65 and D102 in E-PPase), that provide two ligands for one of the bound $Mn^{2+}$ sites and is considerably further away from those residues involved in the binding of the other two $Mn^{2+}$. In the recent structure of E-PPase carried out in the absence of divalent metal ion or phosphoryl ligand (Kankare *et al.*, 1994), residues D65 and D102 are in close proximity. Given the overall similarity in mechanism and active site structure between E-PPase and *S. cerevisiae* PPase (Cooperman *et al.*, 1992), we speculate that these residues form part of either site A or site B within E-PPase and that D97E substitution distorts the geometry of this site. D97E substitution also raises $K_{m3}$ 7-fold, suggesting that residue 97 may be involved in either or both of sites C1 and C2.

As discussed in the accompanying paper (Salminen *et al.*, 1995), a hydroxide ion, presumably stabilized by interaction of its three lone pairs of electrons with one or more $Mg^{2+}$ ions and possibly with active site Lys or Arg residues as well, appears to be the essential basic group modulating both productive $Mg_2PP_i$ binding and catalytic turnover of the $EMg_4PP_i$ complex, as determined from the pH dependencies of $k_{cat}/K_m$ and $k_{cat}$, respectively. D97E-PPase shares with all other active site variants so far examined, some 12 in all, the property that the p$K_a$s of the essential basic group are raised considerably as compared with the corresponding values for WT-PPase. For D97E-PPase, $k_3$ dominates $k_{cat}$ so that the increase in p$K_{ESH_2}$ on mutation (Table 5) reflects the rise in the p$K_a$ for this rate constant. $k_{cat}/K_m$ is dominated by $k_1$ for WT-PPase but is given by $K_1k_3/(K_3/k_2 + 1)$ for D97E-PPase. The rise in the values of both $K_1$ and $k_3$ with pH for this variant (Table 2) is thus likely to account for the observed rise in p$K_{EH_2}$ on mutation, although full confidence in this conclusion will require reliable estimates of $k_2$ as a function of pH.

Summarizing the results discussed above, the major effects of D97E substitution in E-PPase are to (a) markedly decrease the pH-independent rate constants for both $PP_i$ hydrolysis and, especially, $PP_i$ resynthesis on the enzyme, (b) selectively destabilize both the $EMg_4PP_i$ complex and the transition state between this complex and the $EMg_2(MgP_i)_2$ complex, (c) raise the p$K_a$ of a basic group (or groups) "essential" for $PP_i$ hydrolysis and for productive $PP_i$ binding by 1.5 and >2.2 log units, respectively, (d) distort site B such that $Mg^{2+}$ binding to it no longer confers enzymatic activity, and (e) decrease the affinity of one of the two $Mg^{2+}$ ions that binds to enzyme in the presence of substrate.

That this multiplicity of effects arises from a single Asp to Glu substitution suggests, in the absence of any evidence for a generalized structural change, a tightly integrated active site, in which the perturbation induced by conservative

substitution at a single location can have widespread functional effects. On the other hand, it is important to stress that many important properties of E-PPases are unaffected or little affected by D97E substitution, including its dissociation constants for sites A and B and for both $MgP_i$ complexes as well as the $pK_a$s of the essential acidic groups that modulate $k_3$ and $k_{cat}/K_m$. Thus, it may be possible to divide the active site into different segments, with strong functional linkage within segments and only weak functional linkage between segments. Detailed studies of the kind presented here on other PPase variants should permit critical evaluation of this notion.

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