Probing Essential Water in Yeast Pyrophosphatase by Directed Mutagenesis and Fluoride Inhibition Measurements*

Received for publication, August 14, 2000, and in revised form, October 6, 2000
Published, JBC Papers in Press, October 12, 2000, DOI 10.1074/jbc.M007360200

Pekka Pohjanjoki, Igor P. Fabrichiy, Vladimir N. Kasho, Barry S. Cooperman, Adrian Goldman, Alexander A. Baykov, and Reijo Lahti

From the Department of Biochemistry, University of Turku, FIN-20014 Turku, Finland, the Center of Ulcer Research and Education, Department of Medicine, University of California, Los Angeles, California 90073, the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the Institute of Biotechnology, University of Helsinki, PO Box 56, FIN-00014 Helsinki, Finland

The pattern of yeast pyrophosphatase (Y-PPase) inhibition by fluoride suggests that it replaces active site Mg$^{2+}$-bound nucleophilic water, for which two different locations were proposed previously. To localize the bound fluoride, we investigate here the effects of mutating Tyr$^{93}$ and five dicarboxylic amino acid residues forming two metal binding sites in Y-PPase on its inhibition by fluoride and its five catalytic functions (steady-state PP$\_i$ hydrolysis and synthesis, formation of enzyme-bound PP$\_i$, at equilibrium, phosphate-water oxygen exchange, and Mg$^{2+}$ binding). D117E substitution had the largest effect on fluoride binding and made the P-O bond cleavage step rate-limiting in the catalytic cycle, consistent with the mechanism in which the nucleophile is coordinated by two metal ions and Asp$^{117}$. The effects of the mutations on PP$\_i$ hydrolysis (as characterized by the catalytic constant and the net rate constant for P-O bond cleavage) were in general larger than on PP$\_i$ synthesis (as characterized by the net rate constant for PP$\_i$ release from active site). The effects of fluoride on the Y-PPase variants confirmed that PPase catalysis involves two enzyme-PP$\_i$ intermediates, which bind fluoride with greatly different rates (Baykov, A. A., Fabrichiy, I. P., Pohjanjoki, P., Zyryanov, A. B., and Lahti, R. (2000) Biochemistry 39, 11939–11947). A mechanism for the structural changes underlying the interconversion of the enzyme-PP$\_i$ intermediates is proposed.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) catalyzes reversible phosphoryl transfer from pyrophosphate (PP$\_i$) to water, a metabolically important reaction chemically similar to that catalyzed by numerous ATPases and GTPases. Yeast PPase is a homodimer containing 286 amino acid residues/monomer (1) and requiring three or four divalent metal ions for catalysis, with Mg$^{2+}$ conferring the highest activity (2–5). Two divalent metal ions (M1 and M2) per active site have been identified in the “resting” enzyme by x-ray crystallography and four metal ions (M1–M4) and two phosphates (P1 and P2) in the product complex of Y-PPase (6, 7).

PP$\_i$ hydrolysis by PPase occurs via direct attack of water without formation of a phosphorylated enzyme intermediate (8). Modeling the transition state of the chemical step from the structure of the enzyme-product complex Y-PPase$\cdot$Mn$\_i$(MnP)$_2$ has led to two models that differ in the identity of the water nucleophile placed between metal ions M1 and M2 in the model of Heikinheimo et al. (6) or in the vicinity of Tyr$^{93}$ in the model of Harutyunyan et al. (7, 9). Although the former model requires an additional “relaxation” step, in which a new water molecule displaces P$_i$ oxygen from the position between M1 and M2, it has an advantage of providing an efficient mechanism for nucleophile activation through combined action of the two metal ions and an adjacent Asp$^{117}$ residue (see Fig. 1).

In aqueous solution and even in crystalline state, proteins are surrounded with water shells, making identification of function-related water molecules a difficult task. Use of fluoride, a potent and most specific inhibitor of cytoplasmic pyrophosphatase, provides a convenient approach to detect such water molecules because molecules of HF and H$_2$O are isoelectronic and of similar size, as are the anions derived therefrom. Fluoride inhibition of yeast PPase during PP$\_i$ hydrolysis and synthesis involves a rapid and slow phases (10), which refer to F$^-$ binding to enzyme-PP$\_i$ and enzyme-PP$\_i$ intermediates, respectively (Scheme I). The rapid binding decelerates PP$\_i$ hydrolysis 10-fold at pH 7.2, whereas the slow binding arrests it completely. These characteristics of the inhibition are consistent with fluoride replacing an essential metal-bound water molecule/OH$^-$ ion, acting as nucleophile in the PP$\_i$ hydrolysis step (11).

In the present work, we employed fluoride inhibition in combination with site-directed mutagenesis to identify the fluoride binding site and, hence, the essential water molecule in the active site of PPase. Because fluoride inhibition of Y-PPase is closely associated with Mg$^{2+}$ binding (10, 12), the effects of mutating five amino acid residues forming the M1 and M2 sites (Glu$^{48}$, Asp$^{115}$, Asp$^{120}$, Asp$^{117}$, and Asp$^{152}$) on fluoride inhibition were studied. A Y93F variant was also included in this list, because in the PPase mechanism suggested by Harutyunyan et al. (9) a water molecule associated with Tyr$^{93}$ is assumed to be the nucleophile and, hence, might be replaced by F$^-$. X-ray crystallographic analysis of three relevant variants, D117E Y-PPase (13) and D65N and D70N Escherichia coli PPase (Asp$^{65}$ and Asp$^{70}$ in E. coli PPase correspond to Asp$^{115}$ and

---

*This work was supported by Academy of Finland Grants 35736 and 47513 and Russian Foundation for Basic Research Grants 00-04-48310 and 00-15-97907. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

††To whom correspondence may be addressed. Tel.: 95-939-5541; Fax: 95-939-3181; E-mail: baykov@genebee.msu.su.

§§To whom correspondence may be addressed. Tel.: 358-02-333-6845; Fax: 358-02-3336860; E-mail: reijo.lahti@utu.fi.

§The abbreviations used are: PPase, inorganic pyrophosphatase; E-PPase, E. coli PPase; PP$\_i$, pyrophosphate; WT, wild type; Y-PPase, yeast (Saccharomyces cerevisiae) PPase; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; TAPS, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl}amino]1-propanesulfonic acid.

This paper is available on line at http://www.jbc.org
Asp$^{120}$ in Y-PPase, respectively) (14), indicated no marked structural changes induced by the mutations.

**EXPERIMENTAL PROCEDURES**

The expression and purification of wild type Y-PPase and its active site variants from overproducing *E. coli* XL2blue$^{®}$ strain transformed with suitable plasmids were carried out as described by Heikinheimo et al. (15).

PP$_i$ hydrolysis was measured continuously with an automatic P$_i$ analyzer (16). The assay medium contained, except as noted, 0.32 mM (at pH 7.2) or 0.28 mM (at pH 8.5) total PP$_i$ (corresponding to 0.2 mM Mg$_2$PP$_i$ complex), 5.5 mM MgCl$_2$ (corresponding to 5 mM free Mg$^{2+}$), 0–20 mM F$^-$ (added as NaF) and buffer. The following pH buffers were used (0.1 M ionic strength, 50 mM K$_3$EGTA, 50 mM EGTA (pH 7.2), or 90 mM TAPS/KOH, 5 mM EGTA (pH 8.5)). Enzyme-bound PP$_i$ formation was assayed luminometrically with ATP-sulfurylase and luciferase (17, 18) using 60–260 μM Y-PPase concentration. PP$_i$ synthesis in solution was measured continuously by the same coupled enzyme assay (11). P$_i$,H$_2$O oxygen exchange was measured by gas chromatography/mass spectrometry (19). The assay medium used to measure fluoride effects on PP$_i$ase in the presence of F$^-$ contained 13.4 mM MgCl$_2$ (5 mM free Mg$^{2+}$), 20 mM total P$_i$, 55 mM TES/KOH buffer, 11 mM KCl, and 33 mM EGTA. Mg$^{2+}$ binding was assayed by equilibrium microdialysis in combination with atomic absorption spectroscopy to measure Mg content in the dialysis chambers (20). All experiments were performed at 25 °C.

Effects of fluoride on PP$_i$ase hydrolysis were analyzed in terms of Scheme II, a simplified version of Scheme I. $K_{d1}$ is the dissociation constant governing rapid fluoride binding in the presence of PP$_i$, $k_{i, app}$ and $k_1$ are the second-order and first-order rate constants for slow fluoride binding and release, respectively.

Equations 1 and 2 describe product (P) formation curves at a fixed fluoride concentration, where $a$ is the fraction of enzyme that has not yet undergone slow conversion into $K_{d1}$, and $v_0$,app is the initial velocity of product formation.

\[
\frac{da}{dt} = k_1(1-a) - k_{i, app}[F]a
\]  
(Eq. 1)

\[
\frac{d[P]}{dt} = v_{0, app}a
\]  
(Eq. 2)

**Scheme I. Interactions between Y-PPase and fluoride during catalysis.** F, F$^-$; M, Mg$^{2+}$; P, PP$_i$; PP$_i$ase (11).

**Scheme II. A minimal kinetic scheme of Y-PPase inactivation by fluoride during catalysis.**

Fitting [P] as a function of both time and [F] was accomplished by making the following substitutions ($v_0$ and $v_0'$ are the initial velocities of product formation observed at [F] equal to zero and infinity, respectively).

\[
v_0, app = v_0' + \frac{v_0 - v_0'}{1 + [F]/K_{i, app}}
\]  
(Eq. 3)

\[
h_{i, app} = \frac{k_i}{1 + [F]/K_{i, app}}
\]  
(Eq. 4)

Equations 1–4 were simultaneously fit, with the program SCIENTIST (MicroMath), to sets of product formation curves, each represented by 90–200 pairs of [P] and $t$ values. The calculated and measured curves agreed within 3%.

Values of the dissociation constants $K_{d1}$ and $K_{d2}$ for Mg$^{2+}$ binding to two sites on Y-PPase were estimated by fitting equilibrium dialysis data to Equation 5, where $n$ measures the number of Mg$^{2+}$ ions bound per monomer (21).

\[
n = \frac{1 + 2[Mg^{2+}]/K_{d2}}{1 + [Mg^{2+}]/K_{d1} + [Mg^{2+}]/K_{d2}}
\]  
(Eq. 5)

**RESULTS**

**Mg$^{2+}$ Binding.—** The effects of conservative mutations of Tyr$^{93}$ and five amino acid ligands to Mg$^{2+}$ (Fig. 1) on the binding of two activating metal ions in Y-PPase active site were studied by equilibrium dialysis at pH 7.2 (Fig. 2). Interestingly, every substitution suppressed Mg$^{2+}$ binding to the low affinity site, the lowest (1.8-fold) effect seen with Y93F, and the highest (147-fold) with E48D substitutions (Table I). Binding of Mg$^{2+}$ to the high affinity site was markedly suppressed in both Asp$^{120}$ variants (34- and 16-fold effects with D120E and D120N, respectively) and less markedly (4.0-fold effect) in the D120L variant. These data are consistent with M1 being the high affinity site and M2 being the low affinity site, as measured by equilibrium dialysis.

Importantly, all the variants tested still exhibited appreciable affinity for Mg$^{2+}$, such that both sites were almost saturated at 5 mM Mg$^{2+}$ concentrations used in the fluoride inhibition studies described below. The E48D variant, which exhibited the highest $K_{d2}$ value (5.0 mM), is expected to be 50% saturated at the M2 site in the absence of substrate, but keeping in mind that substrate strengthens metal ion binding to the corresponding E-PPase E20D variant (22) and WT-E-PPase (23), at least, 90% saturation is expected in the presence of 200 μM Mg$_2$PP$_i$ used in fluoride inhibition studies. This rules out a possibility that the effects of the mutations on fluoride inhibition reported below result from incomplete metal binding. Fluoride added at a 10 mM concentration suppressed binding of M1 and stimulated binding of M2 approximately 2-fold in WT-Y-PPase (Table I).

**Catalytic Properties.—** Values of the catalytic constant for PP$_i$ hydrolysis ($k_{i}$), rate of PP$_i$ synthesis ($v_0$), rate of P$_i$,H$_2$O oxygen exchange ($v_{o-ex}$), the distribution of five P$_i$ “isotopomers” during
but the values of $K_E$ (E48D, Y93F, D115E, and D117E) resembled WT-Y-PPase (11). All the variants could be classified into three classes: (i) group I (Y93F, D115E, and D117E variants at pH 7.2 and 8.5 (Table II). Such measurements were not repeated for the D120N variant, which displayed very low hydrolytic activity (<0.005 s⁻¹). These data allowed evaluation of two important parameters: $k_{epp} = v_{epp}(1 - 0.75P_i/K_{app}(1 - P_{app}/[E])$, and $k_{pp,off} = v_{pp,off}/[E]$, the net rate constant for $P_i$ release, i.e., back conversion of EM₄PP to EM₂(21). Equations 6 (11) and 7 (21) relate $k_3$ and $k_{pp,off}$ to the rate constants shown in Scheme I ($K_{AB} = k_3/k_2$). In fact, $k_3$ is the lower limit for $k_3$ (corresponding to $K_{AB} = ∞$), and $k_{pp,off}$ is the lower limit for both $k_2$ and $k_3$. For WT-PPase, $K_{AB}$ is equal to 25–65, and the values of $k_2$ and $k_3$ differ by less than 4% at pH 7.2 (11), but the values of $K_{AB}$ for the variant PPases are unknown. The advantage of using $k_3’$ and $k_{pp,off}$ instead of $v_{epp}/[E]$, and $v_{pp,off}/[E]$, is that the former parameters are independent of the degree of enzyme saturation with $P_i$ in corresponding measurements.

$$k_3’ = k_3/1 + 1/K_{AB}$$  \(\text{(Eq. 6)}\)

$$k_{pp,off} = 1/(k_{pp,off} + 1/k_2 + 1/k_3)$$  \(\text{(Eq. 7)}\)

**Fluoride Inhibition of PP, Hydrolysis**—According to the inhibition pattern, all the variants could be classified into three groups. Typical examples are shown in Fig. 3. Group I variants (E48D, Y93F, D115E, and D117E) resembled WT-Y-PPase (11) by exhibiting a virtually instant decrease in the initial slope of the P_i production curve, followed by a slower inactivation step. The group II variant (D120E at pH 7.2) exhibited only slow inactivation, without the instant decrease in the initial slope, and the group III variant (D152E) exhibited only instant inactivation. At pH 8.5, D120E-PPase also behaved like a group III member, whereas other variants did not change their behavior. It should be noted that the $P_i$ productions curves look very similar for group I and group II variants (Fig. 3), and they could be only distinguished based on the fitting results shown below; the best-fit value of $K_{p,i}$ tended to approach infinity for D120E-PPase, clearly indicating no change in $v_{pp,off}$ in the presence of fluoride. Values for the rate constants $k_i$ and $k_{pp,off}$ describing the slow phase of the inhibition and for the equilibrium constant $K_{p,i}$ describing the fast step of the inhibition (Scheme II) were obtained by simultaneous fitting of Equations 1–4 to series of product formation curves measured at different fluoride concentrations. The most drastic effects were observed on $k_i$ with the D117E and D152E variants (the latter exhibited no slow inactivation step at any pH value), on $k_{pp,off}$ with the D115E and D152E variants, and on $K_{p,i}$ with the D117E variant (Table III).

**Fluoride Effect on Enzyme-bound PP_i Formation**—The Y-PPase active site is preformed to bind $P_i$. Although the concentration of $P_i$ present at equilibrium with 20 mM $P_i$ and 5 mM Mg²⁺ at pH 7.2 is <1 μM, i.e., the ratio [P_i]/[PP_i] > 20,000 (5, 24, 25), the fraction of enzyme containing bound $P_i$ ($f_{pp} = ([EMPP]/[EMPP])/[E_i]$ in these conditions is as high as 16% (Table II), in agreement with previous estimates (5, 26, 27). This remarkable feature of the active site was completely retained in D117E and D120E variants, partially retained in the D48E, Y93F, and D115E variants and almost completely lost in the D152E variant (Table II and Fig. 4). As shown in Fig. 4, addition of 2 mM fluoride to E48D, Y93F, and D115E variants at pH 7.2 caused a rapid decrease in enzyme-bound PP_i, not resolved in time (F⁻ binding to enzyme-P_i complex in Scheme I, followed by a slow increase to a higher level (F⁻ binding to enzyme-PP_i complex) (Fig. 4), like in wild type Y-PPase (11). The time course of the slow increase could be described by Equations 8 and 9 (11), where $f_{pp}^{slow}$ refers to the amount of enzyme-bound PP_i after completion of the fast phase of the interaction with F⁻ (i.e., the zero time point for the slow phase), and $f_{pp}^{slow}$ is the amount of the enzyme-bound PP_i accumulated at time $t$ during the slow phase. Fluoride binding was assumed to be a second-order reaction (see also below).

$$f_{pp} = f_{pp}^{slow} + f_{pp}^{slow}(100 - f_{pp}^{slow})/100$$  \(\text{(Eq. 8)}\)

$$df_{pp}^{slow}/dt = k_{pp,off} [P_i] (100 - f_{pp}^{slow}) - k_f f_{pp}^{slow}$$  \(\text{(Eq. 9)}\)

For the D117E and D120E variants, the initial decrease in $f_{pp}$ could be resolved in time. Moreover, no slow increase in $f_{pp}$ followed after its rapid drop in the D120E variant. Values of $k_{pp,off}$, $k_f$, and $f_{pp}^{slow}$ for the D117E variant were obtained with Equations 8 and 9 using the points collected at $t = 2$ min on the curve shown in Fig. 4. The curve for the D120E variant and the initial part of the curve for the D117E variant (≤2 min) could be described by Equations 10 and 11 for a reversible binding, where $r$ is the percentage of free enzymes-P_i complex containing bound fluoride and the primed rate constants refer to F⁻ binding to and release from enzyme-P_i complex. As the enzyme-P_i complex binds two fluoride ions (see below), Equations 11 contains the second power of fluoride concentration. The effect of F⁻ on PP_i bound to D152E-PPase was quite small, if there was any.

$$f_{pp} = f_{pp}^{slow}d(100 - r)/100$$  \(\text{(Eq. 10)}\)

$$df_{pp}^{slow}/dt = k_{pp,off} [P_i] (100 - r) - k_f r$$  \(\text{(Eq. 11)}\)

The ratio of $k_{pp,off}/k_f$ the true rate constant for F⁻ binding to enzyme-P_i complex ($k_{pp,off}$ refers to total enzyme), is de-
TABLE II
Catalytic properties of Y-PPase variants

| Enzyme variant | pH | $k_h$  | $v_0/E_0$  | $v_0$/$E_0$  | $P_e$  | $f_{app}$ | $k_{pp,cat}$ | $k_3^*$ |
|----------------|----|--------|-------------|--------------|--------|-----------|-------------|--------|
| WT            | 7.2 | 214 ± 5 | 6.0 ± 0.5  | 170 ± 6  | 0.30 ± 0.01 | 16 ± 2    | 38 ± 9 | 1200 ± 200 |
| E48D          | 7.2 | 66 ± 1  | 1.7 ± 0.2  | 7.9 ± 0.2 | 0.025 ± 0.002 | 8 ± 1     | 21 ± 5 | 90 ± 10  |
| Y93F          | 7.2 | 6.0 ± 0.1 | 0.24 ± 0.01 | 0.36 ± 0.01  | 0.097 ± 0.003 | 3.7 ± 0.6 | 6.5 ± 1.4 | 10 ± 2 |
| D115E         | 7.2 | 27 ± 1  | 0.81 ± 0.07 | 2.9 ± 0.1 | 0.11 ± 0.01 | 5.7 ± 1.5 | 14 ± 5 | 50 ± 20 |
| D117E         | 7.2 | 1.28 ± 0.05 | 0.36 ± 0.04  | 0.294 ± 0.005 | 0.052 ± 0.005 | 19 ± 1 | 1.9 ± 0.3 | 1.9 ± 0.1 |
| D120E         | 7.2 | 0.132 ± 0.004 | 0.018 ± 0.001 | 0.085 ± 0.001 | 0.276 ± 0.002 | 15 ± 1 | 0.12 ± 0.02 | 0.63 ± 0.02 |
| D120N         | 7.2 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| D152E         | 7.2 | 0.103 ± 0.05 | 0.0019 ± 0.0001 | 0.012 ± 0.001 | 0.25 ± 0.02 | 0.7 ± 0.2 | 0.27 ± 0.07 | 2.2 ± 0.6 |
| WT            | 8.5 | 108 ± 4 | 1.4 ± 0.1  | 54 ± 0.3 | 0.44 ± 0.01 | 124 ± 0.8 | 11 ± 2 | 520 ± 50 |
| E48D          | 8.5 | 123 ± 8 | 3.6 ± 0.7  | 15.6 ± 0.4 | 0.09 ± 0.01 | 7.9 ± 0.7 | 46 ± 13 | 200 ± 30 |
| Y93E          | 8.5 | 14.2 ± 0.2 | 0.25 ± 0.05  | 0.74 ± 0.05 | 0.105 ± 0.01 | 1.8 ± 0.2 | 19 ± 5 | 41 ± 7 |
| D115E         | 8.5 | 13.5 ± 0.1 | 0.41 ± 0.05  | 1.17 ± 0.01 | 0.14 ± 0.01 | 2.0 ± 0.2 | 21 ± 4 | 62 ± 5 |
| D117E         | 8.5 | 9.6 ± 0.1 | 0.45 ± 0.04 | 0.57 ± 0.10 | 0.035 ± 0.005 | 7.3 ± 0.8 | 6 ± 1  | 8 ± 2 |
| D120E         | 8.5 | 0.32 ± 0.01 | 0.028 ± 0.001 | 0.088 ± 0.001 | 0.114 ± 0.003 | 12 ± 1 | 0.22 ± 0.03 | 0.73 ± 0.08 |
| D152E         | 8.5 | 0.85 ± 0.04 | 0.010 ± 0.001 | 0.014 ± 0.002 | 0.088 ± 0.006 | 0.5 ± 0.1 | 2.0 ± 0.6 | 2.9 ± 1.0 |

* From Baykov et al. (11).

FIG. 3. The effects of NaF on the progress curves for PP1 hydrolysis catalyzed by four variant Y-PPases at pH 7.2. F– concentrations (in mmol/liter) are indicated at the curves. Curves were normalized to 0.008 μM (E48D), 0.27 μM (D117E), 2.9 μM (D120E), or 3.5 μM (D152E) enzyme concentration.

TABLE III
Parameters for fluoride inhibition of PP1 hydrolysis

| Enzyme variant | pH | $K_{f}^*$ | $K_{FF}$ | $K_3^*$ | $k_{cat}/k_{cat}$ |
|----------------|----|-----------|---------|---------|-------------------|
| WT            | 7.2 | 2.5 ± 0.3 | 0.02    | 0.008   | 2.6 ± 0.2 | 0.05 ± 0.01 |
| E48D          | 7.2 | 2.5 ± 0.2 | 0.07 ± 0.01 | 0.025 ± 0.005 | 3.0 ± 0.1 | 1 ± 0.02 |
| Y93E          | 7.2 | 0.83 ± 0.04 | 0.03 | 0.036 | 5.3 ± 0.3 | 0.02 |
| D115E         | 7.2 | 5.0 ± 0.4 | 0.34 ± 0.04 | 0.07 ± 0.01 | 0.74 ± 0.03 | 0.02 |
| D117E         | 7.2 | 0.054 ± 0.003 | 0.01 | 0.2 | 50 ± 0.10 | 0.1 |
| D120E         | 7.2 | 0.06 ± 0.02 | 0.01 | 0.2 | 35 ± 0.10 | 0.1 |
| D152E         | 7.2 | 2.8 ± 0.1 | 0.19 ± 0.04 | 0.07 ± 0.01 | 0.18 ± 0.02 |

$K_{f}^* = k_h / k_r$.

$K_3^*$ Measured with 50 μM Mg2PPi.

This variant exhibited no slow inactivation step.

Increased only in the D117E variant by comparison with wild type Y-PPase (Table IV).

Inactivation by Fluoride in the Presence of $P_1$—Upon the incubation with $P_1$ and F– at pH 7.2, the variant PPases were diluted 900–4500-fold into assay medium, and the initial velocity of PP1 hydrolysis was estimated from the slope of the $P_1$ production curve 1 min after the dilution (the dead-time of the $P_1$ analyzer increased by comparison with Fig. 3 because of the $P_1$ present in the enzyme solution). The extensive dilution and the 1-min delay were expected to completely reverse the fast
fluoride binding step seen in Fig. 4 for the E48D, Y93E, and D115E variants and to partially reverse the slower initial binding step observed with the D120E and D117E variants. In agreement with the data reported above, the preincubation with fluoride did not affect the activity of the D152E variant, slightly inactivated the D115E and D120E variants and more deeply inactivated the E48D, Y93E, and D117E variants (Figs. 5 and 6). Low degree of the inactivation seen with the D115E variant is consistent with its high $k_h$ value (Table IV). For all the variants, the degree of the inactivation observed after 80 min was smaller than for wild type Y-PPase (11), consistent with lower $k_{i,app}$ or higher $k_h$ value (Table IV). The values of these parameters estimated with Equation 1 for the two variants exhibiting $>30\%$ inactivation in Figs. 5 and 6 (Y93F and D117E) were similar to those derived from Fig. 4 (Table IV). For the D117E variant, only points with $t > 5$ min were used in this estimation.

Increasing the $F^-$ concentration decreased D117E-PPase inactivation (Fig. 6), a phenomenon previously observed with wild type Y-PPase and explained by binding of two $F^-$ ions to the enzyme-P$_i$ complex (11). Such binding decreases the concentration of the enzyme-P$_i$ complex (the true binding species) proportionally to $[F]^2$, and this is only partly compensated by an increase in the fluoride binding rate, which is proportional to $[F]$. $k_h$.

**Fluoride Inhibition of D117E-PPase during PP$_i$ Synthesis**—Studies of PP$_i$ synthesis provided further evidence for the ability of D117E-PPase to bind two $F^-$ ions in the presence of P$_i$ (Fig. 7). The effect of $[F]$ on the initial velocity of PP$_i$ synthesis was only poorly described by Equation 3 with $v'_0 = 0$, implying binding of only one $F^-$ ion but was well described by Equation 12, implying binding of two $F^-$ ions (Fig. 7, right panel).

$$v_{i,app} = \frac{v_0}{1 + [F]^2/K_F} \quad (\text{Eq. 12})$$

The nonlinearity of the luminescence versus time curves (Fig. 7, left panel) was similar at different $[F]$ and could be fully explained by fluoride-independent inactivation of luciferase (11). This means that the slow fluoride binding step is not evident over the 1–4.5 min time interval in the presence of $P_i$, consistent with the data in Fig. 4, showing no significant increase in $v_{i,pp}$ during this time interval.

**DISCUSSION**

**Roles for the Six Residues in Metal Binding and Catalysis**—There is a substantial increase in $K_{M2}$ with five of the six variants under study (Table I), indicating the M2 site to be highly sensitive to changes in the delicate network of interactions in the active site of Y-PPase (Fig. 1). The most drastic effects on $K_{M2}$ are seen with the E48D and D120E variants, which is consistent with the x-ray crystallographic data showing Glu$^{48}$ and Asp$^{120}$ to be most closely associated with M2 (Fig. 1). The M1 site is clearly much more resistant to amino acid substitutions than the M2 site; a significant effect is only seen with the D120E(N), D152E, and D117E variants. This is compatible with changes seen between the EM$_0$ and EM$_0$(MP)$_0$ complexes (6). In these structures, the M2 site showed itself to be more rigidly fixed than the M1 site, so that the loops carrying Asp$^{115}$ and Asp$^{152}$ swing in toward a rigid M2 binding site in the product complex structure. Consequently, variation at the M1 ligands Asp$^{115}$ and Asp$^{152}$ is more readily tolerated than variation at the M2 ligands Asp$^{120}$ and Glu$^{48}$. However, it is unclear why D115E and D152E substitutions have much larger effect on M2 than on M1 binding (Table I). One possible explanation is that the substitutions effectively misposition M1 and Asp$^{120}$ and so prevent the rigid M2 binding site from forming properly. Consistent with this notion is the fact that the changes seen at M2 for the D115E and D152E substitutions, although large, are smaller than that seen for directly mutating Asp$^{120}$, as if the effects of the D115E and D152E substitutions were to misalign Asp$^{120}$.

In general, the effects of the mutations on PP$_i$ hydrolysis (as characterized by $k_h$) and $k_{i,app}$ are larger than the effects on PP$_i$ synthesis (as characterized by $k_{pp,off}$) (Table II). All the mutations, with the only exception of E48D at pH 8.5, decrease $k_h$, whereas $k_{pp,off}$ is increased in three variants (E48D, Y93F, and D115E) at pH 8.5. The latter effect appears to result from increased $k_{pp}$ (see Equation 7), because the $k_h$ step is rate-limiting in PP$_i$ synthesis by wild type PPase (11). A similar stimulating effect of an E20D substitution on PP$_i$ synthesis was earlier observed with E. coli PPase (Glu$^{20}$ in E. coli PPase corresponds to Glu$^{48}$ in Y-PPase) (22).

Combining Equation 6 with the expression for $k_h$ (11), one obtains

$$\frac{1}{k_h} = \frac{1}{k_A} + \frac{1}{k_{1}(1-P_{1})} + \frac{1}{k_{3}} + \frac{1}{k_{7}} \quad (\text{Eq. 13})$$

At pH 7.2, $k_A$ (840–2200 s$^{-1}$), $k_{3}'$ (1200 s$^{-1}$), $k_{5}$ (800 s$^{-1}$), and $k_{7}$ (960–3300 s$^{-1}$) are nearly equal for wild type Y-PPase (11), and $(1 - P_{1})$ does not differ much from unity (Table II). Therefore, a mutation decreasing only $k_{3}'$ by a factor of $n$ would decrease $k_h$ by a factor approaching $(n + 3)/4$. This criterion is met for most mutations, except for D120E and D152E, at pH 7.2 (Table II). For the latter mutations, the effect on $k_h$ is nearly equal to (D120E) or exceeds (D152E) the effect on $k_{3}'$, which can only result if, at least, one of $k_A$, $k_{3}$, and $k_{7}$ is also decreased. The anomalous behavior of the D152E variant is preserved at pH 8.5. Moreover, despite a decrease in $k_{3}'$, $k_h$ is slightly increased in the E48D variant at pH 8.5, suggesting an increase in $k_A$, $k_{3}$, and/or $k_{7}$. Thus, both PP$_i$ hydrolysis ($k_{3}'$) and P$_i$ release are decelerated in the D152E variant. PP$_i$ hydrolysis is decelerated, and P$_i$ release is accelerated in the E48D variant, and only PP$_i$ hydrolysis is affected in the other four variants. According to Equation 6, the effects on $k_{3}'$ may result from a decrease in $k_{3}$ or $K_{M2}$. At present, we cannot distinguish between these alternatives. The invariance of $k_h$ in the Y93F, D115E, and D117E variants would mean that the decrease in
with 2 mM FEM4PP predominate during hydrolysis and are at equilibrium also larger at pH 7.2 (Table III), where $k_d$ is the largest with the D117E variant (6–13-fold) but, never-
the less, is nearly unchanged (28). The shift in the presence of 20 mM P$_i$ and 5 mM Mg$^{2+}$ at pH 7.2. The incubation was carried out in a total volume of 70 μl at 6–850 μm enzyme concentration. Aliquots of 2–12 μl were withdrawn at indi-
cated time intervals and immediately added to the activity assay con-
taining 1 mM P$_i$, 0.2 mM MgCl$_2$, 10 μM EGTA, and 0.05 mM Tris-HCl, pH 8.5. The line for the Y93F variant was obtained with Equation 1 using parameter values found in Table IV, the line for wild type Y-PPase is from Baykov et al. (11), and the other lines were drawn by eye.

Their $P_e$ values (Table II) results from a drop in $k_r$. This effect is the largest with the D117E variant (6–13-fold) but, never-
theless, is much less than the effect on $k_{\text{app}}$

The fluoride inhibition data support the notion that PP$_i$ conversion to P$_i$ becomes rate-limiting in some variants. For D117E-PPase, values of $k_{\text{app}}$, $f_{\text{app}}$, and $k_{i\text{app/f}}$ (Table IV) are the same. This is possible if EM$_4$PP and EM$_2$PP predominate during hydrolysis and are at equilibrium with each other, which is equivalent to $k_{\text{app}}$ being the rate-
determining step in hydrolysis. This is consistent with high $K_{\text{pi}}$ value for this variant (Table III), indicating that the fraction of EM$_4$P is quite low during hydrolysis. That EM$_3$P, when present, binds fluoridewell is indicated by its large effect on PP$_i$ synthesis by D115E-PPase (Fig. 7) by comparison with wild type PPase (11). The effect of the Y93F substitution on $K_{\text{pi}}$ is also larger at pH 7.2 (Table III), where $k_{\text{app}}$ is nearly rate-
determining (Table II), than at pH 8.5, where $k_{\text{app}}$ is not such.

Interestingly, changes in pH had an opposite effect on $k_{\text{app}}$ for all the variants, except for D115E, by comparison with wild

type Y-PPase (Table II). This appears to result from a substan-
tial alkaline shift in the bell-shaped pH profile for $k_{\text{app}}$ (28). As a result, the pH 7.2 and 8.5 points are on the descending limb of the pH rate profile for wild type Y-PPase and on its ascending limb for most variants. By contrast, the D115E substitution

leaves the pH profile for $k_{\text{app}}$ nearly unchanged (28). The shift may be explained by a change in the $pK_a$ of the nucleophilic water (28, 29) or by inhibition of the three-metal pathway in

### Table IV

| Enzyme variant | $k_{\text{app}}$ | $k_r$ | $f_{\text{app}}$ | $k_{i\text{app/f}}$ | $k_{i\text{app}}$ | $k_r$ |
|---------------|-----------------|------|---------------|------------------|-----------------|------|
| WT           | 0.0137 ± 0.0005 | <0.02 | 4.8 ± 0.3    | 0.29             | 0.027 ± 0.002   | 0.016 ± 0.003 |
| E48D         | 0.006 ± 0.002   | 0.08 ± 0.03 | 2.0 ± 0.6    | 0.31             | 0.013 ± 0.004   | 0.025 ± 0.008 |
| Y93F         | 0.006 ± 0.001   | 0.026 ± 0.006 | <0.5        | >1               | 0.0031 ± 0.0002 | <0.001 |
| D115E        | 0.032 ± 0.007   | 0.40 ± 0.09   | 2.0 ± 0.5    | 1.6              |                  |      |
| D117E        | 0.0003 ± 0.0001 | <0.002       | 7.8 ± 1      | 0.05             | 0.0031 ± 0.0002 | <0.001 |
| D120E        | 0.6 ± 0.1       | 1.1 ± 0.2     | 3.4 ± 0.5    |                  |                  |      |
| D152E        | 0.7 ± 0.1       | 0.81 ± 0.08   | 3.4 ± 0.5    |                  |                  |      |

*Parameters obtained with Equations 8 and 9 from the fast step of the pH rate profile for wild type Y-PPase and on its ascending

### FIG. 5

Inactivation of Y-PPase variants during incubation with 2 mM F$^-$ in the presence of 20 mM P$_i$ and 5 mM Mg$^{2+}$ at pH 7.2. The line for wild type Y-PPase is from Baykov et al. (11), and the other lines were drawn by eye.

### FIG. 6

Inactivation of D117E-PPase during incubation with F$^-$ in the presence of 20 mM P$_i$ and 5 mM Mg$^{2+}$ at pH 7.2. F$^-$ concentrations in mmol/liter are shown at the curves. Other conditions were as for Fig. 5, except that the activity assay was started by adding PP$_i$ 1 min after enzyme was added; this 1-min delay was introduced to completely reverse the first, faster step of F$^-$ binding. The lines are the best fits of Equation 1.

### FIG. 7

The effects of NaF on PP$_i$ synthesis catalyzed by D117E-

PPase. Left panel, progress curves at indicated F$^-$ concentrations (in mmol/liter). Curves were normalized to 1.2 mmol/liter. Right panel, dependence of $v_\text{app}$ on NaF concentration. The dotted and solid lines show the best fits of Equations 3 (with $v_\text{app} = 0$) and 12, respectively.
the variant PPases (21).

**Fluoride Binding Site**—The slow inhibition step results in the incorporation of one fluoride ion per substrate molecule, which remains intact and tightly bound to PPase (30), suggesting that fluoride replaces the nucleophilic water. Substitutions mispositioning its ligands are thus expected to affect fluoride binding. Computer modelling of the enzyme-PP complex from the X-ray structure of Y-PPase and E-PPase suggested that the nucleophile is either Wat1, located between metal ions M1 and M2 and further liganded by Asp (Fig. 1 and Ref. 6) or the water molecule associated with M2 and Tyr (7). The results presented in Tables III and IV clearly favor Wat1 as the nucleophile, because they show that the D117E substitution does have the most drastic effect on F binding, as characterized by \( k_{f} \), \( K_{F} \), \( K_{P} \) and \( k_{i, app} / f_{app}^{fast} \). Furthermore, a strong effect of the D120E substitution on F binding is consistent with Asp being important for exact placing M1 and M2 and, hence, Wat1 (Fig. 1). The substitutions of Asp and Asp, which are in the first coordination sphere of M1, and of Glu, which is in the second coordination sphere of M2, apparently have smaller effects on Wat1 position, which correlates with much greater activity of the D115E, D152E and D48E variants by comparison with the D120E or D120N variants (Table II; see also Ref. 28). The Y93F substitution has only a moderate effect on fluoride inhibition, despite a more drastic nature of this substitution (the hydroxy group interacting with water is eliminated) by comparison with the D117E substitution (the side chain is elongated). For the same reason, similar effects of the two substitutions on different catalytic properties in Y-PPase (Table II; see also Ref. 28) and of the corresponding substitutions in E-PPase (29, 31) also point to a greater importance of Asp by comparison with Tyr.

Several other lines of evidence support the identification of Wat1 as the water molecule substituted by fluoride. First, Wat1 is replaced with a Glu carbonyl oxygen in D117E-PPase (13), showing the largest decrease in fluoride binding. Second, as measured by differential UV spectroscopy, fluoride binding to Y-PPase in the absence of substrates requires metal ions M1 and M2 (10), the ligands to Wat1. Third, F binding becomes entrapped together with two Mg ions (11, 12) in the stable enzyme-F-PP complex (12). Fourth, F- inhibition of E-PPase, in which the carbonylate of Asp (equivalent of Asp in Y-PPase) is moved away from M1 and M2 by about 1 Å by comparison with Y-PPase (7), exhibits only a fast step. Moreover, a D67N substitution results in appearance of the slow inhibition step in E-PPase (31), which is similar in this respect to Y-PPase. In a series of D/N substitutions in the active site of E-PPase, the D67N substitution had the largest effect on fluoride binding (31). Fifth, other dimetal hydrolases, including enolase (32, 33), aminopeptidase (34), purple acid phosphatase (35), and urease (36), presumably generating a hydroxide ion in the dimetal active site, are similarly inhibited by fluoride.

**Mechanism of Fluoride Inhibition and Catalysis**—According to Scheme I, derived from studies of wild type Y-PPase (11), fluoride binds with different rates and stoichiometry to two enzyme intermediates. Effects of fluoride on PPase variants provide further support to Scheme I. Thus, five of the six variants exhibit a biphasic transition in the amount of enzyme-bound PP, upon addition of fluoride (Fig. 4; compare values for \( f_{app} \) and \( f_{app}^{fast} \) in Tables II and IV), the rapid drop and slow rise corresponding to fluoride binding to EM-P and EM-PP, respectively. These data are inconsistent with a consecutive binding model, in which rapid preassociation of a single enzyme form (EM-PP* in our case) with the inhibitor is followed by a slow conformational change, because such a model predicts an increase in the amount of enzyme-bound PP during both rapid and slow phases. Besides, the involvement of two different intermediates is supported by the observation that the D120E (at pH 8.5) and D152E substitutions cancel the slow inhibition phase without significantly affecting the fast phase. These substitutions appear to greatly reduce the steady-state concentration of EM-P, which binds fluoride rapidly. The ability of EM-P to bind two F ions, another important feature of Scheme I, is confirmed by the inactivation patterns seen in Figs. 6 and 7 for the D117E variant.

The fluoride binding data obtained for the E48D and D115E variants confirm the occurrence of two PP-containing intermediates (EM-P* and EM-PP in Scheme I) in PPase catalysis. For both variants, \( k_{i} \) values (Table III) are greater than \( k_{i, app} / f_{app}^{fast} \) values (Table IV), despite the fact that the former parameter refers to the total enzyme, of which only a fraction contains bound PP, during steady-state hydrolysis, and therefore underestimates the rate constant for fluoride binding. Importantly, the \( k_{i, app} / f_{app}^{fast} \) values are true rate constants for fluoride binding with the sum of EM-P* and EM-PP. Like with wild type Y-PPase, this difference can be only explained by existence of two PP-containing intermediates that react with fluoride at substantially different rates (11). Independent evidence for two PP-containing intermediates was obtained in presteady-state measurements of PP, hydrolysis.²

² P. Halonen et al., manuscript in preparation.

³ P. Heikinheimo, V. Tuominen, A.-K. Ahonen, A. Tepljakov, B. S. Cooperman, A. A. Baykov, R. Lahti, and A. Goldman, submitted for publication.
constant of $K'_p$, to a form having a fluoride ion between M1 and M2. However, the two mechanisms are kinetically equivalent, provided that the bottom left species in Scheme III is stoichiometrically insignificant, i.e., $K'_p \gg [F]$. In terms of Scheme III, $k$ is equal to $k'_p/K'_p$. Given the chemistry of fluoride, the M1-M2 complex is likely to be very stable, making the reverse reaction, which requires insertion of a water molecule, very slow. For this reason, it is not shown in Scheme III. On the other hand, the bound PP cannot hydrolyze and leave as P.

This explains why fluoride stabilizes the enzyme-substrate intermediate to an extent allowing its isolation by gel filtration (30). At neutral pH, the bridging water molecule exists predominantly as OH⁻ in EM₃PP and, presumably, EM₄P₂ (21), which two water molecules are expected to be present between M1 and M2 because fluoride binding to this species is relatively weak and proceeds in a rapidly reversible manner with a stoichiometry of two per active site. The unsurpassed stability of the F-H-F hydrogen bond (38) would favor the M1-F-H-F-M2 structure, despite the low $pK_a$ value (3.2) for hydrofluoric acid.

REFERENCES

1. Kolakowski, L. F. Jr., Schloesser, M., and Cooperman, B. S. (1988) Nucleic Acids Res. 16, 10441–10452
2. Kunitz, M. (1952) J. Gen. Physiol. 35, 423–450
3. Baykov, A. A., and Avava, S. M. (1973) Eur. J. Biochem. 32, 136–142
4. Welsh, K. M., Jacobiansky, A., Springs, B., and Cooperman, B. S. (1983) Biochemistry 22, 2243–2248
5. Baykov, A. A., and Shestakov, A. S. (1992) Eur. J. Biochem. 206, 463–470
6. Heinkinheimo, P., Lehtonen, J., Baykov, A., Lahti, R., Cooperman, B., and Goldman, A. (1996) Structure 4, 1491–1508
7. Harutyunyan, E. H., Kur'anova, I. P., Vainshtein, B. K., Hohne, W. E., Lamin, V. S., Dauter, Z., Teplyakov, A. V., and Wilson, K. S. (1996) Eur. J. Biochem. 239, 220–228
8. Gonzalez, M. A., Webb, M. R., Welsh, K. M., and Cooperman, B. S. (1984) Biochemistry 23, 787–801
9. Harutyunyan, E. H., Oganesian, V. Yu., Oganesyan, N. N., Avava, S. M., Nazarova, T. I., Vorobyeva, N. N., Kurilo, S. A., Huber, R., and Mather, T. (1997) Biochemistry 36, 7754–7760
10. Smirnova, I. N., and Baykov, A. A. (1983) Biokhimija 48, 1643–1653
11. Baykov, A. A., Fabrichnyi, I. P., Pohjankoski, P., Zyryanov, A. B., and Lahti, R. (2000) Biochemistry 39, 11939–11947
12. Baykov, A. A., Tam-Villoloso, J. J., and Avava, S. M. (1979) Biochim. Biophys. Acta 509, 228–238
13. Tsoumas, V., Heinkinheimo, P., Kajander, T., Torkkel, T., Hitittia, T., Kapyla, J., Lahti, R., Cooperman, B. S., and Goldman, A. (1998) J. Mol. Biol. 284, 1565–1580
14. Avava, S. M., Rodina, E. V., Vorobyeva, N. N., Kurilo, S. A., Nazarova, T. I., Sklyankina, V. A., Oganesyan, V. Yu., Samygina, V. R., and Harutyunyan, E. G. (1998) Biochemistry (Moscow) 63, 671–684
15. Heinkinheimo, P., Pohjankoski, P., Helimen, A., Tasanen, M., Cooperman, B. S., Goldman, A., Baykov, A., and Lahti, R. (1996) Eur. J. Biochem. 238, 138–143
16. Baykov, A. A., and Avava, S. M. (1981) Anal. Biochem. 116, 1–4
17. Nyren, P., and Lundin, A. (1985) Anal. Biochem. 151, 504–509
18. Fabrichnyi, I. P., Kasho, V. N., Hitittia, T., Salminen, T., Halonen, P., Dudarenkov, V. Yu., Heinkinheimo, P., Chernyak, V. Ya., Goldman, A., Lahti, R., Cooperman, B. S., and Baykov, A. A. (1997) Biochemistry 36, 7746–7750
19. Hackney, D. D. (1980) J. Biol. Chem. 255, 5320–5328
20. Kapyla, J., Hytti, T., Lahti, R., Goldman, A., Baykov, A. A., and Cooperman, B. S. (1995) Biochemistry 34, 792–800
21. Belogurov, G. V., Fabrichnyi, I. P., Pohjankoski, P., Kasho, V. N., Lehtihuta, E., Turkina, M. V., Cooperman, B. S., Goldman, A., Baykov, A. A., and Lahti, R. (2000) Biochemistry 39, 13931–13938
22. Volk, S. E., Dudarenkov, V. Yu., Kapyla, J., Kasho, V., Valoshina, O. A., Salminen, T., Goldman, A., Lahti, R., Baykov, A. A., and Cooperman, B. S. (1996) Biochemistry 35, 4662–4669
23. Baykov, A. A., Hitittia, T., Volk, S. E., Kasho, V. N., Vener, A. V., Goldman, A., Lahti, R., and Cooperman, B. S. (1996) Biochemistry 35, 4655–4661
24. Day, L. A., Renosto, P., and Segel, I. H. (1986) Anal. Chem. 58, 305–305
25. de Meis, L. (1989) Biochim. Biophys. Acta 973, 333–349
26. Janson, C. A., Degani, C., and Boyer, P. D. (1979) J. Biol. Chem. 254, 3743–3749
27. Springs, B., Welsh, K. M., and Cooperman, B. S. (1981) Biochemistry 20, 6384–6391
28. Pohjankoski, P., Lahti, R., Goldman, A., and Cooperman, B. S. (1998) Biochemistry 37, 1754–1761
29. Salminen, T., Kapyla, J., Heinkinheimo, P., Kankare, J., Goldman, A., Heinonen, J., Baykov, A. A., Cooperman, B. S., and Lahti, R. (1995) Biochemistry 34, 782–791
30. Baykov, A. A., Bakuleva, N. P., Nazarova, T. I., and Avava, S. M. (1977) Biochim. Biophys. Acta 481, 184–194
31. Avava, S., Ignatov, P., Kurilo, N. S., Nazarova, T., Rodina, E., Vorobyeva, N., Oganesyan, V., and Harutyunyan, E. (1996) FEBS Lett. 399, 99–102
32. Lebioda, L., Zhang, E., Lewinski, K., and Brewer, J. M. (1993) Proteins Struct. Funct. Genet. 16, 219–225
33. Wedekind, J. E., Purner, R. R., Reed, G. H., and Rayment, I. (1994) Biochemistry 33, 9333–9342
34. Chen, G., Edwards, T., D’souza, V. M., and Holz, R. C. (1997) Biochemistry 36, 4278–4286
35. Pinkoe, M. W. H., Merks, M., and Averill, B. A. (1999) Biochemistry 38, 9920–9936
36. Todd, M. J., and Hausinger, R. P. (2000) Biochemistry 39, 5389–5396
37. Kaminskaia, N. V., Spingler, B., and Lippard, S. J. (2000) J. Am. Chem. Soc. 122, 6411–6422
38. Emaley, J., Hoyte, O. P. A., and Overill, R. E. (1977) J. Chem. Soc. Perkin Trans. 2, 2079–2081