The Electrophilic and Leaving Group Phosphates in the Catalytic Mechanism of Yeast Pyrophosphatase*

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Binding of pyrophosphate or two phosphate molecules to the pyrophosphatase (PPase) active site occurs at two subsites, P1 and P2. Mutations at P2 subsite residues (Y93F and K56R) caused a much greater decrease in phosphate binding affinity of yeast PPase in the presence of Mn$^{2+}$ or Co$^{2+}$ than mutations at P1 subsite residues (R78K and K193R). Phosphate binding was estimated in these experiments from the inhibition of ATP hydrolysis at a sub-K_m concentration of ATP. Tight phosphate binding required four Mn$^{2+}$ ions/active site. These data identify P2 as the high affinity subsite and P1 as the low affinity subsite, the difference in the affinities being at least 250-fold. The time course of five "isotopomers" of phosphate that have from zero to four $^{18}$O during $^{[18O]}$Py$_{2}$-H$_2$O oxygen exchange indicated that the phosphate containing added water is released after the leaving group phosphate during pyrophosphate hydrolysis. These findings provide support for the structure-based mechanism in which pyrophosphate hydrolysis involves water attack on the phosphorus atom located at the P2 subsite of PPase.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) is a ubiquitous enzyme catalyzing interconversion of PP$_i$ and P$_i$. Soluble PPase provides a thermodynamic pull for biosynthetic reactions by removing PP$_i$ formed when nucleoside 5'-triphosphates are converted to the corresponding monophosphates (1). The PPase reaction involves, in the direction of hydrolysis, PP$_i$ binding, isomerization of the resulting complex, PP$_i$, hydrolysis, and the stepwise release of two P$_i$ molecules (Scheme I) (2). The hydrolysis step proceeds via direct attack of water on PP$_i$ without formation of a covalent intermediate (3). Numerous mechanistic studies of PPase have been carried out (for reviews, see Refs. 4 and 5), making this enzyme the best characterized among the catalysts of phosphoryl transfer from various polyphosphates (including ATP and GTP) to water.

X-ray crystallographic studies of PPase complexed with phosphate have identified two P$_i$ binding subsites, P1 and P2, within the active site (6, 7) (Fig. 1). In addition, an activated water molecule, placed between two metal ions in the vicinity of P2, was considered the only logical candidate for nucleophile (6). This supposition was confirmed by the structure of the F- inhibited complex (8), which showed a fluoride ion replacing that water molecule. Solution confirmation of this interpretation has been harder to achieve, however. The oxygen exchange measurements done in the presence of Mg$^{2+}$ as the activator have shown unequivocally that the P$_i$ containing the electrophilic phosphorus is released first after PP$_i$ hydrolysis (9). In terms of the structure-based mechanism (6, 8, 10), this would mean that P$_i$ is first released from P2, where it is more buried than at P1. Resolving this issue requires knowledge of the relative affinities of P1 and P2 for P$_i$, because it is logical to expect faster release from a weaker binding subsite. Earlier attempts to compare the affinities of P1 and P2 used three types of data (5), each of which has been subject to criticism. First, preferential binding of sulfate, an analog of P$_i$, occurred at P1 in the PPase crystals grown in the presence of sulfate (11, 12). However, the crystallization medium in these studies contained no metal ions, the major P$_i$ ligands at the P2 subsite (Fig. 1). Second, x-ray data indicate more extensive hydrogen bonding between the enzyme and P$_i$ at P1 than at P2 (Fig. 1), which was thought to provide greater binding strength at P1. We will show below that this is not the case for Mn$^{2+}$ and Co$^{2+}$ as cofactors. Third, protection of Arg78, located at P1, against chemical modification upon binding of 1 mol of P$_i$/mol of subunit in the presence of Mn$^{2+}$ was interpreted as showing that P1 binds first (13). However, because of the close proximity of P1 and P2, the protection could equally result from binding to P2. We will show below that P2 does in fact exhibit a far greater affinity for P$_i$ in the presence of Mn$^{2+}$ and Co$^{2+}$.

We addressed the correct assignment of the phosphate binding subsites by employing site-directed mutagenesis of P$_i$ ligands together with P$_i$ binding and P$_i$-water oxygen exchange measurements in the presence of Mn$^{2+}$ and Co$^{2+}$. By comparison with Mg$^{2+}$, these cations induce a much greater difference in the binding affinities of P1 and P2, as evidenced by the fact that only one P$_i$ binding site/subunit was observed over a wide range of P$_i$ concentrations (13, 14). In the presence of Mg$^{2+}$, the affinities of P1 and P2 for MgP$_i$ differ only 1–9-fold (9, 15, 16); for comparison, macroscopic binding constants for two sites with equal microscopic constants differ 4-fold (17). The results reported below demonstrate that the relative affinities of P1 and P2 and the order in which these sites release P$_i$ during PP$_i$ hydrolysis depend on the nature of the metal ion cofactor used.

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† The abbreviations used are: PPase, inorganic pyrophosphatase; Y-PPase, yeast (Saccharomyces cerevisiae) inorganic pyrophosphatase; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
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and provide support for the mechanism currently proposed for this reaction.

MATERIALS AND METHODS

The expression and purification of wild type Y-PPase and its active site variants from overproducing Escherichia coli XL2blue strain transformed with suitable plasmids were carried out as described by Heinikin et al. (18). Enzyme concentration was calculated on the basis of a subunit molecular mass of 32 kDa (19) and an A_{260}^\text{nm} equal to 14.5 (20).

Disodium ATP (Sigma Chemical Co.) was freed from Pi and PPi by crystallization from water-ethanol (21). Stock solutions of ATP were standardized by measuring light absorbance at 260 nm (ε = 15, 400 m M^-1 cm^-1) (22). 15O-enriched potassium phosphate (96.4%) was prepared according to Hackney et al. (23).

ATP hydrolysis was assayed luminometrically. The assay mixture of a 0.2–1-ml volume contained 1 μM ATP, 83 mM TES/KOH (pH 7.2), 17 mM KCl, and varied amounts of potassium phosphate and MnCl2. Assays done with Co2+ employed a 100 mM MOPS/KOH buffer (without KCl) because TES was reported to bind Co2+ appreciably (24). Bovine serum albumin (0.01 mg/ml) was also added for enzyme stability in the assays done with R78K-PPase. The reaction was initiated by adding PPase. Aliquots (20 μl) of the assay mixture were withdrawn at 2–3-min intervals over 20–25 min and added to 0.18 ml of 0.1M Tris acetate KCl) because TES was reported to bind Co2+ and EDTA, 1 mg/ml bovine serum albumin, and 1 mM dithiothreitol. The luminescence was then measured with an LKB model 1250 luminometer. The concentration of the luciferin/luciferase reagent was sufficient to produce a 100-mV signal for samples containing 1 μM ATP.

The procedures used to measure enzyme-bound PPi formation at equilibrium (25) and enzyme-catalyzed P_i-water oxygen exchange (26) were as described previously. The media used in the incubations for oxygen exchange and synthesis of enzyme-bound PPi were prepared by mixing appropriate volumes of 100 mM potassium phosphate, 100 mM MOPS/KOH buffer (pH 7.2), and 100 or 3 mM CoCl2, respectively. Although the solubility products for CoHPO4 (1.91 × 10^-17 M3) (Ref. 27) and MnHPO4 (1.40 × 10^-13 M3) (Ref. 28)) were exceeded in many of the incubations, no incipient precipitation occurred in the concentration ranges of the metal ions and P_i used in this study. That the precipitation in these systems was quite slow was confirmed by the following data. First, no decrease in P_i concentration was detected when solutions containing 1 mM Mn2+ plus 200 μM P_i or 20 mM Mn2+ plus 20 μM P_i were incubated for 30 min and centrifuged for 15 min at 4,000 × g. Similarly, no change in Co2+ concentration, measured with Arsenazo III (28), was detected at 100 mM P_i.

RESULTS

P_i Inhibition of Mn2+–supported ATP Hydrolysis—P_i was found to be a very potent inhibitor of wild type Y-PPase in the presence of Mn2+ (Fig. 2). The inhibition constant calculated from the time courses of ATP hydrolysis, as described under “Materials and Methods,” decreased with increasing [Mn2+], approaching a constant level of about 0.04 μM (Fig. 3). The mutations decreased the affinity of Y-PPase to P_i, the effect being moderate with R78K and K193R variants and quite large with the K56R, and especially Y93F variant (Fig. 3).

The Mn2+ concentration dependences of K_p shown in Fig. 3 could be well described by Scheme III. This implies that two enzyme species, E_Mn and E_MnP, bind MnP, with different affinities. Y-PPase is known to have three binding sites for divalent metal ions; P_i complex. Y-PPase converts ATP into ADP and P_i, and further hydrolysis of ADP to yield AMP and P_i proceeds at a negligible rate (30).

The rate of ATP hydrolysis obeyed Equation 1, where t is time, K_m is the Michaelis constant, and [E] is free enzyme concentration. In the absence of P_i (added externally or formed from ATP), [E] could be equated to the total enzyme concentration, [E].

\[ \frac{d[ATP]}{dt} = \frac{k_{cat}[ATP][E]}{K_m} \] (Eq. 1)

Equation 2 was obtained by solving the equation for K_p (Equation 3) together with Equations 4 and 5, describing mass balance for enzyme and inhibitor, respectively.

\[ [E] = 0.5([E]_0 - [P_i]_0 + K_p + \sqrt{([E]_0 - [P_i]_0)^2 + 4K_p[E]_0}) \] (Eq. 2)

\[ [P_i][E]/[E] = K_p \] (Eq. 3)

\[ [E] = [E]_0 - [P_i]_0 - [ATP] \] (Eq. 4)

\[ [P_i] + [ATP] - [ATP] \] (Eq. 6)

It should be noted that [P_i] in Equations 2 and 5 refers to the sum of the added and enzymatically generated P_i, and is therefore given by Equation 6, where [P_i]_0 and [ATP]_0 are the initial concentrations of P_i and ATP, respectively.

In theory, the value for K_p could be obtained from the time course of ATP hydrolysis measured in the absence of added P_i ([P_i]_0 = 0), thus evaluating the effect of P_i produced enzymatically. However, much more accurate estimates for this parameter were obtained from fittings that simultaneously employed the time courses measured in the absence and presence of added P_i (Fig. 2). When such time courses were measured at varied [E], it was treated as a variable along with t and [P_i]_0.

The rate of P_i-water oxygen exchange, \( \nu_{\text{cat}} \), was calculated as 4[P_i]ln[E]/t, where \( E_0 \) and E are the average 18O enrichments of P_i before and after incubation with Y-PPase, and t is the time of the incubation. Values of the partition coefficient \( P_c \) were calculated using the program written by Hackney (31).

All the fittings were performed using the program SCIENTIST (MicroMath).

\[ K_p = \frac{K_{cat}^2}{K_{cat}^1} \frac{(1 + [M]_0)}{(1 + [M]_0)} \] (Eq. 7)

Values of \( k_{cat}/K_m \) also obtained from Equations 1, 2, and 6, using the time courses of ATP hydrolysis (Fig. 2), either increased with Mn2+ until a constant level was attained (wild type, K56R) or passed through a maximum at 5–10 mM Mn2+ and decreased slightly (by 15–20%) at 20 mM Mn2+ (R78K, Y93F, K193R) (data not shown). The maximum values of \( k_{cat}/K_m \) observed for wild type Y-PPase and each variant are listed in Table I.

Mn2+ Binding in the Presence of P_i—Equilibrium dialysis

2 A. B. Zyryanov, manuscript in preparation.
measurements of Mn\(^{2+}\) binding in the presence of Pi provided direct support for Scheme III. In these experiments, Y-PPase was equilibrated with 50 \(\mu\)M MnCl\(_2\) at varied concentrations of Pi, and the manganese content of the two chambers separated by a dialysis membrane was measured by atomic absorption spectroscopy. As shown in Fig. 4, the stoichiometry of Mn\(^{2+}\) binding approached 2 in the absence of Pi, indicating nearly full occupancy of the two high affinity sites, consistent with earlier data (13, 14, 34), and was above 3 at the highest Pi concentration. The theoretical curve obtained using Equation 8 with \(K_{p}^{M4} = 0.041 \text{ mM}, K_{p}^{M3} = \infty, K_{M3} = 1.7 \text{ mM}\) (Table I) and [M] and [MP] given by Equations 9 and 10 is in satisfactory agreement with the measured data. Parameter \(K_{M2}\) (equal to 20 \(\mu\)M\(^3\)) in Equation 8 is the dissociation constant characterizing the equilibrium \(EM = EM_{2}\) (not shown in Scheme III).

\[
E + ATP \rightleftharpoons [EATP] \rightarrow E + ADP + (P)_{h}
\]

\[
\frac{K_{p}}{E(P)_{h}}
\]

**Scheme II. ATP hydrolysis in the presence of Pi.** Subscript t refers to the sum of free and metal-bound phosphate.

**Fig. 1.** Coordination of two phosphate ions in the active site of Y-PPase (6). Black circles (M1–M4) are metal ions, the gray circle (W1) is a water oxygen, and the two black molecules (P1 and P2) are phosphates. Hydrogen bonds and electrostatic interactions are shown by dashed lines. The metal ions are further coordinated by one (M2 and M3), two (M4), or three (M1) amino acid side chains (not shown).

**Fig. 2.** Time courses of Mn\(^{2+}\)-supported ATP hydrolysis by wild type Y-PPase in the absence and presence of 1 \(\mu\)M phosphate. Conditions: 0.3 \(\mu\)M enzyme, 10 mM Mn\(^{2+}\). The lines show the best fits of Equations 1, 2, and 6.

**Fig. 3.** Values of \(K_{p}\) for wild type and variant Y-PPase as a function of Mn\(^{2+}\) concentration. The lines are drawn according to Equation 7, using parameter values given in Table I.

**Scheme III.** Phosphate and metal ion binding to Y-PPase.

\[^{3}\text{P. Pohjanjoki, unpublished data.}\]
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**Table I**

| Enzyme form | $K_{M3}$ | $K_{M4}$ | $K_{M3}^{\text{Mn}}$ | $K_{M4}^{\text{Mn}}$ | $K_{\text{cat}}/K_m$ |
|-------------|----------|----------|----------------------|----------------------|---------------------|
|             | $\text{mM}$ | $\mu\text{M}$ | $\text{mM}$ | $\text{mM}$ | $\text{mM}^{-1} \cdot \text{s}^{-1}$ |
| WT          | $1.7 \pm 0.5$ | $>1$ | $0.041 \pm 0.006$ | $21 \pm 2$ |
| K58R        | $10 \pm 5$ | $7.1 \pm 3.5$ | $0.5 \pm 0.1$ | $0.84 \pm 0.03$ |
| R78K        | $3.8 \pm 0.7$ | $>1$ | $0.072 \pm 0.008$ | $95 \pm 5$ |
| Y93F        | $\leq 65 (24 \pm 40)$ | $47 \pm 6$ | $15 \pm 10$ | $0.90 \pm 0.02$ |
| K193R       | $\leq 23 (11 \pm 12)$ | $0.43 \pm 0.14$ | $0.08 \pm 0.03$ | $8.2 \pm 0.9$ |

$^a$ WT, wild type.

**Table II**

| Enzyme form | $K_p$ | $K_{\text{cat}}/K_m$ |
|-------------|--------|---------------------|
|             | $\mu\text{M}$ | $\text{mM}^{-1} \cdot \text{s}^{-1}$ |
| WT$^a$      | $12.5 \pm 0.7$ | $1.28 \pm 0.03$ |
| K56R        | $270 \pm 20$ | $0.044 \pm 0.001$ |
| R78K        | $40 \pm 2$ | $1.05 \pm 0.02$ |
| Y93F        | $2300 \pm 200$ | $0.053 \pm 0.001$ |
| K193R       | $41 \pm 2$ | $0.46 \pm 0.01$ |

$^a$ WT, wild type.

**Fig. 4.** Mn$^{2+}$ binding to wild type Y-PPase in the presence of P$_i$ as measured by equilibrium dialysis. Total metal concentration was fixed at 50 μM. The line is drawn according to Equations 8–10, using parameter values derived from P$_i$ inhibition measurements (Table I).

\[ n = \frac{K_p^{\text{Mn}}K_{M3} + 2K_p^{\text{Mn}}K_{M4}[M] + 3K_p^{\text{Mn}}[M]^2 + 4[M]^3[MP]}{[MP] = [M]_t - [M]} \] (Eq. 8)

**Fig. 5.** Formation of enzyme-bound PP$_i$ by wild type Y-PPase in the presence of 0.5 mM Co$^{2+}$. Enzyme concentration was 30 μM. The line shows the best fit to a simple hyperbolic saturation function with parameter values given under “Results.”

**Formulation of Enzyme-bound PP$_i$ in the Presence of Co$^{2+}$—**

The Y-PPase active site contains two P$_i$ binding subsites. P$_i$ binding to both of these subsites can be accessed by measuring the fraction of the enzyme containing bound PP$_i$ ($f_{\text{app}}$) as a function of P$_i$ concentration in solution (9) because PP$_i$ synthesis requires occupancy of both subsites. Values of $f_{\text{app}}$ measured in the presence of 0.5 mM free Co$^{2+}$ ion exhibited a hyperbolic dependence on [P$_i$] (Fig. 5), allowing calculation of the limiting value of $f_{\text{app}}$ at infinite [P$_i$]$_t$ (0.18 ± 0.03) and the dissociation constant for P$_i$ binding ($3 \pm 1$ mM). The latter value exceeds $K_p$ (Table II) by a factor of 240 and therefore characterizes binding of the second P$_i$ molecule.

**P$_i$-Water Oxygen Exchange in the Presence of Co$^{2+}$—**

Y-PPase catalyzes rapid exchange of oxygen between P$_i$ and water in the presence of Mg$^{2+}$ and other metal ions that activate PP$_i$ hydrolysis (30, 35–37). This exchange results from a dynamic reversal of the steps characterized by $k_3$ and $k_4$ in Scheme I (36, 37): one oxygen originally present in P$_i$ is released as water when the two bound P$_i$ molecules dehydrate forming PP$_i$ ($k_4$ step) and is subsequently replaced by an oxygen from water when the PP$_i$ is converted back into P$_i$ ($k_3$ step). The exchange is conveniently followed by mass spectrometry, starting from $[^{18}\text{O}]P_i$ and $[^{16}\text{O}]\text{H}_2\text{O}$ (23).

Table III shows two examples of the observed distributions of the five P$_i$ species that have from 0 to 4 exchanged oxygens in the Co$^{2+}$-supported reaction catalyzed by wild type Y-PPase. Such distributions are characterized by two parameters: the exchange rate $v_{\text{ex}}$ and the partition coefficient $P_c$, which equals the probability of bound phosphate conversion into EM$_4$PP versus its release into solution in Scheme I (31). As shown in Table III and Fig. 6A, $P_c$ exhibited a strong dependence on [P$_i$] in the Co$^{2+}$-supported reaction, changing from less than 0.3 at low [P$_i$] to ~0.9 at its saturating concentration. By contrast, $P_c$ is independent of [P$_i$] in the Mg$^{2+}$-supported reaction (9, 15, 31). $v_{\text{ex}}/|E|_t$ increased with [P$_i$], reaching a maximum at about 1.5 mM P$_i$, and dropped slightly at higher [P$_i$] (Fig. 6B). By
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The 18O isotomers containing from four to zero 18O atoms are designated as P18O4, P18O3, P18O2, P18O1, and P18O0. The initial distribution of the 18O isotomers in this order was 91.19, 7.31, 0.35, 0.06, and 1.09%. The enzyme concentration was 0.62 μM (0.125 mM Pi) or 6.2 μM (5 mM Pi). The observed distributions shown were measured after a 10-min incubation. The best fit theoretical distributions are shown in the upper row for each Pi concentration. Two more theoretical distributions, calculated assuming the same average 18O enrichment in the final Pi, show that the final distribution is highly sensitive to Pi concentration.

**TABLE III**

| [Pi]c | m膳 | P18O4 | P18O3 | P18O2 | P18O1 | P18O0 |
|-------|------|-------|-------|-------|-------|-------|
| 0.125 | Observed | 13.30 | 24.79 | 29.18 | 22.47 | 10.26 |
|       | Theory | P = 0.31 | 13.34 | 24.86 | 29.03 | 22.66 | 10.18 |
|       |       | P = 0.84 | 35.92 | 10.37 | 9.98  | 13.89 | 29.90 |
|       |       | Pc = 0.01 | 7.65  | 27.24 | 36.91 | 22.54 | 5.74  |
| 5     | Observed | 50.69 | 11.72 | 8.06  | 10.38 | 19.15 |
|       | Theory | P = 0.84 | 59.00 | 10.76 | 8.40  | 10.69 | 19.31 |
|       |       | P = 0.31 | 27.34 | 30.75 | 24.28 | 13.24 | 4.47  |
|       |       | P = 0.09 | 61.13 | 5.47  | 0.72  | 1.02  | 31.73 |

**FIG. 6.** P2-water oxygen exchange catalyzed by wild type Y-PPase in the presence of 0.5 mM Co2⁺ as a function of Pi concentration. A, Pi values. The line shows the best fit of Equation 11 multiplied by 0.88, the limiting value of Pi at infinite phosphate concentration. The exchange rate, shown as a function of exchange rate constant. Open circles, 18O/[E]I; closed circles, 18O(4-3P)/4P[E]I. The line shows the best fit of the equation 18O(4-3P)/4P[E]I = a + b/(1 + [Pi]/Kp), with the following best fit values of the parameters: a = 0.52 ± 0.11 s⁻¹ b, 2.04 ± 0.09; Kp, 1.2 ± 0.4 mM.

The Pi binding capacity of four of these variants was estimated here from the inhibition of ATP hydrolysis in the presence of Mn2⁺ and Co2⁺ as cofactors. The advantage of these cations over Mg2⁺ is 3-fold. First and most important, the binding affinities of the subsites P1 and P2 differ much more in the presence of Mn2⁺ or Co2⁺ (13, 14). Second, these cations support hydrolysis of ATP (Mg2⁺ does not), a substrate with a high K values. At 1 mM ATP, more than 99% of Y-PPase is substrate-free in the assay medium. Therefore, the true Pi binding constant can be estimated directly from the effect of Pi on activity at this fixed ATP concentration. Third, the structure shown in Fig. 1 was, in fact, determined for M = Mn2⁺, although similar radii and coordination chemistry for Mn2⁺ and Mg2⁺ suggest that this structure should be largely preserved with Mg2⁺.

**P2 Is the Tighter Binding Subsite for Phosphate in the Presence of Mn2⁺ and Co2⁺—**

The data in Fig. 3 and Tables I and II indicate that the two mutations at subsite P2 have a much greater effect on Pi binding to Y-PPase than the two mutations at subsite P1. The measured binding clearly refers to the tighter binding subsite because occupancy of any one of the two subsites by Pi will suffice for inhibition. Furthermore, the data in Fig. 5 demonstrate that the dissociation constant for the weaker binding subsite is as high as 3 mM in the presence of Co2⁺, 240 times larger than for the tighter binding site (Table II). The weaker binding site was thus essentially empty during K values measurements, consistent with the earlier equilibrium dialysis and Pi titration data revealing only one Pi binding subsite in the presence of Mn2⁺ and Co2⁺ and up to 0.1 mM Pi (13, 14).

Other data support the identification of P2 as the tighter binding subsite in the presence of Mn2⁺ and Co2⁺. First, the tighter binding site requires four Mn2⁺ ions for optimal Pi binding (Scheme III), as one would expect for P2 that has four metal ligands, rather than P1 that has only two (Fig. 1). Second, P1 was observed only in P2 in the manganese structure of the R78K variant (38), whose Pi binding affinity is similar to that of the wild type enzyme. Finally, mutations at the P2 subsite (R292Y and Y55F) in E. coli PPase have also been shown to have greater effects on Pi binding measured in the presence of 0.1 mM Mn2⁺ than mutations at the P1 subsite (R43K, Y141F, and K142R).4

Interestingly, the effects of the mutations on ATP binding, as characterized by kcat/Km (Tables I and II), parallel those on Pi binding (Fig. 3 and Table II). This indicates that ATP binding is dominated by interactions at subsite P2. One of the P1 mutations (R78K) increases kcat/Km for Mn2⁺-supported ATP hydrolysis nearly 5-fold (Table I), suggesting that the interac-

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is consistent with the observation that the value of $v_{\text{c}}(4 - 3P)AP[E_i]$ tends to reach a constant non-zero value with increasing $P_i$ concentration (Fig. 6B) rather than approach 0, as expected for a strictly ordered release.

To sum up, these data provide strong support for the mechanism of PP hydrolysis (6, 8), involving water/hydroxide addition to the phosphorus atom located in the site P2 of PPase.

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