Modulation of Dimer Stability in Yeast Pyrophosphatase by Mutations at the Subunit Interface and Ligand Binding to the Active Site*

Yeast (Saccharomyces cerevisiae) pyrophosphatase (YPase) is a tight homodimer with two active sites separated in space from the subunit interface. The present study addresses the effects of mutation of four amino acid residues at the subunit interface on dimer stability and catalytic activity. The W52S variant of Y-PPase is monomeric up to an enzyme concentration of 300 μM, whereas R51S, H87T, and W279S variants produce monomer only in dilute solutions at pH ≥ 8.5, as revealed by sedimentation, gel electrophoresis, and activity measurements. Monomeric Y-PPase is considerably more sensitive to the SH reagents N-ethylmaleimide and p-hydroxymercuribenzenesulfonate than the dimeric protein. Additionally, replacement of a single cysteine residue (Cys83), which is not part of the subunit interface or active site, with Ser resulted in insensitivity of the monomer to SH reagents and stabilization against spontaneous inactivation during storage. Active site ligands (Mg²⁺ cofactor, P₆ product, and the PPᵢ analog imidodiphosphate) stabilized the W279S dimer versus monomer predominantly by decreasing the rate of dimer to monomer conversion. The monomeric protein exhibited a markedly increased (5-9-fold) Michaelis constant, whereas kcat remained virtually unchanged, compared with dimer. These results indicate that dimerization of Y-PPase improves its substrate binding performance and, conversely, that active site adjustment through cofactor, product, or substrate binding strengthens intersubunit interactions. Both effects appear to be mediated by a conformational change involving the C-terminal segment that generally shields the Cys83 residue in the dimer.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) is an essential enzyme that catalyzes the interchange between pyrophosphate and phosphate (1, 2). Due to its relatively simple structure and high catalytic efficiency (kcat/Km = 10⁸ M⁻¹ s⁻¹), PPase has become a paradigm for mechanistic and structural studies of enzymatic phosphofructokinase transfer from phosphoric acid anhydrides to water (3, 4). Two nonhomologous families of soluble PPase have been identified to date. Yeast (Saccharomyces cerevisiae) PPase (Y-PPase) is a member of family I, which is fairly widespread in all types of organisms (5). Family I PPases are homohexamers of ~20-kDa subunits in prokaryotes and homodimers of ~32-kDa subunits in eukaryotes with highly conserved active sites and mechanisms of action (3, 4). Family II PPases have been discovered more recently (6, 7). All the established and putative members of this family belong to bacteria but are homodimers of ~33-kDa subunits (8), in contrast to bacterial PPases of family I. Although family II members have yet to be characterized in detail, available data suggest that the active sites of family I and family II PPases are quite similar, presenting a remarkable example of convergent enzyme evolution (9, 10).

The extensively studied Y-PPase enzyme exists as a very tight dimer in a wide range of conditions. The active site and subunit interface are separated by about 5 Å (11–13) and do not share common amino acid residues. All intersubunit interactions involve side chain atoms (Fig. 1). Core intersubunit contact is formed by a three-layer-stacking of the aromatic rings of Trp52, His87, His87, and Trp279, with His87 and His87 forming the central layer (represents residues of the second subunit). Trp279 and Trp279 pack perpendicular and on the outside of this three-layer stack. Polar contacts between subunits include hydrogen bonds His87—His87, Arg51 side chain—Asp277, main chain oxygen, and a symmetrical Arg87—Asp772 interaction. The interface is essentially conserved in other fungal and animal PPases, except for a His87 to Lys replacement in four of the nine known sequences (5). Active monomeric Y-PPase was previously obtained upon covalent immobilization on Sepharose beads, followed by denaturation with guanidine hydrochloride and renaturation of the protein (14).

Here we describe the effects of Arg51, Trp52, His87, and Trp279 substitutions on the quaternary structure and activity of Y-PPase. Our results indicate that mutation of Trp279 has the most significant effect on dimerization and that active site ligands enhance dimer stability.

EXPERIMENTAL PROCEDURES

Enzymes—The production and purification of wild-type and variant Y-PPase from overproducing Escherichia coli XL2blue® strains transformed with suitable plasmids were performed as described by Heikinheimo et al. (15), except that the Stratagene QuikChange mutagenesis kit was used. Enzyme concentration was calculated on the basis of the subunit molecular mass of 32.0 kDa (16) and the specific absorbance...
A $^{280}$ of 1% equal to 14.5 for wild-type PPase (17). Substitution of each Trp residue with Ser decreased $A^{280}$ by 1.7 (18).

**Methods** — The initial rates of PP i hydrolysis were measured using a continuous Pi assay (19). The assay medium contained 6 $\mu$M PPi, 20 m\textbf{M} Mg2+, 0.15 M Tris/HCl, pH 7.2, and 40 $\mu$M EGTA, except where specified. The reaction was initiated by adding enzyme and continued for 3–4 min at 25°C. Polyacrylamide gel electrophoresis under nondenaturing conditions was done with a 12.5% gel in a Sigma-Aldrich vertical electrophoresis unit. The gel buffer and the running buffer were 25 mM Tris-glycine, pH 9.3, 0.5 m\textbf{M} dithiothreitol (20). Electrophoresis in the presence of 0.55% dodecyl sulfate was performed with an 8–25% gradient gel, using the Phast System (Amersham Biosciences). Analytical ultracentrifugation was carried out in a Spinco E instrument (Beckman-Spinco), with scanning at 280 nm. Sedimentation velocity was measured at 48,000 rpm, and the sedimentation coefficient, $s_{20,w}$, was calculated using standard procedures (21). A partial specific volume of 0.730 cm$^3$/g at 25°C was calculated from the amino acid composition.

The following pH buffers were used for enzyme incubations: (a) 0.1 M citric acid/NaOH and 50 $\mu$M EGTA (pH 4.5); (b) 0.083 M TES/KOH, 0.017 M KCl, and 50 $\mu$M EGTA (pH 7.2); (c) 0.09 M TAPS/KOH and 5 $\mu$M EGTA (pH 8.5); and (d) 0.052 M TAPS/NaOH and 0.048 M CAPS/KOH (pH 9.3). All measurements were performed at 25°C.

**Data Analysis** — Eqs. 1 and 2 (derived from Scheme I) describe time-courses of activity (A) resulting from dimer (D) conversion into monomer (M) and the reverse reaction, as well as the equilibrium activity (at $t = \infty$ and $dA/dt = 0$) as a function of enzyme concentration (22). $A_D$ and $A_M$ are the specific activities of the dimer and monomer, respectively; $k_2$ is the fraction of the dimer at time $t$; $[E]_0$ is total enzyme subunit concentration; $k_1$ and $k_2$ are the apparent rate constants for association and dissociation, respectively. Eqs. 1 and 2 were simultaneously fit to data with the SCIENTIST program (MicroMath).
RESULTS

Production of Y-PPase Variants—The primary goal of this work was to redesign the subunit interface to yield nonassociating and stable monomer. Low probability substitutions (23) of dimer-forming residues were specifically selected (W/S, H/T, and R/S) to induce structural changes within this region of the protein. Three single variants (W52S, H87T, and W279S) and one double variant (H87T/W279S) were expressed and isolated in amounts ranging from 90 to 200 mg/liter culture medium. However, the R51S variant, as well as the R51G and R51L variants, could not be expressed, possibly as a consequence of replacing the buried and charged Arg51 side chain with uncharged side chains. A more conservative R51K replacement preserving the positive charge on the side chain resulted in yield improvement. Another major problem was the low stability of the W52S variant during short-term incubations in solution or long-term storage as a frozen solution, which ultimately resulted in a large scatter in data for this variant. This behavior was dithiothreitol-dependent, suggesting the involvement of SH groups. The problem was solved by replacement of the single Cys residue in Y-PPase (Cys83) with Ser.

All preparations of the variant proteins used in this study were >95% homogeneous, as observed by SDS-PAGE analysis.

Effect of Substitutions on Quaternary Structure—The first indication of altered quaternary structure in Y-PPase interface variants was evident upon native polyacrylamide gel electrophoresis, which revealed that they migrated faster than the wild-type enzyme and the noninterface variant, C83S (Fig. 2).

Direct evidence for changes in the quaternary structure was additionally provided by sedimentation data (Table I). The W52S variant exhibited a lower $s_{20,w}$ value (2.4 S) than wild-type PPase (4.0 S) at both pH 7.2 and pH 9.3, indicating monomeric protein as a result of the mutation. The other single- and double-substituted variants were dimers at pH 7.2 and mixtures of monomer and dimer at pH 9.3 and 5 $\mu$M enzyme, as indicated by the $s_{20,w}$ values. The W52S substitution had the most significant effect on the quaternary structure of the protein. A combination of two substitutions (H87T and W279S), each inadequate in yielding monomers at pH 7.2, also resulted in monomeric protein at both pH values.

Wild-type PPase remained dimeric at pH values as low as 4.5, in both the absence and presence of Mg$^{2+}$, as indicated by the unchanged $s_{20,w}$ value (4.0 S).

Effects of Active Site Ligands on the Equilibrium and Rates of the Dimer-Monomer Interconversion in W279S-PPase—Dimer-monomer interconversion was conveniently monitored by activity measurements because activities of the dimer and monomer were different, and they converted into each other slowly on the time scale of the enzyme assay. This approach was used previously in studies on E. coli PPase variants with weakened quaternary structure (22, 24, 25, 27); here, we use it to characterize the W279S variant, which exists as either a dimer or a monomer, depending on specific conditions (Table I). In accordance with the sedimentation data, the specific activity of W279S-PPase (measured with 6 $\mu$M substrate) increased with enzyme concentration in a stock solution preincubated at pH 9.3 (Fig. 3), as expected for a slow equilibrium between dimer and less active monomer. No such inactivation of W279S-PPase at low enzyme concentrations was observed upon preincubation at pH 7.2. In contrast, the specific activity of wild-type Y-PPase and its W52S/C83S variant remained constant (240 ± 15 and 29 ± 2 s$^{-1}$, respectively) on preincubation with 1 mM Mg$^{2+}$ at both pH 7.2 and pH 9.3 at the same range of enzyme concentrations (data not shown). The inactivation of the W279S variant observed at pH 9.3 was completely reversed by decreasing the pH to 7.2 (Fig. 4).

The shift in the activity versus enzyme concentration profile to the left caused by the active site ligands Mg$^{2+}$, P$_i$, and imidodiphosphate (a PP$_i$ analog containing N instead of O at the bridge position) (Fig. 5) indicated that the ligands stabilize the dimer rather than the monomer. The values of the equilibrium dissociation constant for dimer ($K_d = k_d/k_a$) derived from these profiles with Eq. 1 and 2 decreased by 4 orders of magnitude in the presence of Mg$^{2+}$ and imidodiphosphate. The effects of the active site ligands on the rate of W279S-PPase dissociation (Fig. 5) paralleled their effects on $K_d$ (Table II). Because imidodiphosphate is a tightly bound ($K_a < 1 \mu$M) and slowly converted ($k_{cat} = 0.01$ s$^{-1}$) substrate for Y-PPases (28), enzyme concentration was limited to 0.6 $\mu$M in the experiments.

FIG. 4. Reversibility of W279S-PPase inactivation at pH 9.3. Enzyme solution (16 nM) was pre-equilibrated at pH 9.3 in the presence of 1 mM Mg$^{2+}$ as described in the Fig. 3 legend, and the pH was lowered to 7.2 with 0.5 M TES at a specific time (indicated by the arrow). Aliquots were withdrawn as a function of time, and PPase activity was assayed at pH 7.2. The line was obtained with Eqs. 1 and 2 using $k_a$ value specified in Table II.

\[
A = A_D + (A_D - A_M)a_D \\
\frac{dA_M}{dt} = 2k_a[E_l(1 - A_M)^2 - k_dA_M] 
\]

FIG. 5. Effects of ligands on W279S-PPase dissociation into monomers at pH 9.3. Stock enzyme solution (22 $\mu$M) pre-equilibrated at pH 7.2 to convert essentially all enzyme into dimeric form was diluted to 0.2 $\mu$M with the pH 9.3 buffer containing the indicated ligands, 0.5 mM dithiothreitol, and 1 mg/ml bovine serum albumin. Aliquots were withdrawn as a function of time, and PPase activity was assayed at pH 7.2. The lines represent a reversible first-order reaction with the $k_a$ and $k_d$ values specified in Table II.

FIG. 5. Monomeric Yeast Pyrophosphatase
illustrated in Figs. 3 and 5 to ensure that at least 50% of imidodiphosphate remained intact at the end of the incubation.

Fitting Eqs. 1 and 2 with \( k_d = k_m K_d / K_m \), values specified in Table II) to the time-courses shown in Fig. 5 allowed the estimation of \( k_m \). The value of \( k_m \) at pH 7.2 was obtained by fitting Eqs. 1 and 2 to the time-course of the association reaction (Fig. 4), which was essentially irreversible (i.e. \( k_d \) could be set to 0) because the enzyme was predominantly monomeric at the start of the reaction and dimeric at the end of the reaction.

A similar analysis was performed for W279S-PPase at a wide range of Mg\(^{2+}\) concentrations for yielding \( k_m \) and \( k_a \) dependences, as shown in Fig. 6. The dependence of \( k_m \) may be described by Scheme II, which implies an effect of two metal ions bound sequentially with dissociation constants of \( K_{M1} \) and \( K_{M2} \). The shape of the profile indicates that \( k_d2 \) is the lowest of the three individual dissociation rate constants shown in Scheme II. Fitting the data of Fig. 6 to Eq. 3 allowed the evaluation of all parameters in Scheme II: \( K_{M1} = 19 \pm 8 \mu M \), \( K_{M2} = 20,000 \mu M \), \( k_{d1} = 2.1 \pm 0.3 \) min\(^{-1} \), \( k_{d2} = 0.12 \pm 0.08 \) min\(^{-1} \), and \( k_{d3} > 1 \) min\(^{-1} \).

The less significant effect of [Mg\(^{2+}\)] on \( k_m \) (Fig. 6) is described by Eq. 4, which indicates one metal binding site/subunit. The corresponding dissociation constant, \( K_{MX} \), and the values of the rate constants \( k_{cat} \), for free monomers and \( k_{cat} \) for its magnesium complex were found to be 1600 \( \pm 600 \mu M \), 4.1 \( \pm 0.1 \) \mu M\(^{-1} \) min\(^{-1} \), and 6.9 \( \pm 0.2 \) \mu M\(^{-1} \) min\(^{-1} \), respectively.

\[
\frac{k_m}{1 + [Mg^{2+}] / [K_{MX}]} = k_{cat} + \frac{k_{d} [Mg^{2+}] / [K_{MX}]}{1 + [Mg^{2+}] / [K_{MX}]} \quad \text{(Eq. 3)}
\]

\[
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\]

\[\text{Sensitivity to SH Reagents—} \text{Additional evidence for the different quaternary structures of the W279S and W52S variants at pH 7.2 was obtained by comparing the effects of SH reagents on different enzyme forms. In dimeric Y-PPase, the single Cys residue/subunit, located at \( \sim 5 \) Å from the subunit interface, is shielded by C-terminal residues 280–284 (13), which protect the residue from modification by bulky reagents (29). Consistent with this structure, N-ethylmaleimide had a minimal effect on the activity of wild-type Y-PPase and its dimeric W279S variant at pH 7.2, as confirmed by the \( k_{cat} \) values, but inactivated the monomeric W52S variant (Fig. 7). p-HMBS, a more reactive SH reagent, inactivated the dimeric PPases appreciably, but again, the effect on the monomeric W52S variant was much more significant. The Cys residue was not appreciably modified by N-ethylmaleimide in the dimeric PPases but was nearly completely modified in monomeric W52S-PPase at pH 7.2, as confirmed by a greater inactivating effect of p-HMBS on the N-ethylmaleimide-treated wild-type PPase and W279S-PPase compared with W52S-PPase (Fig. 7). The monomeric double variant (W52S/C83S) lacking Cys was not inactivated by N-ethylmaleimide.

At pH 9.3, both p-HMBS and N-ethylmaleimide modified wild-type PPase significantly, causing partial dissociation into monomers, as indicated by the decrease in \( k_{20,w} \) (Fig. 7). Again, the W279S variant that is predominantly monomeric at this pH value displayed significantly greater reactivity to these reagents and decreased \( k_{20,w} \) values upon the modification.

\[\text{Michaelis-Menten Parameters for Dimer and Monomer—} \text{Only minor changes in } K_m \text{ and } K_m \text{ values were observed in the dimeric variant PPases, compared with wild-type protein (Table III), indicating no significant alterations in the active site. The monomeric forms of all variant PPases exhibited markedly increased } K_m \text{ values, whereas } k_{cat} \text{ values decreased significantly in only two variants (W52S and W279S).}

\[\text{DISCUSSION}\]

\[\text{Contribution of Different Residues to Dimerization—} \text{X-ray crystallography has led to the identification of four critical residues at the subunit interface of Y-PPase, specifically Arg}^{21}, \text{Trp}^{52}, \text{His}^{87}, \text{and Trp}^{279} (\text{Fig. 1}). \text{ Sedimentation, gel electrophoresis, and activity measurements reveal that the W52S substitution has a greater effect on dimer stability than the R51K, H87T, and W279S substitutions. Among other factors, the nature of a substitution largely influences the extent of the effects on protein structure and function. With this in mind, one can conclude that Trp}^{52} \text{ contributes more to dimer formation than Trp}^{279} (\text{identical substitution}) \text{ and perhaps His}^{87} \text{ as a function of Mg}^{2+} \text{ concentration. Experiments were performed as described in the Fig. 4 legend. Lines were obtained with Eqs. 3 and 4, using the parameter values specified under "Results."}

\[\text{FIG. 6. Rate constants for W279S-PPase dissociation into monomers at pH 9.3 (○) and reassociation into dimers at pH 7.2 (●) as a function of Mg}^{2+} \text{ concentration. Experiments were performed as described in the Fig. 4 legend. Lines were obtained with Eqs. 3 and 4, using the parameter values specified under "Results."}

\[\text{TABLE II}\]

Parameters for dimer-monomer equilibrium in W279S-PPase

| pH     | Ligands                | \( K_d \) (µM) | \( k_d \) (min\(^{-1} \)) | \( k_a \) (µM/min\(^{-1} \)) |
|--------|------------------------|---------------|--------------------------|-----------------------------|
| 7.2    | 1 mM Mg\(^{2+}\)       | <0.01         | 5.4 ± 0.8                |
| 8.5    | 1 mM Mg\(^{2+}\) + 0.01 mM Mg\(^{2+}\) | 0.020 ± 0.003 | 0.016 ± 0.002       | 0.2 ± 0.8         |
| 9.3    | 1 mM Mg\(^{2+}\)       | 27 ± 3        | 1.40 ± 0.15              | 0.05 ± 0.01       |
| 9.3    | 1 mM Mg\(^{2+}\) + 3 mM Pi | 1.6 ± 0.4     | 0.30 ± 0.04              | 0.19 ± 0.08       |
| 9.3    | 1 mM Mg\(^{2+}\) + 0.1 mM imidodiphosphate | 0.23 ± 0.03  | 0.057 ± 0.003          | 0.25 ± 0.05       |

\[\text{Scheme II. Dissociation of dimeric W279S-PPase in the presence of metal ions.}\]
Factors Affecting Dimer Stability—Whereas wild-type Y-PPase exists as dimer in a wide range of conditions, the W279S variant is a mixture of dimer and monomer, facilitating analyses of the effects of various stimuli on the dimer = monomer equilibrium. The results of these analyses indicated that substrate (imidodiphosphate), product (Pi), and Mg$^{2+}$ and Mg$^{2+}$ concentrations (Fig. 6) are associated with the binding of the second and third Mg$^{2+}$ ions, respectively. It should be noted that the destabilizing effect of Mg$^{2+}$ on the dimer is insignificant at physiological concentrations of the ion (≈1 mM). The Mg$^{2+}$ concentration dependence of $k_{cat}$ in Fig. 6 yielded an Mg$^{2+}$ binding constant of 1600 ± 600 μM for monomeric Y-PPase at pH 7.2. Our recent kinetic analysis of the activating effect of Mg$^{2+}$ on monomeric W279S-PPase$^2$ suggests that this constant refers to binding of the second Mg$^{2+}$ ion and thus implies an 80-fold decrease in affinity for monomer, compared with dimer. Accordingly, Mg$^{2+}$ stimulates dimer formation via tighter binding. Because the catalytic active site and subunit interface are separated in space, the effect of Mg$^{2+}$ implies a conformational difference between monomer and dimer and between dimers with vacant and occupied M2 sites. The same considerations apply to imidodiphosphate and P$^\gamma$ binding.

The effects of Mg$^{2+}$ on dimer stability are mainly a result of its effect on $k_d$ (however, it should be taken into account that the $k_d$ and $k_a$ values were obtained at different pH conditions) and are reversed at high Mg$^{2+}$ concentrations (Fig. 6). Four metal binding sites/subunit have been identified in the phospho complex of dimeric wild-type Y-PPase by x-ray crystallography (11). Three of them bind Mg$^{2+}$ in the absence of phosphate (30, 31), with dissociation constants of 2.3, 15, and >5000 μM at pH 9.3 (31). A comparison of these values with $K_{M}^{2+}$ of 19 ± 8 μM and $K_{M}^{2+}$ of >20,000 μM in Scheme II indicates that the decrease in $k_d$ and reversal of this effect with increasing Mg$^{2+}$ concentrations (Fig. 6) are associated with the binding of the second and third Mg$^{2+}$ ions, respectively. It should be noted that the destabilizing effect of Mg$^{2+}$ on the dimer is insignificant at physiological concentrations of the ion (≈1 mM). The Mg$^{2+}$ concentration dependence of $k_{cat}$ in Fig. 6 yielded an Mg$^{2+}$ binding constant of 1600 ± 600 μM for monomeric Y-PPase at pH 7.2. Our recent kinetic analysis of the activating effect of Mg$^{2+}$ on monomeric W279S-PPase$^2$ suggests that this constant refers to binding of the second Mg$^{2+}$ ion and thus implies an 80-fold decrease in affinity for monomer, compared with dimer. Accordingly, Mg$^{2+}$ stimulates dimer formation via tighter binding. Because the catalytic active site and subunit interface are separated in space, the effect of Mg$^{2+}$ implies a conformational difference between monomer and dimer and between dimers with vacant and occupied M2 sites. The same considerations apply to imidodiphosphate and P$^\gamma$ binding.

In addition to confirming that the W52S variant is monomeric, Cys$^{83}$ modification helped to identify the conformational differences between dimer and monomer. The increased reactivity of Cys$^{83}$ in monomer clearly indicates that the C-terminal segment that normally shields Cys$^{83}$ in dimer (Fig. 8) becomes more mobile or possibly adopts a completely different conformation, making the SH group accessible to modifying agents. This displacement is not a specific effect of the W279S substi-
dimerization (8). This may be a consequence of the two-domain structure of family II PPases as active site location at the domain interface (9, 10). In this case, dissociation into monomers may cause domain flexibility that cannot be overcome by substrate binding. In contrast, Y-PPase is a one-domain protein, like all family I PPases.

Another interesting comparison may be made with E. coli PPase, which, like Y-PPase, belongs to family I. The active sites of the two PPases are nearly identical, but the subunit size is much smaller for E. coli PPase (20 versus 32 kDa). Accordingly, the E. coli enzyme is inactive as a monomer but fully functional as a hexamer, similar to other prokaryotic PPases of family I (27). For E. coli PPase, $k_{cat}$ values are similar in dimer, trimer, and hexamer, whereas $K_m$ values decrease progressively (10-fold) from dimer to hexamer (22, 27). This comparison suggests that the critical mass required to form a well-ordered active site in homo-oligomeric PPases is inversely proportional to monomer mass, i.e. larger monomers lead to smaller homooligomers. This information is crucial in the design of other polypeptides with enzymatic activity de novo.

The structure of the subunit interface is highly conserved in other fungal and animal family I PPases (5) but is significantly different in prokaryotic PPases of both families (5, 9, 10, 26). This allows the design of selective inhibitors of PPases of families I and II that would interfere with oligomerization in pathogenic prokaryotes. In this context, it is important to note that subunit contacts are weaker in prokaryotic than in eukaryotic PPases.

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