Reciprocal Effects of Substitutions at the Subunit Interfaces in Hexameric Pyrophosphatase of Escherichia coli

DIMERIC AND MONOMERIC FORMS OF THE ENZYME*

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A homohexameric molecule of Escherichia coli pyrophosphatase is arranged as a dimer of trimers, with an active site present in each of its six monomers. Earlier we reported that substitution of His136 and His140 in the intertrimeric subunit interface splits the molecule into active trimers (Velichko, I. S., Mikalahti, K., Kasho, V. N., Dudarenkov, V. Y., Hyytiä, T., Goldman, A., Cooperman, B. S., Lahti, R., and Baykov, A. A. (1998) Biochemistry 37, 734–740). Here we demonstrate that additional substitutions of Tyr77 and Gln80 in the intratrimeric interface give rise to moderately active dimers or virtually inactive monomers, depending on pH, temperature, and Mg2+ concentration. Successive dissociation of the hexamer into trimers, dimers, and monomers progressively decreases the catalytic efficiency (by 106-fold in total), and conversion of a trimer into dimer decreases the affinity of one of the essential Mg2+-binding sites/monomer. Disruptive substitutions predominantly in the intratrimeric interface stabilize the intertrimeric interface and vice versa, suggesting that the optimal intratrimeric interaction is not compatible with the optimal intertrimeric interaction. Because of the resulting “conformational strain,” hexameric wild-type structure appears to be preformed to bind substrate. A hexameric triple variant substituted at Tyr77, Gln80, and His136 exhibits positive cooperativity in catalysis, consistent with this model.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) catalyzes the interconversion between pyrophosphate and orthophosphate and is essential for life (1, 2). Because of its relative simplicity and high efficiency (kcat/Km = 107 M−1 s−1), PPase has become a paradigm for mechanistic and structural studies of enzymatic phosphoryl transfer from phosphoric acid anhydrides to water. The best studied PPases are those from Escherichia coli and Saccharomyces cerevisiae (3, 4).

A molecule of E. coli PPase (E-PPase) is formed by six identical subunits, 20 kDa each, arranged with D3 symmetry in two layers of trimers (Fig. 1A). The three or four contact zones (see below), which are not well separated, cover about 24% of the accessible surface area of a monomer. The intertrimer contacts are of a circular “head-to-tail” type, meaning that each monomer has two different intertrimer contact regions (Fig. 1B) (5, 6). These involve a mixture of hydrophilic and hydrophobic interactions that include Tyr77 and the backbone NH of Gln80 (5); the total surface area buried per monomer is about 1300 Å2 (6).

There are also two different interfaces between trimers. The smaller one (140 Å2) includes a Tyr77–Asn24 hydrogen bond (primed and unprimed numbers refer to different monomers). Tyr77 acts as a link between the intertrimer interface, the minor intertrimer interface, and the major intertrimer interface. The larger intertrimer interface in E-PPase (640 Å2) chiefly involves α-helix A, including an ion-triple formed between His140, Asp143, and His136 (5, 8). Replacing either His136 or His140 with Gln destabilizes the E-PPase hexamer (9), whereas replacing both makes trimers the dominant species in solution even at millimolar protein concentrations (10). Another important interaction occurs through Mg2+ bound at the intertrimeric interface. The Mg2+ ion is octahedrally associated with six water molecules, which in turn hydrogen bond to the side chains of Asn24 and Asp26, as well as to the backbone carbonyls of Asn24 and Ala25 (8, 11). Substitution of Asp26 with Asn or Ser eliminates Mg2+ binding to the intertrimeric site and somewhat decreases hexamer stability but hardly affects catalysis (12). Hexamer dissociation into trimers greatly decreases the rate constant for substrate binding to enzyme but has no effect on the catalytic constant (10, 12).

Here we describe the effects of substitutions of Tyr77 and Gln80, predominantly at the intertrimeric interface, on the quaternary structure and catalytic activity of E-PPase. This interface mainly consists of hydrophobic contacts between strands and contains few hydrogen bonds between monomers (Fig. 1C). The side chains of Tyr77 and Gln80 are thus in a rather hydrophobic environment. We show that Y77D and Q80E substitutions markedly destabilize the intertrimeric contact and, in combination with substitutions at the intertrimeric contact, yield moderately active dimeric and virtually inactive monomeric E-PPase. The results of this study also shed light on the interactions between different subunit contacts in E-PPase.

MATERIALS AND METHODS

Enzymes—Wild-type E-PPase (13) and E-PPase variants free of contamination with wild-type enzyme (14) were prepared and purified as described elsewhere. The final preparations were homogeneous according to SDS-polyacrylamide gel electrophoresis. Enzyme concentrations are expressed in terms of monomer (15).

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¶ These abbreviations used are: PPase, inorganic pyrophosphatase; E-PPase, E. coli PPase; Mg2+PPi, dimagnesium pyrophosphate; WT, wild type; MES, 4-morpholinolinesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amine-1-propanesulfonic acid.
Methods—The initial rates of PP, hydrolysis were measured by a continuous P, assay (16). The reaction was initiated by adding enzyme and was carried out for 3–4 min at 25 °C. No appreciable conversion among enzyme forms was observed during the assay, as evidenced by nearly linear product formation curves. Analytical ultracentrifugation, chemical cross-linking, and electrophoresis were performed as described previously (9).

Data Analysis—Equations 1 and 2, derived from Scheme I, describe time courses of activity (A) resulting from enzyme (E) dissociation into two equal parts (E) (i.e. hexamer to trimers or dimer to monomers) and the reverse reaction, as well as the equilibrium activity (at t = ∞, \( dA/dt = 0 \)) as a function of enzyme concentration (10).

\[ k_d E_i = 2E \]

\[ \frac{dA}{dt} = \frac{n}{k_d} E_i (1 - \alpha)^2 - k_d A \]

A and A0 are the specific activities of the associated and dissociated enzyme, respectively; \( \alpha \) is the fraction of the associated enzyme at time t; \( [E_i] \) is total enzyme concentration expressed in monomers; \( k_d \) and \( k_a \) are the apparent rate constants for the association and dissociation, respectively; \( K_d = k_d/k_a \), n is 3 for hexamer-trimer and 1 for dimer-monomer conversions. Equations 1 and 2 were simultaneously fit to data with the program SCIENTIST (MicroMath).

RESULTS

Effect of Substitutions on Quaternary Structure—The effects of substitutions at the subunit interfaces on the quaternary structure of E-PPase were studied as a function of pH. Direct evidence for changes in the quaternary structure was provided by sedimentation data (Table I). The data were collected at 2–4 °C, because both intratrimeric (see below) and intertrimeric (17) interactions are weakened at low temperature. At pH 7.2, all variants having no substitutions at the intertrimeric interface (Y77D, Q80E, and Y77D/Q80E) and the Q80E/H136Q variant retained their hexameric structure (\( s_{20,w} \approx 6.0 \) S). Lowering the pH to 3.8–4.6, which disrupts the intertrimeric contact (12, 18), decreased \( s_{20,w} \) for these variants to either 3.7–4.2 or 2.6–3.1 S. Based on the \( s_{20,w} \) values previously measured for wild-type and variant E-PPase trimers whose structures were confirmed by independent methods (9, 10, 12, 19, 20), the corresponding enzyme forms were identified as trimer and dimer, respectively. The variants with modified intertrimeric interface behaved as dimers (Y77D/H136Q and Y77D/H136Q/Q140Q) or as a mixture of hexamer and dimer/monomer (Y77A/Q80E/H136Q and Y77D/Q80E/H136Q) at pH 7.2. One variant (Y77D/Q80E/H140Q) exhibited an \( s_{20,w} \) value as low as 2.0 S and was tentatively identified as monomer.

The designation of the enzyme species with \( s_{20,w} \) of about 3.0 S as a dimer is supported by several lines of evidence. First, the molecular mass of Y77D/Q80E/H136Q/H140Q-PPase determined from sedimentation equilibrium was 41 ± 1 kDa under conditions (20 °C, pH 7.2, 10 mM enzyme, 1 mM MgCl2) where the \( s_{20,w} \) is 2.8 S (Table I). At 1 mM initial enzyme concentration, the average molecular mass was estimated to be 29 ± 2 kDa (1 mM Mg2+, pH 7.2), indicating that the quadruple variant exists as a mixture of dimer and monomer under these conditions. Second, SDS-polyacrylamide gel electrophoresis of the Y77D/Q80E/H136Q/H140Q variant cross-linked with glutaraldehyde revealed two major protein bands attributable to monomer and dimer (Fig. 2). The minor band corresponding to trimer was much less intense than that from H136Q/H140Q-PPase, which is essentially trimeric (10), and may have resulted from inter-
molecular cross-linking. These data indicate that the trimer is either absent or present in only small quantities in the Y77D/Q80E/H136Q/H140Q variant. The identification of different enzyme species in the variant PPases is further supported by activity data presented in the next section.

A comparison of $s_{20,w}$ values indicates that WT-PPase is fully trimeric by pH 5.5 but that lower pH is required for full conversion of the Q80E variant to trimer. This was somewhat unexpected, as $O_{20,w}$ is in the intertrimeric interface. The stabilizing effect of the Q80E substitution on the intertrimeric contact also explains why H136Q-PPase is trimeric, whereas Q80E/H136Q-PPase is hexameric at pH 7.2 (Table I). These data also indicate that the Q80E substitution stabilizes the enzyme over a wide pH range, not only below pH 7. Conversely, the H136Q substitution, which destabilizes the intertrimeric contact (9), clearly strengthens the intratrimeric contact; the H136Q substitution, which destabilizes the intertrimeric con-

| Enzyme variant       | pH 3.8 | pH 4.6 | pH 5.5 | pH 7.2 |
|----------------------|--------|--------|--------|--------|
| WT                   | 3.9 ± 0.1 | 4.2 ± 0.2 | 3.8 ± 0.1 | 6.0 ± 0.2 |
| Y77D                 | 2.7 ± 0.1 | 3.0 ± 0.1 | 2.6 ± 0.1 | 6.6 ± 0.1 |
| Q80E                 | 4.0 ± 0.2 | 3.7 ± 0.1 | 6.3 ± 0.4 | 6.6 ± 0.2 |
| H136Q                | 4.0 ± 0.1a | 3.6 ± 0.1a | 6.5 ± 0.1 | 6.2 ± 0.1 |
| H140Q                |        |        |        |        |
| Y77D/Q80E            | 2.6 ± 0.1 | 2.8 ± 0.1 | 2.6 ± 0.1 | 6.5 ± 0.1 |
| Y77D/H136Q           | 3.1 ± 0.1 | 3.0 ± 0.1 | 3.0 ± 0.1 | 3.0 ± 0.1 |
| Q80E/H136Q           | 3.8 ± 0.0/2.6 ± 0.2b | 3.8 ± 0.1 | 6.3 ± 0.1 | 3.8 ± 0.1 |
| Q80E/H140Q           | 2.3 ± 0.1 | 2.6 ± 0.1 | 2.6 ± 0.1 | 2.6 ± 0.1 |
| H136Q/H140Q          |        |        |        |        |
| Y77A/Q80E/H136Q      | 6.3 ± 0.2/1.0 ± 0.5b | 6.4 ± 0.2/4.2 ± 0.5b | 6.5 ± 0.1 | 6.2 ± 0.1 |
| Y77D/Q80E/H136Q      |        |        |        |        |
| Y77D/Q80E/H140Q      | 2.3 ± 0.1 | 2.6 ± 0.1 | 2.6 ± 0.1 | 2.6 ± 0.1 |
| H136Q/H136Q/H140Q    | 2.8 ± 0.1 | 2.5 ± 0.1 | 2.5 ± 0.1 | 2.5 ± 0.1 |

**From Ref. 9.**

**Two forms of enzyme are present.**

**From Ref. 10.**

**Measured at 20 °C.**

Effect of Substitutions on Activity—Earlier we showed that rates and equilibria of hexamer-trimer interconversion in E-PPase can be monitored by activity measurements at low (20 μM) substrate concentration, because the Michaelis constant is drastically greater in trimer than in hexamer and interconversion between these species occurs slowly on the time scale of the enzyme assay (12). The data in Fig. 3 confirm this for WT-, Q80E-, and Q80E/H136Q-PPase and further suggest that this approach can be used to monitor hexamer-dimer interconversion in Y77D- and Y77D/Q80E-PPase. Similarly to the $s_{20,w}$ values, the activities of WT-, Y77D-, and Y77D/Q80E-PPases drop to their lower levels at pH 5.5, but Q80E-PPase requires pH < 5.0 to be converted to its low activity form (Fig. 3A). The activity of Q80E/H136Q-PPase, which is substantially greater than that of H136Q-PPase at pH 7.2 (Fig. 3B), further demonstrates the hexamer-stabilizing effect of the Q80E substitution in accordance with Table I. The activities presented in Fig. 3 represent equilibrium values, no changes being observed during longer incubations. Furthermore, the inactivation observed at low pH could be reversed by at least 80% in all cases by adjusting the pH in the enzyme solution to 7.2 and incubating it for 30 min at 25 °C.
activity was assayed at 25 °C with 1 mM Mg$_2$PPi, 20 mM MgCl$_2$ (pH 7.2), and 10 mM EGTA. Aliquots were withdrawn as a function of time, and PPase activity was assayed as for Fig. 3. The lines are drawn according to Equations 1 and 2, using values of 350, 0.16, and 15 s$^{-1}$ for WT-, Y77D-, Y77D/Q80E-, and Q80E-PPase, respectively. Curve labels are as described in the legend to Fig. 3.

Monomeric and Dimeric E. coli Pyrophosphatase

The activities of the variants carrying the Y77D substitution indicate that the intratrimeric contact is stabilized with increasing pH, Mg$^{2+}$ concentration, or temperature (Table II).

Functional Properties of Dimeric and Monomeric E-PPase—The activity versus substrate concentration profiles for WT-PPase or its variants with one or both of His$_{136}^\alpha$ and His$_{140}^\alpha$ at the intertrimeric interface indicated the presence of a small fraction of enzyme that is saturated at micromolar substrate concentration (Fig. 6). This is most likely due to trace amounts of hexamers, because it did not occur with the Y77D/H136Q/H140Q variant. The ratio of the catalytic constant to the Michaelis constant for WT-PPase or its variants with one or both of His$_{136}^\alpha$ and His$_{140}^\alpha$ at the intertrimeric interface is calculated from the slope of the lines in Fig. 6, indicating Michaelis constants of greater than 1.5 mM. This is at least 3 orders of magnitude weaker binding than for hexameric E-PPase (21).

As shown in Fig. 4, the Q80E substitution appears to stabilize the hexamer at equilibrium (Table I and Fig. 3) by slowing down its dissociation. Surprisingly, the hexameric Y77D and Y77D/Q80E variants also exhibit much greater kinetic stability than WT-PPase under the conditions tested. At first glance, this contradicts the data in Fig. 3, which indicate inferior thermodynamic stability of these variants compared with WT-PPase. However, unlike WT-PPase, these variants dissociate into dimers (Table I), making hexamer reformation a slow trimolecular reaction.

The activities of the variants carrying the Y77D substitution decrease with decreasing enzyme concentration in pre-equilibrated stock solutions (Fig. 5) in a manner consistent with an equilibrium between active dimers and inactive monomers (Scheme I). Equilibrium between dimers and monomers in the quadruple variant is supported by the sedimentation equilibrium data presented above. In addition, consistent with Scheme I, concentrating a 0.5 μM solution of the quadruple variant to 20 μM (Centricron 10 ultrafilter, Amicon) reversed by at least 60% the inactivation resulting from dilution at pH 7.2. $K_d$ values obtained by fitting Equations 1 and 2 to the activity versus enzyme concentration profiles (Table II) indicate, in agreement with the sedimentation data, that replacing Gln$^\alpha$ in the Y77D/H136Q/H140Q variant destabilizes the intratrimeric contact. For the Y77D/Q80E/H140Q variant, no $K_d$ value characterizing dimer-monomer equilibrium could be obtained in this way because activity rises sharply at enzyme concentrations above 10 μM, probably because of hexamer formation, which is not prevented by substituting only one His residue in the intertrimeric interface (9). Nevertheless, the profile for the Y77D/Q80E/H136Q/H140Q variant at low enzyme concentration is clearly shifted to the left compared with the Y77D/Q80E/H140Q variant. This indicates that the H136Q substitution stabilizes the dimer, in accordance with the sedimentation data described above. The $K_d$ values measured for the Y77D/Q80E/H136Q/H140Q variant indicate that the intratrimeric contact is stabilized with increasing pH, Mg$^{2+}$ concentration, or temperature (Table II).

Tris buffer, used in the incubation media for Fig. 5 and Tables I and II, has recently been shown to bind to the active site of E-PPase (21). Tris markedly decreases the $k_d$ for H136Q-PPase hexamer, thereby stabilizing the hexameric form (21). In contrast, Tris exerted only a minor effect on the trimer-dimer and dimer-monomer equilibria (data not shown), consistent with the severely decreased substrate binding affinity of these oligomeric forms (see below).

FIG. 4. Kinetics of inactivation of wild-type and variant PPases at pH 4.6, 0 °C. Stock enzyme solution containing 750–900 μM enzyme, 1 mM MgCl$_2$, 0.05 M Tris-HCl (pH 7.2), and 10 mM EGTA. Aliquots were withdrawn as a function of time, and PPase activity was assayed as for Fig. 3. The lines are drawn according to Equations 1 and 2, using $k_v$ values of 350, 0.86, 0.16, and 0.20 h$^{-1}$ for WT-, Y77D-, Y77D/Q80E-, and Q80E-PPase, respectively. Curve labels are as described in the legend to Fig. 3.

FIG. 5. Specific activity of variant PPases preincubated at different enzyme concentrations. Preincubation took place for 3 h at 20 °C, except as noted in the curve label, in the presence of 0.1 mM Tris-HCl (pH 7.2), 1 mM MgCl$_2$, and 40 μM EGTA. Preincubation, enzyme activity was assayed at 25 °C with 1 mM Mg$_2$PP$_i$, 20 mM Mg$_2$Cl$_2$, 0.05 mM Tris/HCl (pH 7.2), and 40 μM EGTA. Preincubation for 24 h gave identical activity values. The lines are drawn according to Equations 1 and 2 with parameter values given in Table II. The activity profiles (Table II) indicate, in accordance with the sedimentation data, that replacing Gln$^\alpha$ in the Y77D/H136Q/H140Q variant destabilizes the intratrimeric contact. For the Y77D/Q80E/H140Q variant, no $K_d$ value characterizing dimer-monomer equilibrium could be obtained in this way because activity rises sharply at enzyme concentrations above 10 μM, probably because of hexamer formation, which is not prevented by substituting only one His residue in the intertrimeric interface (9). Nevertheless, the profile for the Y77D/Q80E/H136Q/H140Q variant at low enzyme concentration is clearly shifted to the left compared with the Y77D/Q80E/H140Q variant. This indicates that the H136Q substitution stabilizes the dimer, in accordance with the sedimentation data described above. The $K_d$ values measured for the Y77D/Q80E/H136Q/H140Q variant indicate that the intratrimeric contact is stabilized with increasing pH, Mg$^{2+}$ concentration, or temperature (Table II).

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Functional Properties of Dimeric and Monomeric E-PPase—The activity versus substrate concentration profiles for WT-PPase or its variants with one or both of His$_{136}^\alpha$ and His$_{140}^\alpha$ at the intertrimeric interface indicated the presence of a small fraction of enzyme that is saturated at micromolar substrate concentration (Fig. 6). This is most likely due to trace amounts of hexamers, because it did not occur with the Y77D/H136Q/H140Q and Y77D/Q80E/H136Q/H140Q variants (Fig. 6A), which do not form hexamers (10). For the variants that are dimeric or monomeric in stock solution (Table I), the bulk of the activity exhibited a nearly linear dependence on the concentration of Mg$_2$PP$_i$ within its solubility range (Fig. 6A), indicating Michaelis constants of greater than 1.5 mM. This is at least 3 orders of magnitude weaker binding than for hexameric E-PPase (10). For the Y77D/Q80E/H136Q/H140Q variant, $K_m$ exceeded 1.5 mM over the pH range 7.2–10.0 (data not shown). In contrast, trimeric forms of wild-type and Q80E-PPase exhibited saturation kinetics with $K_m$ values of 350 ± 80 and 160 ± 20 μM, respectively, and much greater activity values (Fig. 6B).

As shown in Fig. 4, the Q80E substitution appears to stabilize the hexamer at equilibrium (Table I and Fig. 3) by slowing down its dissociation. Surprisingly, the hexameric Y77D and Y77D/Q80E variants also exhibit much greater kinetic stability than WT-PPase under the conditions tested. At first glance, this contradicts the data in Fig. 3, which indicate inferior thermodynamic stability of these variants compared with WT-PPase. However, unlike WT-PPase, these variants dissociate into dimers (Table I), making hexamer reformation a slow trimolecular reaction.

The activities of the variants carrying the Y77D substitution decrease with decreasing enzyme concentration in pre-equilibrated stock solutions (Fig. 5) in a manner consistent with an equilibrium between active dimers and inactive monomers (Scheme I). Equilibrium between dimers and monomers in the quadruple variant is supported by the sedimentation equilibrium data presented above. In addition, consistent with Scheme I, concentrating a 0.5 μM solution of the quadruple variant to 20 μM (Centricron 10 ultrafilter, Amicon) reversed by at least 60% the inactivation resulting from dilution at pH 7.2. $K_d$ values obtained by fitting Equations 1 and 2 to the activity versus enzyme concentration profiles (Table II) indicate, in agreement with the sedimentation data, that replacing Gln$^\alpha$ in the Y77D/H136Q/H140Q variant destabilizes the intratrimeric contact. For the Y77D/Q80E/H140Q variant, no $K_d$ value characterizing dimer-monomer equilibrium could be obtained in this way because activity rises sharply at enzyme concentrations above 10 μM, probably because of hexamer formation, which is not prevented by substituting only one His residue in the intertrimeric interface (9). Nevertheless, the profile for the Y77D/Q80E/H136Q/H140Q variant at low enzyme concentration is clearly shifted to the left compared with the Y77D/Q80E/H140Q variant. This indicates that the H136Q substitution stabilizes the dimer, in accordance with the sedimentation data described above. The $K_d$ values measured for the Y77D/Q80E/H136Q/H140Q variant indicate that the intratrimeric contact is stabilized with increasing pH, Mg$^{2+}$ concentration, or temperature (Table II).

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**Results and Discussion**

**Fig. 6.** Activities of wild-type and variant PPases as a function of substrate (Mg\(_2\)) concentration at pH 7.2. Enzymes were preincubated for 40 min at pH 3.8 (Q80E), 1 h at pH 4.6 (WT, 30 h at pH 4.6 (Y77D, Y77D/Q80E, Y77D/Q80E/H140Q, and Y77D/H136Q/H140Q), or 1 h at pH 7.2 (Y77D/Q80E/H136Q/H140Q) under conditions used for Table I (for the quadruple variant, the preincubation temperature was 20 °C). Following preincubation, enzyme activity was assayed at varied substrate concentration with 20 mM Mg\(_2\) at pH 8.0, but at pH 7.2 Mg\(_2\) binding to both sites is required (10). The subunit interaction site has no catalytic role in hexamers (12), is absent in dimers. The subunit interface site has no catalytic role in hexamers (12), is absent in dimers (10), and is most likely absent in dimers.

As measured by equilibrium dialysis, Mg\(_2\) interaction with the high affinity site is much weaker in the dimer than in the hexamer and trimer (Fig. 8). This is also indicated by the sigmoidal appearance of the activity versus [Mg\(_2\)] profile at pH 7.2 (Fig. 9), which did not occur for the hexamer or trimer in the same range of Mg\(_2\) concentrations. The ascending parts of the profiles shown in Fig. 9 are shifted to higher Mg\(_2\) concentrations compared with the hexamer and trimer, and no drop in activity (i.e. \(k_{cat}/K_m\)) is observed at up to 50 mM Mg\(_2\) concentration. In contrast, \(k_{cat}/K_m\) is reduced 2.5- to 15-fold for the hexamer and trimer in the presence of 20 mM Mg\(_2\) at pH 9.3–9.5 because of competition between the metal ion bound to M2 and the second metal ion of Mg\(_2\)PPase (10, 21). This means that the M2 site also exhibits a markedly decreased affinity in the quadruple variant or that substrate binding becomes dependent on the presence of Mg\(_2\) at both M1 and M2 sites over the whole pH range examined.

**Cooperativity in Catalysis by Hexameric Y77D/Q80E/H136Q-PPase—**Catalysis by wild-type and variant PPases of different oligomeric states follows Michaelis-Menten kinetics, consistent with the notion that all six active sites function independently. The two exceptions were hexameric Y77D/Q80E/H136Q- and Y77A/Q80E/H136Q-PPases, which exhibited positive cooperativity among subunits, as illustrated in Fig. 10 for one of the variants. The profiles shown were analyzed in terms of Scheme II, which assumes two equal populations of

| Enzyme variant | pH | Temperature °C | [Mg\(_2\)] mm | \(K_d\) \(\mu\)M | \(A_1\) s\(^{-1}\) | \(A_2\) s\(^{-1}\) |
|----------------|----|---------------|----------------|----------------|----------------|----------------|
| Y77D/H136Q/H140Q | 7.2 | 20 | 1 | 0.39 ± 0.06 | 2.9 ± 0.1 | <0.5 |
| Y77D/Q80E/H136Q/H140Q | 7.2 | 2 | 1 | 200 ± 40 | 2.5 ± 0.2 | 0.25 ± 0.01 |
| Y77D/Q80E/H136Q/H140Q | 7.2 | 20 | 1 | 5 ± 1 | 3.7 ± 0.2 | <0.5 |
| Y77D/Q80E/H136Q/H140Q | 8.5 | 20 | 1 | 2.2 ± 0.5 | 4.0 ± 0.1 | <0.5 |
| Y77D/Q80E/H136Q/H140Q | 8.5 | 20 | 50 | 0.04 ± 0.02 | 4.1 ± 0.1 | |

**Fig. 7.** Value of \(k_{cat}/K_m\) for dimeric Y77D/Q80E/H136Q/H140Q-PPase measured in the presence of 20 mM Mg\(_2\) as a function of pH. Stock enzyme solution containing 50 \(\mu\)M enzyme, 1 mM Mg\(_{2}\)-ly in 0.1 mM Tris-HCl (pH 7.2), and 40 \(\mu\)M EGTA was preincubated for 1 h at 20 °C to convert the enzyme into dimers. The buffers were: pH 7.2, 83 mM TES/KOH, 17 mM KCl; pH 8.5, 90 mM CAPS/KOH, 5 mM EGTA; pH 9.3, 52 mM TAPS/KOH, 48 mM CAPS/KOH; pH 10.0, 70 mM CAPS/KOH, 30 mM KCl. Previously measured data for hexameric WT-PPase (21) and trimeric H136Q/H140Q-PPase (10) are also shown.

**Fig. 8.** Mg\(_2\) binding to dimeric Y77D/Q80E/H136Q/H140Q-PPase as measured by equilibrium dialysis at pH 7.2. The lines show the binding profiles for hexameric WT-PPase and trimeric H136Q/H140Q-PPase (10); \(n\) indicates the number of Mg\(_2\) ions bound per monomer. [PPase] was 0.5–0.7 mM; the buffer was as for Fig. 7.
assay medium contained 1 mM Mg$_2$PPi and the buffers used for Fig. 7. A function of Mg$^{2+}$ hexameric Y77D/H136Q-PPase at 20 mM Mg$^{2+}$.

The enzyme was converted into hexameric form Q80E/H136Q-PPase. (9–28% of wild-type PPase) compared with ours (Table II). Our results are consistent with the V75D variant of E-PPase (Val75 in B. stearothermophilus PPase). The change from Tyr77 to Asp is measured.

**Catalytic Properties of Different Oligomeric Forms of E-PPase**—The results of this study identify Tyr$^{77}$ as an important residue in the intratrameric interface of E-PPase. Replacing Tyr$^{77}$ with Asp is enough to dissociate hexamers into dimers at pH ≤ 5.5 (Table I). The Q80E substitution also destabilizes this interface (Table II), but the effect is smaller and does not result in dimers or monomers under the same conditions (Table I). It is also, however, true that the change from Tyr$^{77}$ to Asp is larger than from Gln$^{80}$ to Glu, which is isosteric. The effects of the Y77D and Q80E substitutions on the intratrameric interface may result because the charged side chains introduced in these substitutions are not favored in a relatively hydrophobic environment (Fig. 1C). Cavity formation and loss of the Tyr$^{77}$–Asn$^{24}$ hydrogen bond in the minor intertrimeric interface are two other important factors that may contribute to the effects of Tyr$^{77}$ substitutions. With the Q80E substitution, electrostatic repulsion of charged Glu$^{80}$ side chains may play a role, as the distances between Gln$^{80}$ side chains within trimer are only 3.8 Å (22).

In addition, hexamers cannot be dissociated even by extensive substitutions at the intratrameric interface unless the intertrimeric contact is broken. This can be achieved by incubating the enzyme at pH < 6 (12, 18) or by substituting at least one His residue monomer in the intratrameric contact (10). Thus, the Y77D and Y77D/Q80E variants remained hexameric at pH 7.2 but could be dissociated into dimers by adjusting the pH to ≤ 5.5 or by making additional mutations of His$^{136}$ and/or His$^{140}$ (Table I). Consequently, the monomer–monomer interactions building the dimer are not from the intertrimeric interface, which is completely destabilized under these conditions (10, 12).

The intertrimeric interaction in *Bacillus stearothermophilus* PPase is weak even at neutral pH, which allowed Shinoda et al. (23) to dissociate this enzyme into monomers by making a single substitution (V75D or V75K) at the intratrameric interface. However, the variant enzymes contained some of the trimeric form, which may explain their rather high activity (9–28% of wild-type PPase) compared with ours (Table II). Our attempts to incorporate the V84D or V84K mutation into the trimeric HI136/140Q variant of E-PPase (Val$^{77}$ in B. stearothermophilus PPase) corresponded to Val$^{84}$ in E-PPase) yielded no recombinant enzyme, suggesting severe distortion of the enzyme structure by these mutations.

**DISCUSSION**

**Cooperative kinetics of Mg$^{2+}$ hydrolysis by Y77D/Q80E/H136Q-PPase (S = Mg$_2$PPi).**

$K_{m1}$ and $K_{m2}$ obtained from fitting the data to Equation 3 were $4300 \pm 600$ and $50 \pm 10 \mu M$, respectively, at $2 \mu M$ Mg$^{2+}$ and $1700 \pm 200$ and $19 \pm 4 \mu M$, respectively, at $20 \mu M$ Mg$^{2+}$. In contrast, hexameric WT-, Y77D-, Q80E-, Y77D/Q80E-, and Q80E/H136Q-PPases exhibited single $K_{m}$ values of 1.6, 10, 0.4, 8, and 4 $\mu M$, respectively, in the presence of 2 $\mu M$ Mg$^{2+}$. Kinetic characterization of hexameric Y77D/H136Q-PPase could only be performed at $20 \mu M$ Mg$^{2+}$ because of rapid inactivation during assays at lower Mg$^{2+}$ concentrations. The characterization of hexameric Y77D/H136Q-PPase at $20 \mu M$ Mg$^{2+}$ yielded a $K_{m}$ value of 25 $\mu M$. Furthermore, all variants except for Q80E had greater $K_{m}$ values than the wild-type E-PPase, but none reached the level observed for the Y77D/Q80E/H136Q variant.

$$K_{m1} = K_{m2} = K_{m}$$

**Schema II.** Substrate binding and hydrolysis by Y77D/Q80E/H136Q-PPase (S = Mg$_2$PPi).  

$$V_{h} = k_{cat}[E][S] + 0.5K_{m2}[S] + K_{m2}$$

Equation 3

Four lines of evidence indicate that the apparent cooperativity seen in Fig. 10 does not result from interconversion between different oligomeric forms in the assay medium. First, the enzyme was clearly hexameric in its stock solution from which aliquots were added to the reaction mixture. This is indicated by $r_{0.5}$ values of $6.6 \pm 0.2$ and $6.0 \pm 0.2 S$ measured at 10 and 100 $\mu M$ enzyme concentrations, respectively, and by the independence of measured specific activity on the concentration of stock enzyme solution over the range of 0.05–50 $\mu M$ (the partial dissociation seen at low temperature (Table I) was avoided in this case by keeping the stock enzyme solution at 25 °C). Second, product formation curves were linear in all cases, indicating no interconversion between dimer/trimer and hexamer during measurement. Third, the specific activity of Y77D/Q80E/H136Q-PPase remained constant when its concentration was varied in the range of 0.25–1.75 $\mu M$ in the reaction medium. Finally, the profiles of rate versus substrate concentration do not show enzyme heterogeneity similar to that in Fig. 6, as would have occurred, for example, with a mixture of hexamer and dimer/trimer with different $K_{m}$ values.
charge, and activate the nucleophile and the leaving group (4, 8, 24). Because each PPase monomer carries a full active site capable of binding all these reactants, the changes in catalysis caused by disrupting PPase quaternary structure with point mutations presumably result from distortions of the active site cavity. The major effects of changes to the quaternary structure of E-PPase are on substrate and metal ion binding.

Substrate binding, as characterized by \( k_{cat}/K_m \), is progressively weakened from hexamer to monomer (100, 0.30, 0.018, and <0.0001 \( \mu \)M \(^{-1} \)s\(^{-1} \) for hexamer, trimer, dimer, and monomer, respectively). At least for the trimer, this results from decreased substrate binding affinity, the consequence of a decreased forward rate constant for substrate binding with little or no change in the backward rate constant (12). The similarity of the \( k_{cat}/K_m \) values for wild-type trimer (Fig. 6B) and H136Q/H140Q trimer (10) indicates no direct effect of the H136Q and H140Q substitutions on active site. In contrast, the difference in \( k_{cat}/K_m \) between trimer and dimer may be a direct effect of the Y77D substitution not its effect on oligomeric structure. This is because \( K_m \) value increases in the hexameric Y77D and Y77D/Q80E variables. If so, the trimer to dimer conversion by itself has a smaller effect on catalytic properties than the hexamer to trimer. The behavior of the subunit interface variants thus supports the idea that destabilization of \( \alpha \)-helix A (residues 128–140), which, \( \alpha \)-helix A (residues 128–140), makes very important contributions to the intertrimeric contact and also forms an essential part of the active site cavity (5). Dissociation into dimers and monomers probably mispositions a long excursion (residues 22–53), which, as two basic residues, Lys29 and Arg43, which make important interactions and, consequently, distorted active site cavity exhibit strong positive cooperativity in substrate binding (Fig. 10). In these variants, substrate binding at one monomer causes a conformational change that brings a contacting monomer (presumably in the other trimer) into substrate-binding conformation. An association between cooperativity and an increase in the Michaelis constant has also been reported for variant tyrosyl-tRNA synthetases (25) and glutathione transferases (26).

Partial relaxation accompanied by some distortion to active site may occur in the subunit interface variants even when hexameric. The \( K_m \) value is increased significantly in almost all such variants, which can form hexamers (see also Ref. 9). The largest effect is observed with the hexameric Y77D/Q80E/H136Q and Y77A/Q80E/H136Q variants, for which \( K_m \) values are greater than for other hexameric variant E-PPases and even greater than for the trimeric wild-type E-PPase.

If, as proposed, hexameric wild-type E-PPase is preformed into the substrate/product binding conformation, all six active sites should function independently if the conformational changes during catalysis are small. The same model explains why hexameric Y77D/A/Q80E/H136Q variants with partly impaired subunit interactions and, consequently, distorted active site cavity exhibit strong positive cooperativity in substrate binding (Fig. 10). In these variants, substrate binding at one monomer causes a conformational change that brings a contacting monomer (presumably in the other trimer) into substrate-binding conformation. An association between cooperativity and an increase in the Michaelis constant has also been reported for variant tyrosyl-tRNA synthetases (25) and glutathione transferases (26).

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