Directed Mutagenesis Studies of the Metal Binding Site at the Subunit Interface of *Escherichia coli* Inorganic Pyrophosphatase*

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Irina S. Efimova‡, Anu Salminen§, Pekka Pohjanjoki†, Jukka Lapinniemi†,
Natalia N. Magretova‡, Barry S. Cooperman§, Adrian Goldman§, Reijo Lahti§, and
Alexander A. Baykov++

From the ‡A. N. Belozersky Institute of Physico-Chemical Biology and School of Chemistry, Moscow State University, Moscow 119992, Russia, the §Department of Biochemistry, University of Turku, FIN-20014 Turku, Finland, the ++Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, and the Centre for Biotechnology, University of Turku and Åbo Akademi University, FIN-20251 Turku, Finland

Recent crystallographic studies on *Escherichia coli* inorganic pyrophosphatase (E-PPase) have identified three Mg$^{2+}$ ions/enzyme hexamer in water-filled cavities formed by Asn$^{24}$, Ala$^{25}$, and Asp$^{26}$ at the trimer-trimer interface (Kankare, J., Salminen, T., Lahti, R., Cooperman, B., Baykov, A. A., and Goldman, A. (1996) Biochemistry 35, 4670–4677). Here we show that D26S and D26N substitutions decrease the stoichiometry of tight Mg$^{2+}$ binding to E-PPase by approximately 0.5 mol/mol monomer and increase hexamer stability in acidic medium. Mg$^{2+}$ markedly decelerates the dissociation of enzyme hexamer into trimers at pH 5.0 and accelerates hexamer formation from trimers at pH 7.2 with wild type E-PPase and the N24D variant, in contrast to the D26S and D26N variants, when little or no effect is seen. The catalytic parameters describing the dependences of enzyme activity on substrate and Mg$^{2+}$ concentrations are of the same magnitude for wild type E-PPase and the three variants. The affinity of the intertracer site for Mg$^{2+}$ at pH 7.2 is intermediate between those of two Mg$^{2+}$ binding sites found in the E-PPase active site. It is concluded that the metal ion binding site found at the trimer-trimer interface of E-PPase is a high affinity site whose occupancy by Mg$^{2+}$ greatly stabilizes the enzyme hexamer but has little effect on catalysis.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase$^1$) belongs to a group of enzymes catalyzing phosphoryl transfer from phosphoric acid anhydrides to water, a principal reaction of cellular energetics. Soluble PPases hydrolyze PP$_i$ to Pi, with release of energy as heat and provide in this way a thermodynamic pull for many biosynthetic reactions (1). PPases are essential in many other prokaryotic PPases (5–8). The active site, present in each 20-kDa monomeric unit, contains 14 polar amino acid residues that are completely conserved in all known soluble PPases, despite only moderate sequence similarity in the rest of the molecule (9). The overall folding motifs are very similar in E-PPase (10, 11) and *Thermus thermophilus* PPase (8) as well as in the core part of the larger (32 kDa per subunit) PPase of *Saccharomyces cerevisiae* (12, 13).

The E-PPase hexamer is arranged as a dimer of trimers (11, 14–16). The principal trimer-trimer interaction involves a three-center ionic, hydrogen-bonding interaction among the residues His$^{140}$-Asp$^{143}$-His$^{136}$ (11, 14). Another important trimer-trimer interaction occurs among Asn$^{24}$, Ala$^{25}$, and Asp$^{26}$ (Fig. 1). The cavity formed by Asn$^{24}$-Asp$^{26}$ and Asn$^{24}$-Asp$^{26}$ contains several water molecules one of which is replaced by Mg$^{2+}$ when the crystals are soaked in a decimolar concentration of a magnesium salt (15, 17). As a result of the Mg$^{2+}$ binding, the side chains of the Asn$^{24}$ residues reorient, and the trimers move toward each other by about 0.4 Å.

Metal ions, including Mg$^{2+}$, are rare but not uncommon at subunit interfaces (18–21). Brinen et al. (22) were able to engineer a metal binding site at the interface formed by trypsin and its protein inhibitor ecotin. However, in all known instances the intersubunit metal ion is coordinated directly to protein residues except for E-PPase where it is connected through water molecules (15, 17). On the other hand, water-filled cavities often occur at protein interfaces (23) and in protein interiors (24). Because even large molecules can rapidly penetrate deep into proteins and bind to seemingly inaccessible cavities (25), it is not unlikely that, in vivo, protein cavities can contain metal ions, especially Mg$^{2+}$, the most abundant divalent cation in the cell. Understanding the effects of these cavities on protein properties may therefore be important in understanding how proteins function in vivo as well as in designing interacting proteins and ligands that can disrupt protein-protein interactions.

The present work addresses two major questions: (a) Does the intertrimer cavity in E-PPase bind Mg$^{2+}$ strongly enough to be occupied appreciably at its physiological concentration? (b) What is the role of the unusual binding site in hexamer stability and catalytic activity?

**EXPERIMENTAL PROCEDURES**

**Enzymes**

Wild type and wild type-free variant PPases were expressed using the overproducing *E. coli* strains HB101 (26) and MC1061/YPPA/Δppa (27), respectively. The enzymes were purified by chromatography on Fast Flow DEAE-Sepharose (Amersham Pharmacia Biotech), heat treatment at 65 °C, and gel filtration on a column (2.6 × 60 cm) of Superdex 200 (Amersham Pharmacia Biotech) (27). The final prepara-
Intersubunit Metal Ion in Pyrophosphatase

Methods

Initial rates of PPase hydrolysis were estimated from continuous recordings of phosphate production obtained with an automatic phosphate analyzer (30). The assay medium of 25 ml total volume contained, except as noted, 20 μM Mg2PPi, 20 mM Mg2+, 0.05 M Tris/HCl (pH 7.2), and 40 μM EGTA. The reaction was initiated by adding a suitable aliquot of enzyme solution and carried out for 3–4 min at 25 °C. No appreciable interconversion between the hexamer and less active trimer was observed during the assay, as evidenced by nearly linear product formation curves.

Differential spectra of PPase induced by Mg2+ were recorded with a computer-controlled LKB Ultraspec Plus spectrophotometer in a 1-cm cuvette containing 0.7 ml of enzyme solution. After the base line stabilized, stock MgCl2 solution was added in 0.7-ml increments, and the spectra were recorded.

Equilibrium microdialysis (31) and analytical ultracentrifugation (32) were performed as described.

Calculations and Data Analysis

Hexamer-Trimer Equilibration—Equations 1 and 2, derived from Scheme I, describe time courses of activity (a) resulting from hexamer (E6) dissociation into trimers (E3) and the reverse reaction, as well as the equilibrium activity (at t = ∞, da/dt = 0) as functions of enzyme concentration, A4 and A0 are specific activities of the hexamer and trimer, respectively, aH is the fraction of enzyme in hexameric form at time t, [E] is total enzyme concentration, expressed in monomers, k+ and k− are the apparent rate constants for hexamer formation and breakdown, respectively, measured at a fixed pH and Mg2+ concentrations. Equations 1 and 2 were fitted to data simultaneously with SCIENTIST (MicroMath).

\[
2E6 \rightleftharpoons E3 + E6
\]

Scheme I. Hexamer-trimer equilibrium

\[
A = A_0 + (A_H - A_0)a_H
\]

\[
\frac{da_H}{dt} = \frac{2}{3k_{12}}[E]_t^1(1 - a_H)^2 - k_{-12}a_H
\]

The pH dependence of PPase activity at hexamer-trimer equilibrium is described by Equations 1 and 2 with da/dt = 0 and the apparent \( k_{12} \) given by Equation 3, where \( K'_{H} \) is the pH-independent dissociation constant for the hexamer, \( K'_H \) is the microscopic dissociation constant for H+ binding to the trimer, and m is the number of protons bound per hexamer.

\[
K'_H = K'_H \left( 1 + \frac{[H^+]}{K_H} \right)^m
\]

The effect of Mg2+ on \( k_a \) at fixed pH is described by Equation 4, derived for the simplified model shown in Fig. 2. The model assumes that \( a \) a hexamer is formed from two trimers containing in total from zero to three metal ions bound at the trimer-trimer interface and \( b \) the association rate constant is equal to \( k_{a0} \) for trimers without metal ions and increases by the same factor \( p \) with each added metal ion. Here \( t = 1/K_m(M) \) is the probability of finding a metal ion (M) in one of the three metal ion binding sites/trimer, and \( K_{M} \) is the respective metal binding constant for the trimer. Statistical factors of 12 (6 + 6) and 4 (2 + 2) were used when more than one metal ion was present in two interacting trimers, taking into account that the combination of two metal-bound monomers within a single subunit-subunit interface is not allowed (Fig. 2).

\[
k_a = k_{a0}[1 - (1 - p)^3] + 6p[1 - (1 - p)^2] + 12p^2[1 - (1 - p)] + 12p^3[1 - (1 - p)^2] + 6p^3[1 - (1 - p)] + p^4[1 - (1 - p)]
\]

Equilibrium Dialysis—Values of the dissociation constants for the successive binding of two Mg2+ ions to the E-PPase active site (K_{M1} and K_{M2}) and for Mg2+ binding to the subunit interface ([K_{M}]) were estimated by fitting Equation 5 to measured values of n, the number of Mg2+ ions bound per monomer, as a function of free Mg2+ concentration. The first term in Equation 5 describes binding to the active site, and the second binding to the intertrimer site. The second term was ignored for variants that exhibited no binding to the intertrimer site (K_{M} = ∞). Scheme II assumes that the binding reactions in the active site and in the intertrimer site are mutually independent.

\[
\eta = \frac{K_{M1}[M] + 2[M]^2}{K_{M1}[M] + [M]^2} + \frac{K_{M2}[M] + 3[M]^2}{K_{M2}[M] + [M]^2}
\]

Spectral Titration—The dependence of \( \Delta A_{245} \) on Mg2+ concentration at 245 nm, on Mg2+ concentration was fitted with Equation 6, where \( \Delta A \) is the change in the extinction coefficient at saturating [M]. Equation 6 implies \( K_{M1} >> K_{M2} \).

\[
\Delta A_{245} = \frac{\Delta \epsilon [E][M]}{K_{M1}[M] + [M]^2}
\]

PPase, Hydrolysis—Values of the apparent catalytic constant \( h_a \) and the apparent Michaelis constant \( K_{M,a} \) were obtained from rate versus [PPase] dependence measured at fixed Mg2+ concentrations. Values of \( h_a/K_{M,a} \) as a function of [Mg2+] were fitted to Equation 7, allowing an estimation of \( k_{-12}, k_{12}, k_{-32}, k_{32} \), the rate constants for substrate binding to the ME, ME, ME(M), and ME(M) species (M in parentheses denotes the metal ion that is bound at the subunit interface), respectively (Scheme II). The assumption in deriving Equation 7 is that \( h_a \) greatly exceeds the rate constant for Mg2+ release from the enzyme, has been shown elsewhere for wild type E-PPase (33). The values of \( K_{M1}, K_{M2}, \) and \( K_{M,a} \) used in this fitting were those estimated by equilibrium dialysis (see above).

\[
\frac{k_a}{K_{M,a}} = \frac{k_{-12}K_{M1}[M] + k_{12}K_{M2}[M] + k_{-32}K_{M2}[M] + k_{32}K_{M1}[M] + k_{i2}K_{M2}[M] + k_{i3}K_{M1}[M] + [M]^2}{K_{M1} + [M][K_{M1}K_{M2} + K_{M1}[M] + [M]^2} \]

Fig. 1. Part of the trimer-trimer interface in E-PPase, containing an Mg2+ ion (black sphere) surrounded by six water molecules (15). Hydrogen bonds are shown as lines. The two monomers that contribute to the interface are distinguished by the primed and unprimed labels.
The concentrations of free Mg sup+ and Mg,PP, at pH 7.2 were calculated using the dissociation constants of 0.112 and 2.84 mM for the MgPP, and Mg,PP, complexes, respectively (34).

RESULTS

pH Effects on Quaternary Structure and Enzyme Activity—
The effects of substitutions at both Asn sup24 and Asp sup26 on quaternary structure and enzyme activity were studied as a function of pH. Direct evidence for shifts in the hexamer-trimer equilibrium was obtained from sedimentation data (Table I). At pH 7.2, s 20,w for WT-PPase, and the three variants studied here (N24D, D26N, D26S) fell in the range 6.3–6.7 S, characteristic of a hexamer (28, 32). Lowering the pH to 5.0 had differential effects. s 20,w decreased to 4.3 for both WT-PPase and N24D-PPase, decreased only to 5.2 for D26S-PPase, and was unchanged for D26N-PPase. Further lowering the pH to 3.8 led to s 20,w values of 3.3–4.0 for all four PPases. Because the molecular mass of WT-PPase at pH 5.0 estimated by sedimentation equilibrium measurements (20 °C, 20 μM initial enzyme concentration) was 63 ± 1 kDa, these results indicate that WT-PPase and N24D-PPase are fully in the trimeric form by pH 5.0 but that lower pH is required for full conversion to trimer of the D26S and D26N variants. That such dissociation is reversible is shown by the increase in s 20,w back to 6.7 ± 0.2 S when the pH of a WT-PPase sample is raised from 5.0 to 7.2. At pH 3.8, the variant PPases may undergo further dissociation to dimers or monomers as suggested by lower s 20,w values compared with WT-PPase.

Earlier we had shown for other variants with weakened trimer-trimer interaction (32) that trimers had lower activity than hexamers at low (20 μM) substrate concentration as a result of a drastic increase in K dis, (10 3-10 5-fold), with little change in k h. Trimers of WT-PPase and the three variants investigated in this paper also had lower activity, and studies of the effect of pH on enzyme activity, paralleling those on s 20,w, demonstrated conclusively that rates and equilibrium of hexamer-trimer interconversion could be monitored by activity measurements. The activities presented in Fig. 3 represent equilibrium values, no changes being observed on longer incubation at the final pH values. Matching the s 20,w values, they demonstrate that as pH is lowered from 7.2 to 5.0, WT- and N24D-PPase are fully converted to less active forms, D26S-PPase is partially converted, whereas D26N-PPase retains almost full activity, and, in addition, lowering the pH further to 3.8 converts the latter two variants virtually completely to their lower activity forms. Furthermore, WT-PPase activity increases with enzyme concentration at pH 5.0 (Fig. 4, inset), as expected for an equilibrium between an active hexamer and less active trimer, and activity is a fully reversible property of pH (Fig. 4). The N24D, D26S, and D26N variants, inactivated at pH 5.0, 4.8, and 3.9, respectively, could also be reactivated at pH 7.2, affording > 80% of original activity. Finally, in all cases, inactivation and reactivation followed strict first- and second-order kinetics, respectively.

The data in Fig. 3 could be fit quantitatively to a model (Equations 1–3) in which deprotonation of a group within trimers (which has a much lower pK a in a hexamer) is required for hexamer formation. Satisfactory fits were obtained when the number of bound protons per hexamer (m) was equal to 6, 2, 2, or 12 for WT-, D26N-, D26S-, and N24D-PPase, respectively. From earlier work (35), K a for WT-PPase is < 1 mM, allowing a lower limit estimate of pK a of 6.7.

Mg sup2+ Effects on Rates and Equilibrium of Hexamer ↔ Tri- mer Transition—Mg sup2+ has been shown to stabilize the hexameric structure of variant E-PPases with weakened trimer-trimer interactions (32, 35). As estimated from time courses of activity loss at pH 5.0 and restoration at pH 7.2, added Mg sup2+ markedly decreased k d (Fig. 5) and increased k a (Fig. 6) for WT- and N24D-PPases. The sigmoidal dependence of k a on [Mg sup2+] was described satisfactorily by Equation 4, yielding fitted val-
Parameters describing Mg\(^{2+}\) effects on trimer reactivation at pH 7.2, 25 °C, (0.1 Tris/HCl, 40 μM EGTA).

| Enzyme variant | \(k_d\) | \(k_{d,0}\) | \(p\) |
|---------------|--------|----------|-----|
| WT           | 42 ± 6 | 0.9 ± 0.1 | 6.0 ± 0.4 |
| N24D         | 43 ± 6 | 0.5 ± 0.1 | 8.9 ± 0.8 |
| D26S         | 4.2 ± 0.4 |        |     |

dialysis, providing the macroscopic binding constants for all three types of the metal binding sites present in E-PPase, M1 and M2 at the active site and M\(_m\) at the trimer-trimer interface, and by differential spectroscopy (16), which, as shown recently, provides the binding constant for M2.

The dialysis data indicated a decrease in the stoichiometry of tightly binding sites by approximately 0.5 mol/mol monomer in the D26S and D26N variants, but not in the N24D variant, when compared with WT-PPase (Fig. 7). These data are thus consistent with a loss of M\(_m\) upon Asp\(^{90}\) substitution and also suggest that M\(_m\) displays high affinity for Mg\(^{2+}\). These conclusions were supported by quantitative analysis of the binding data. The binding affinity of M1 was only slightly affected by the mutations, whereas the affinity of M\(_m\) was increased 3-fold after N24D substitution, which introduced a negatively charged ligand into the intertrimer cavity. Accordingly, in the N24D variant sites M1 and M\(_m\) have similar affinity for Mg\(^{2+}\), whereas in WT-PPase the order in which the three sites become occupied at increasing Mg\(^{2+}\) concentration is M1, M\(_m\), M2 (Table III).

As measured by spectral titrations conducted at pH 8.5 (the effect of Mg\(^{2+}\) binding on absorbance was quite small at pH 7.2 used in the dialysis experiments; see Ref. 16) the affinity at site M2 appeared unaffected by the substitutions (Table III). Moreover, the values obtained for the three variants are essentially identical to those measured by dialysis at pH 7.2. The one apparent inconsistency in the data concerns the considerably higher value obtained by dialysis for WT-PPase at pH 7.2. However, the error in this value is quite high because it greatly exceeds the enzyme concentration used in these measurements.

Kinetics of PP Hydrolysis—As measured by \(k_1\), \(k_{h/K_m,h}\), and \(k_1\), substitution at the subunit interface has only minor effects on PPase catalytic function determined at pH 7.2 as a function of [Mg\(^{2+}\)]. Values of \(k_{h/K_m,h}\) (Fig. 8) were generally quite similar for WT, N24D-, D26N-, and D26S-PPases, with the exception that \(k_{h/K_m,h}\) was somewhat lower for N24D-PPase. Values of \(k_1\) were also determined as a function of [Mg\(^{2+}\)] and interpreted according to Scheme II (Table IV). For WT-PPase and N24D-PPase, binding to the interface site has at most a small effect on \(k_1\) (i.e. \(k_1\) is approximately equal to \(k_1^{(1)}\), but binding to M2 lowers \(k_1\) (\(k_1^{(3)} > k_1^{(1)}\)). Consistent with these results, the two variants lacking a high affinity Mg\(^{2+}\) interface site, D26N- and D26S-PPases, have \(k_1^{(1)}\) values similar to that for WT-PPase, and their \(k_1^{(3)}\) values (reflecting M2 site occupancy) are both lower than their \(k_1^{(1)}\) values and similar to \(k_1^{(4)}\) for WT-PPase. It should be noted that \(k_1^{(2)}\) could not be estimated for WT- and N24D-PPase because the M\(_{E}\) species was stoichiometrically insignificant for these variants.

**DISCUSSION**

Equilibrium dialysis measurements have revealed three types of metal binding sites in E-PPase (33), which we now designate as sites M1, M\(_m\), and M2, following the order in which they become occupied in WT-PPase at an increasing Mg\(^{2+}\) concentration at pH 7.2 (Table III). We assumed previ-
ously that the three binding sites are present in each subunit of homohexameric E-PPase (33). However, crystallographic identification of an Mg\(^{2+}\) ion between pairs of subunits (15, 17) together with the effects of Asn\(^{24}\) and Asp\(^{26}\) mutations on metal binding and hexamer stability reported above suggest a binding stoichiometry of 0.5 mol/molec for one of the sites detected by equilibrium dialysis. Two other lines of evidence support the idea that the site with intermediate affinity is located at the subunit interface. First, X-ray crystallographic data show that E-PPase crystals soaked in 140 mM MgCl\(_2\) contain Mg\(^{2+}\) only at sites M1 and M2 (15), whereas crystals grown at 250 mM MgCl\(_2\) contain Mg\(^{2+}\) at all three sites (17). Also, the site that binds Mn\(^{2+}\) most tightly is located in the active site and exhibits a 1 mol/mol binding stoichiometry (11). Second, site M\(_a\) is apparently absent in trimeric E-PPase prepared by substitution of both His\(^{136}\) and His\(^{140}\) in the trimer-trimer interface (36).

Our results clearly show that [H\(^+\)] and [Mg\(^{2+}\)] determine the quaternary structure of E-PPase and agree with earlier studies showing that (a) low pH induces PPase hexamer dissociation (6, 37) and (b) in variants with weakened trimer-trimer interactions (H136G/PPase, 33; E203D/PPase35) added Mg\(^{2+}\) stabilizes hexamer formation at pH values \(\geq 7.2\). A simple model consistent with our results is that protonation of one or more sites per monomer induces dissociation (Fig. 3). According to this model, added Mg\(^{2+}\) favors the hexamer because it competes with H\(^+\) for a common site (or sites), in addition to stabilizing hexamer structure as such directly. Consistent with this model are (a) the increased stability of the D26S and D26N hexamers because substitution of Asp\(^{26}\) eliminates a potential protonation site, (b) the shift in activity versus pH profiles to lower pH values with increasing Mg\(^{2+}\) concentration, (c) the stability of hexamers at higher pH even in the absence of added Mg\(^{2+}\), and (d) the rise in \(k_a\) for WT- and N24D-PPase with increasing [Mg\(^{2+}\)] at pH 7.2, when the basic group(s) governing hexamer stability is(are) deprotonated (Fig. 6).

This model certainly suggests that Asp\(^{26}\) is at least one, and perhaps the major, basic group controlling hexamer stability in WT-PPase. This would require Asp26 to have a markedly elevated \(pK_a\) of \(> 6.7\), which would not be unexpected, given the extensive hydrogen bonding network at the subunit interface site (see Fig. 1). The value of \(pK_a\) equal to 6 for WT-PPase suggests that all three Asp\(^{26}\) residues/trimer become protonated on subunit dissociation. Following this logic, it is tempting to speculate that the increase in \(m\) value to 12 for the N24D variant reflects protonation of the Asp\(^{26}\) and Asp\(^{26}\) residues in this variant. That \(m\) does not become 0 in the D26S and D26N variants would imply the presence of (an)other, lower \(pK_a\) group(s) whose protonation also destabilizes the hexamer. One possibility is His\(^{140}\), as suggested previously (14, 32). Alternatively, other carboxyl side chains, as yet unidentified, could be involved.

It remains unclear why protonation of Asp\(^{26}\) would destabi-
lize the hexamer, whereas mutation of Asp26 into Asn or Ser does not. The simplest possibility is that the hydrogen bonding network at the subunit interface site (Fig. 1) requires a hydrogen-bond acceptor at position 26, a role that can be filled by a carboxylate anion, by an amide, and by an alcohol, but not by a carboxylic acid. High resolution structures of the variants described in this paper could provide a clear test of the validity of our proposals.

In summary, we have shown that the metal binding site formed by protein-bound water molecules at the trimer-trimer interface of E-PPase is a high affinity site whose occupancy by Mg\(^{2+}\) stabilizes the enzyme hexamer by neutralizing the negative charges and preventing protonation of a site or sites within the binding cavity, without having any substantial effect on catalysis.

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