Dissociation of Hexameric Escherichia coli Inorganic Pyrophosphatase into Trimers on His-136 → Gln or His-140 → Gln Substitution and Its Effect on Enzyme Catalytic Properties*

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Alexander A. Baykov‡§, Valerij Yu. Dudarenkov‡, J armo Käpyläi, Tiina Salminen**, Teppo Huutti, Vladimir N. Kashott, Sari Husgafvel, Barry S. Cooperman¹, Adrian Goldman**, and Reijo Lahti‡‡

From the IA. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia, the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the Department of Biochemistry, University of Turku, FIN-20500 Turku, Finland, the Centre for Biotechnology, FIN-20521 Turku, Finland, and the Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024

Each of the five histidines in Escherichia coli inorganic pyrophosphatase (PPase) was replaced in turn by glutamine. Significant changes in protein structure and activity were observed in the H136Q and H140Q variants only. In contrast to wild-type PPase, which is hexameric, these variants can be dissociated into trimers by dilution, as shown by analytical ultracentrifugation and cross-linking. Mg²⁺- and substrate-stabilized hexameric forms of both variants. The hexameric H136Q- and H140Q-PPases have the same binding affinities for magnesium ion as wild-type, but their hydrolytic activities under optimal conditions are, respectively, 225 and 110% of wild-type PPase, and their synthetic activities, 340 and 140%. The increased activity of hexameric H136Q-PPase results from an increase in the rate constants governing most of the catalytic steps in both directions. Dissociation of the hexameric H136Q and H140Q variants into trimers does not affect the catalytic constants for PP_i hydrolysis between pH 6 and 9 but drastically decreases their affinities for Mg²⁺, Mg⁴⁺, and Mg⁶⁺. These results prove that His-136 and His-140 are key residues in the dimer interface and show that hexamer formation improves the substrate binding characteristics of the active site.

Phosphoryl transfer enzymes form one of the largest classes of enzymes (Knowles, 1980), yet their mechanisms of action are still not fully understood (Herschlag and Jencks, 1990). This class includes soluble inorganic pyrophosphatases (EC 3.6.1.1; PPase),¹ which hydrolyze inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i). These enzymes, essential in both bacteria (Chen et al., 1990) and yeast (Lundin et al., 1991), are ubiquitous and play an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions such as protein, RNA, and DNA synthesis (Kornberg, 1962). According to Peller (1976), nucleic acid synthesis would be energetically impossible in vivo if it were not coupled to the PP_i hydrolysis catalyzed by PPases. The two best-studied soluble PPases are those from the yeast Saccharomyces cerevisiae and E. coli: each accelerates the rate of PP_i hydrolysis by a factor of 10¹⁰ compared with the rate in solution. Detailed understanding of their catalytic mechanisms is important for understanding the class of phosphoryl transfer enzymes as a whole.

E. coli PPase is homohexameric (Wong et al., 1970) and contains 175 amino acid residues per subunit (Lahti et al., 1988). Its three-dimensional structure, recently been determined at 2.5-2.7-Å resolution (Kankare et al., 1994; Oganessian et al., 1994), is very like that of S. cerevisiae PPase (Kuranova et al., 1983; Terzyan et al., 1984), in accord with the conservation of active site residues and mechanism in the two enzymes (Cooperman et al., 1992; Kankare et al., 1994). E. coli PPase requires four Mg⁴⁺ ions per active site for catalysis, as described in Scheme I. This scheme, which fully accounts for the overall catalysis of PP_i catalyzed by E. coli PPase, is a slightly modified version of the one proposed by Baykov et al. (1990) following the approach developed for S. cerevisiae PPase (Springs et al., 1981; Welsh et al., 1983).

The cloning and sequencing of the E. coli ppa gene that encodes PPase (Lahti et al., 1988) together with the analysis of the conservation of functional residues between yeast and E. coli PPase (Lahti et al., 1990a) have made it possible to study the structural and functional relationship of E. coli PPase by site-directed mutagenesis (Lahti et al., 1990b). All 17 polar residues located in the active site cavity have been substituted (Lahti et al., 1990b; Lahti et al., 1991; Cooperman et al., 1992) and some of the variant PPases have been characterized in detail (Salminen et al., 1995; Käpylä et al., 1995).

Earlier chemical modification studies of Samejima et al. (1988) had implicated histidines as being involved in the activity of E. coli PPase. The absence of His residues from the active site cavity (Kankare et al., 1994) and the lack of conserved His residues in soluble PPases (Cooperman et al., 1992) make it clear that His residues have no direct role in catalysis, leaving open the possibility that the chemical modification results arise from an indirect effect. In this work we demonstrate that substitution of each of the five His residues in E. coli PPase with Gln results in substantial retention or even enhancement of catalytic activity, confirming their nonessential character.
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However, in contrast to wild-type enzyme, two variants, H136Q and H140Q, are found to dissociate readily to trimers, providing valuable insight into the details of subunit-subunit interaction.

\[
EMg_5 \rightarrow k_1 \rightarrow EMgPP_6 \rightarrow EMg(MgPi)_7 \rightarrow EMg_5
\]

where \( k_i \) is the catalytic constant for synthesis (see "Results"). Rates of oxygen exchange between \( P_i \) and \( H_2O \) were measured by mass spectrometry as described by Baykov et al. (1990). Other kinetic and binding measurements as well as calculations of various rate and binding constants were carried out as described by Käpylä et al. (1995). Unless otherwise indicated, the media used were buffered with Tris-HCl, the concentration of which was varied to maintain the ionic strength at 0.15–0.20 M. EGTA (typically 50 \( \mu M \)) was included in all solutions containing enzyme. All experiments were carried out at 25 °C.

RESULTS

Preliminary Characterization of Variant PPases—Mutations of the five histidine residues in E. coli PPase to glutamine yielded three variants (H60Q, H110Q, and H119Q) that were unchanged as compared with wild-type enzyme with respect to catalytic activity (measured at pH 8), Nile Red fluorescence (measuring protein surface hydrophobicity; Salminen et al., 1995), and migration as a hexamer on native gel electrophoresis analysis. In contrast, each of the remaining two variants, H136Q and H140Q, showed increased catalytic activity (see below) and much increased Nile Red fluorescence compared with wild-type PPase, as well as the presence of a faster-migrating dissociated form in addition to a hexamer on native gel electrophoresis. Accordingly, only the latter two variants were studied further.

Activity versus Enzyme Concentration Profiles—Specific activities of the H136Q and H140Q variants pre-equilibrated at 25 °C at different enzyme concentrations were measured to define conditions that would favor enzyme dissociation or association. In these experiments, only the composition of enzyme stock solution was varied; the medium for the assays remained the same (20 \( \mu M \) Mg_2PPi, 20 \( \mu M \) Mg_2^2+, 0.15 M Tris-HCl, pH 7.2). The preincubation was carried out for 40–60 min until activity stabilized. The results (Fig. 2) indicate that the specific activity of the enzymes, with the exception of H136Q-PPase at high Mg_2^2+ concentration, decreases with decreasing enzyme concentration. The activity of WT-PPase is not changed on analogous incubations. The effects shown in Fig. 2 are reversible, i.e. the activity could be brought to the levels corresponding to new conditions on changing pH in either direction or increasing Mg_2^2+ concentration from 1 to 50 mM.

These results were analyzed in terms of Scheme II:

\[
\begin{align*}
& k_a H \rightarrow H'; k_d H' \rightarrow H; \quad K_a = k_d/k_a
\end{align*}
\]

using the following relationships (Kurganov, 1982):

\[
A = A_0 + (A_1 - A_0)\alpha_{H,eq}
\]

\[
\alpha_{H,eq} = (z - 1)/(z + 1)
\]

\[
z = \sqrt{1 + 8E/3K_d}
\]

where \( A \) is the observed specific activity, \( A_0 \) and \( A_1 \) are the specific activities of trimeric and hexameric enzyme, respectively; \( \alpha_{H,eq} \) is the fraction of hexameric enzyme at equilibrium (\( \alpha_{H,eq} = E/[E]; [E]_0 \) is total enzyme concentration in terms of monomer. Fitting these equations to the data (Fig. 2) yielded the \( K_d \) values shown (Table I). When fitting the H140Q data obtained at 1 \( \mu M \) Mg_2^2+, the value of \( A_0 \) was fixed at 191 s^−1, the limiting value of activity at infinite enzyme concentration as determined from the profile obtained at 50 \( \mu M \) Mg_2^2+. Measurements of the equilibrium activity at a fixed enzyme concentration (0.11 \( \mu M \)) indicated that...
pH 7.2 is optimal for the stability of H136Q-PPase while the stability of H140Q-PPase increases monotonically up to pH 10.1 (data not shown).

Direct Assessment of the Quaternary Structure of H136Q- and H140Q-PPases—The variant PPases (and WT-PPase as a control) were cross-linked at pH 8.5 and subjected to electrophoresis in the presence of SDS. Only a faint band corresponding to hexameric protein is observed with the variant PPases while WT-PPase yields appreciable amounts of cross-linked hexamers (Fig. 1). Consequently, the variant PPases seem to be predominantly dissociated at pH 8.5 and 17.5 mM enzyme concentration used in cross-linking, in accord with the data in Fig. 2.

Other data are also consistent with the existence of a hexamer-trimer equilibrium in these two variant PPases and support the assumption made above that the effects of enzyme and Mg$^{2+}$ concentrations and pH on their specific activities result from shifts in this equilibrium. Firstly, the sedimentation coefficient ($s_{20,w}$) changes with changing pH and Mg$^{2+}$ concentration (Table I), and this effect correlates with the activity data (Fig. 2). It should be noted that for H1400-PPase, the sedimentation profiles were generally broader than for H136Q-PPase, suggesting that both hexamers and trimers are present in significant, although not equal, amounts. Accordingly, most $s_{20,w}$ values are somewhat lower for hexamer and greater for trimer versus H136Q-PPase (Table I). Secondly, analyzing the electrophoretic mobilities of the H136Q and H140Q variants as functions of polyacrylamide concentration (pH 9.5, in the absence of Mg$^{2+}$) (Hedrick and Smith, 1968) indicated a 2-fold decrease in their molecular weights compared to that of WT-PPase (data not shown). By contrast, a D97E variant, whose specific activity is independent of enzyme concentration, does not show such a decrease (Käpylä et al., 1995).

Rate and Equilibrium Constants for Catalysis by Hexameric Variant PPases—Following the approach devised for S. cerevisiae PPase (Springs et al., 1981), we determined the rate and equilibrium constants of Scheme I for the H136Q- and H140Q-PPases by combining data from equilibrium formation of enzyme-bound PPi with data from the kinetics of PPi hydrolysis, PPi synthesis, and P$_i$/HOH oxygen exchange. From the dependences of hydrolysis rate on [Mg$_2$PPi] and [Mg$^{2+}$] (not shown),
the catalytic constant for exchange at 20 mM Mg\(^{2+}\) values were calculated by extrapolating concentration (Fig. 3 and Equation 3). using the dependence of enzyme-bound PPi formation on MgPi a sufficient amount of P1 was synthesized and liberation of PPi to the medium (medium PPi concentration on the metal ion concentrations (Table III) were used to calculate most, if not all, rate constants for H136Q-PPase were hexameric during these measurements (see “Experimental Procedures”). Additionally, hydrolytic activity was measured at 20 \(\mu\)M Mg\(^{2+}\), (20 mM Mg\(^2+\), pH 7.2) to verify that the hexameric structure is retained by the end of enzyme incubation with P1 and Mg\(^{2+}\) in the oxygen exchange and enzyme-bound PP measurements.

\[ [\text{EPP}_1] = \frac{1}{[\text{E}]_0} \cdot \frac{1}{1 + K_{\text{app}}} \cdot \frac{K_{\text{app}}K_{\text{app}}}{K_{\text{app}}K_{\text{app}}} \quad (\text{Eq. 3}) \]

The results of oxygen exchange measurements at two saturating Mg\(^{2+}\) concentrations (Table IV) were used to calculate the catalytic constant for exchange \(K_{\text{app}}\), and the metal dissociation constants \(K_{\text{M1}}\) and \(K_{\text{M2}}\) (Table III).\(^2\) We also calculated values of \(K_{\text{S1}}, K_{\text{S2}},\) and \(K_{\gamma}\) using the dependence of enzyme-bound PPi formation on MgPi concentration (Fig. 3 and Equation 3).

\[ [\text{EPP}_1] = \frac{1}{[\text{E}]_0} \cdot \frac{1}{1 + K_{\text{app}}} \cdot \frac{K_{\text{app}}K_{\text{app}}}{K_{\text{app}}K_{\text{app}}} \quad (\text{Eq. 3}) \]

\(\text{Table II}\)

| [Enzyme] | pH | [MgCl\(_2\)] | \(s_{\text{Dlow}}\) |
|---------|----|-------------|-----------------|
| \(\mu\)m | mM | \(\mu\)m | WT-PPase | H136Q-PPase | H140Q-PPase |
| 15 | 7.2 | 1 | 5.95 ± 0.2 | 5.77 ± 0.13 | 3.37 ± 0.09 |
| 15 | 7.2 | 50 | 5.85 ± 0.18 | 5.15 ± 0.10 | 2.52 ± 0.03 |
| 5 | 8.5 | 1 | 6.1 ± 0.25 | 3.14 ± 0.09 | 4.62 ± 0.13 |
| 15 | 8.5 | 50 | 5.65 ± 0.12 | 5.23 ± 0.04 |
| 5 | 6.5 | 1 | 3.34 ± 0.04 |

\(^a\) Heterogeneous mixture; the value of \(s_{\text{Dlow}}\) refers to the major component.

\(\text{Table III}\)

| Parameter | pH 7.2 | pH 8.0 | pH 7.2 | pH 7.2 | pH 8.0 | pH 8.0 |
|-----------|-------|-------|-------|-------|-------|-------|
| \(k_{\text{III}}\), s\(^{-1}\) | 210 ± 8 | 480 ± 40\(^b\) | 178 ± 10 | 170 ± 13 | 300 ± 200 | 172 ± 22 |
| \(k_{\text{M1}}\), \(\mu\)M | 2.3 ± 0.2 | 8.0 ± 1.2\(^d\) | 198 ± 6 | 2.7 ± 1.6 | 4200 ± 3400 | 440 ± 110 |
| \(K_{\text{M1}}\), s\(^{-1}\) | 6.8 ± 0.2\(^e\) | 3.6 ± 0.4\(^d\) | >1.0 | 2.8 ± 0.1\(^e\) | 175 ± 9 | 116 ± 21 |
| \(P_1\) | 0.081 ± 0.008 | 0.081 ± 0.005 | 0.157 ± 0.003 | 0.24 ± 0.03 | 0.21 ± 0.03 | 0.6 ± 0.5 |
| \(K_{\text{M1}}\), \(\mu\)M | 6.1 ± 1.0 | 7.9 ± 2.3 | 6.9 ± 0.9 | 2.6 ± 2.3 | 23.6 ± 5.2 | 5.2 ± 3.9 |
| \(K_2\) | 13.4 ± 4.4 | 18.1 ± 9.5 | 8.1 ± 2.7 | 5.8 ± 0.5 | 6.0 ± 2.8 |
| \(K_{\gamma}\) | 2.1 ± 0.7 | 0.27 ± 0.64 (0.78)\(^f\) | 2.3 ± 0.9 | 7.4 ± 1.2 | 13.2 ± 6.7 |
| \(K_{\text{M1}}\), mM (dialysis) | 0.06 ± 0.02 | 0.12 ± 0.04\(^g\) | 0.03 ± 0.04 |

\(\text{Table IV}\)

| Parameter | Hexameric | Trimeric |
|-----------|-----------|---------|
| pH 7.2 | pH 8.0 |

\[^a\] Values from Käpylä et al. (1995).

\[^b\] Measured at 20 mM Mg\(^{2+}\).

\[^c\] Measured at 20 mM concentration of both MgPi and Mg\(^{2+}\) and extrapolated to infinite MgPi concentration with Equation 3.

\[^d\] Measured at 10 mM MgPi and 20 mM Mg\(^{2+}\) and extrapolated to infinite MgPi concentration with Equation 3.

\[^e\] Calculated as \(k_\text{f}/k_\text{d}\) (see Table V).

\[^f\] Value in parentheses calculated from \(K_\gamma = K_{\text{M1}}K_{\text{S1}}K_{\text{S2}}\), where \(K_{\text{S1}} = 199\) is the equilibrium constant for the reaction 2MgPi, \(\leftrightarrow\) MgPPi in solution at pH 8.0 (Käpylä et al., 1995).

\[^g\] Binding is a mixture of hexamer and trimer (see text).
variants have about the same $k_h$ values, but the trimers have
greatly increased $K_{m,h}$, $K_{m,m}$, and $K_{m,a}$ values. Consequently,
they display reduced activity upon dilution (Fig. 2) because the
trimers are not saturated at the 20 $\mu M$ substrate concentration
used in our standard enzyme assay. In fact, virtually no activity
loss was observed with the H136Q variant incubated as for
Fig. 2 but assayed at 1 $\mu M$ Mg$_2$Pi. The binding characteristics
of trimeric H140Q-PPase ($K_{m,h}$ and $K_{m,a}$) were somewhat improved
at higher pH (Table III).

Three lines of evidence rule out the possibility that the
activities measured result from hexamer formation during the
assay. First, hexamer formation is a slow reaction. For H136Q
variant, the association rate constant is only 0.77 $M^{-1} s^{-1}$
at pH 7.2 (see below), which corresponds to half-time of 9 days
formation curves are strictly linear during 3 min of the assay,
we routinely use in hydrolysis assays. Second, the product
for hexamer formation at 0.1 nM enzyme concentration, which
at pH 7.2 (see below), which corresponds to half-time of 9 days
very different from those for the hexameric forms.

The Effects of pH on the Hydrolitic Activities of H136Q and
H140Q Variants—Values of $k_{h,app}$ and $K_{m,h,app}$ for PP hydrolysis
by the variant PPases were determined at 20 and 50 mM Mg$_2$Pi
in the pH range 6–9.6. The pH profile of $K_{m,h,app}$ for H136Q-PPase
is little changed between hexamer and trimer and is virtually
the same at both Mg$_2$Pi concentrations (Fig. 5A). In contrast, the
values of $k_{h,app}/K_{m,h,app}$ are approximately 50–100-fold higher for
hexamer versus trimer and, at pH < 6.5, are considerably
higher at 50 mM Mg$_2$Pi than at 20 mM Mg$_2$Pi (Fig. 5B). By
comparison, for WT-type PPase, $k_{h,app}/K_{m,h,app}$ is practically constant
in the pH range 6–8.5 (Käpylä et al., 1995) and shows little dependence on Mg$_2$Pi concentration above 20 mM.

The results at 50 mM Mg$_2$Pi were used to calculate apparent
ionization constants for enzyme with substrate bound, indicated
as ESH, and for enzyme lacking substrate, indicated as
EH, as well as of pH-independent values for $k_{h,app}$ and $K_{m,h,app}$
at 50 mM Mg$_2$Pi, according to Equations 4a and 4b.

$$\frac{k_{h,app}}{K_{m,h,app}} = \frac{(k_h/K_{m,h})}{1 + [H^+]K_{EH2} + K_{ESH}(H^+)}$$
(Eq. 4a)

$$k_{h,app} = K_h(1 + [H^+]K_{EH2} + K_{ESH}(H^+))$$
(Eq. 4b)

Parameter values are presented in Table VI, along with those
determined from similar data (not shown) for the H140Q
hexamer (we were unable to determine pH-rate profiles for
H140Q trimer). For the most part, values for the hexameric
variants are quite similar to those for wild-type. The only
notable differences are the 2-fold increase in $k_{h}$ for H136Q
variant (see below) and the increases that both variants show
in the values of $p_{K_{EH2}}$ and $p_{K_{ESH2}}$. Mutations of active site residues
also give rise increases in $p_{K_{EH2}}$ and $p_{K_{ESH2}}$, but the magnitude of such increases is considerably greater (Salminen
et al., 1995). The H136Q trimer shows a still larger increase
in $p_{K_{EH2}}$ as well as a large decrease in $K_h/K_{m,h}$.

Mg$_2$Pi Binding to the H136Q and H140Q Variants—Mg$_2$Pi

\[\text{Fig. 3. Formation of enzyme-bound PP} \] by H136Q and H140Q-
\[\text{PPases in the presence of 20 mM free Mg}^{2+}\]. Enzyme concentration
\[\text{was 100–140 }\mu M. \text{ The PP} \] concentration of acid-quenched samples was
determined either from $^{32}$P radioactivity (Springs et al., 1981) (○, △) or
using a coupled enzyme assay (Baykov et al., 1990; Nyren and Lundin,
1985) (■, □, H136Q, pH 7.2; ■, H136Q, pH 8.0; □, H140Q, pH 7.2). The lines are drawn according to Equation 3 using best-fit values for the
parameters (Table III).

\[\text{Fig. 4. Rates of PP} \] synthesis to medium by wild-type and
\[\text{variant PPases as functions of Mg}^{2+}\] concentration. Mg$_2$Pi
\[\text{concentration was fixed at 20 mM (○, △, □) or 10 mM (▲). Rates were measured by a coupled enzyme assay (Baykov and Shestakov, 1992). ○, wild-type PPase, pH 7.2; △, H136Q-PPase, pH 7.2; ▲, H140Q-PPase, pH 8.0; □, H140Q-PPase, pH 7.2.}

| pH      | [Mg$^{2+}$] | [Mg$_2$Pi] | $n^a$ | $P_c$ | $v_{ex}$ | $k_{ex}$ |
|---------|-------------|------------|-------|------|---------|---------|
| H136Q-PPase |
| 7.2     | 20          | 20         | 7     | 0.078 ± 0.010 | 98.0 ± 6 | 169 ± 10 |
| 7.2     | 10          | 20         | 7     | 0.084 ± 0.005 | 92 ± 4 | 157 ± 7 |
| 8.0     | 20          | 12         | 3     | 0.083 ± 0.005 | 93 ± 5 | 235 ± 13 |
| 8.0     | 10          | 12         | 3     | 0.078 ± 0.004 | 85 ± 1 | 213 ± 3 |
| H140Q-PPase |
| 7.2     | 20          | 20         | 3     | 0.155 ± 0.003 | 115 ± 6 | 166 ± 9 |
| 7.2     | 10          | 20         | 3     | 0.159 ± 0.001 | 126 ± 1 | 183 ± 2 |

$^a$ Number of independent determinations.
DISCUSSION

Hexamer Stability and the Interface between Monomers—PPase can either be considered as a dimer of trimers or a trimer of dimers centered around a local 2-fold axis (Fig. 7; Kankare et al. (1994)). The main components of the interface around the local 2-fold axis are the two antiparallel symmetry-related α-helices A (Fig. 7) and a short loop (residues 46 to 50) connecting strands 4 and 5 (data not shown). His-136 and His-140 are located at the C terminus of helix A (Fig. 7A), at the center of a network of intra- and intersubunit interactions, a network so clearly important that we predicted that mutating His-136 and His-140 would affect the oligomeric structure of the enzyme. Starting from His-136 in the first monomer (136 in the right-hand monomer in Fig. 7B), the Nε2 of His-136 forms an interchain hydrogen bond to Oδ2 of Asp-143 in monomer 2 (143' in Fig. 7B). The same Asp-143 Oδ2 also forms an intrachain hydrogen bond to Nε1 of His-140 in monomer 2 (140' in Fig. 7B). The above, of course, describes a half-interface: His-136 → Asp-143' → His-140'. There is another half-interface that runs His-136' → Asp-143 → His-140. The network of interactions is completed by hydrophobic contacts between the almost-parallel His-140 and His-140' which face each other, about 3.5 Å apart, across the intermolecular 2-fold (x in Fig. 7B). A similar arrangement of two antiparallel α-helices also forms the bulk of the homologous trimer-trimer interface in the crystal structure of Thermus thermophilus PPase (Teplyakov et al., 1994). His-136 and His-140 in E. coli PPase align with His-134 and Thr-138 in T. thermophilus PPase, respectively, suggesting that the binding by the variant PPases in the absence of substrate was characterized in two ways. Firstly, equilibrium dialysis measurements (Käyplä et al., 1995) yielded dissociation constants for two Mg2+ sites per subunit indistinguishable from those for WT-PPase (Table III). These experiments were conducted at 750 μM enzyme concentration. In the Mg2+ concentration range used (0.03–2.5 mM), the H136Q variant is predominantly hexameric while the H140Q variant is a mixture of hexameric and trimeric forms (Fig. 2). Secondly, by the protective effect of Mg2+ ions on enzyme dissociation upon dilution. The time course of trimer formation from hexamers is given by Equations 5a and 5b (Kurganov, 1982).

\[
A = A_1 + (A_1 - A_0)a_H \quad \text{(Eq. 5a)}
\]

\[
a_H = (a_{Mg2} + e^{-3.4})/(1 + a_{Mg2}e^{-3.4}) \quad \text{(Eq. 5b)}
\]

Fitting Equations 5a and 5b, in combination with Equations 2b and 2c, to the data shown in Fig. 6A generated \( k_d \) values shown in Fig. 6B. \( k_d \) values paralleled with \( k_d \), indicating that the effect of metal concentration on \( k_d \) is small. The dissociation constant for Mg2+ of 2.4 mM was obtained by fitting \( k_d \) to Equation 6:

\[
k_d = \frac{k_{d,lim}}{1 + \frac{\text{Mg}^{2+}}{K_{Mg}}}
\]

where \( k_{d,lim} \) is \( k_d \) at zero Mg2+ concentration and \( K_{Mg} \) is the metal binding constant.

A similar analysis was carried out for the H140Q variant (data not shown). For this variant \( k_{d,lim} \) was as high as 0.6 s−1, and the data were more scattered. Nevertheless, the value of \( K_{Mg} \) we obtained, 1.8 mM, was again close to the \( K_{M2} \) values derived above from hydrodynamics experiments and equilibrium dialysis measurements (Table III). Despite the high rate constant for hexameric H140Q dissociation, the Pi liberation curves in the assay of residual activity were linear for at least 3 min, indicating that substrate profoundly stabilizes the hexameric form.

**Table V**

Calculated rate constants for H136Q and H140Q E. coli PPase mutants versus wild-type enzyme

| Rate constant | H136Q-PPase | H140Q-PPase | WT-PPase
|---------------|-------------|-------------|--------|
|               | pH 7.2 | pH 8.0 | pH 7.2 | pH 8.0 | pH 7.2 | pH 8.0 |
| \( k_1 \times 10^{-7}, \text{M}^{-1}\text{s}^{-1} \) | 9.5 ± 1.2 | 6.1 ± 1.4 | 6.4 ± 4.2 | 4.6 ± 0.5 | 5.6 ± 0.4 |
| \( k_9, \text{s}^{-1} \) | 50 ± 9 | 33 ± 13 | 25 ± 6 | 20 ± 5 | 11 ± 9 |
| \( k_{90}, \text{s}^{-1} \) | 1200 ± 300 | 2000 ± 800 | 1450 ± 240 | 800 ± 180 | 800 ± 400 |
| \( k_{91}, \text{s}^{-1} \) | 200 ± 20 | 260 ± 30 | 210 ± 15 | 140 ± 30 | 130 ± 20 |
| \( k_{92}, \text{s}^{-1} \) | 2200 ± 300 | 2900 ± 400 | 1130 ± 100 | 440 ± 110 | 500 ± 140 |
| \( k_{94} \times 10^{-4}, \text{M}^{-1}\text{s}^{-1} \) | 17 ± 8 | 16 ± 10 | 14 ± 6 | 5.9 ± 1.7 | 3.8 ± 2.2 |
| \( k_{a} \times 10^{-5}, \text{M}^{-1}\text{s}^{-1} \) | 260 ± 30 | 850 ± 130 | 240 ± 80 | 400 ± 100 | 560 ± 310 |

*Values from Käyplä et al. (1995).
*Calculated using the \( K_{Mg} \) value shown in parentheses in Table III.
It seems very likely that, per monomer at neutral pH, a structure may be formed in which the monomers interact such that the active site conformation. Second, weakening intersubunit interactions may increase conformational flexibility of the protein molecule, which may be important provided that some reaction steps involve rate-limiting conformational changes.

Finally, the trimers in WT-PPase may interact such that the perfect hexameric symmetry breaks down and one trimer is always inactive, while in the H136Q-PPase the trimers are perfect hexameric symmetry.}

Based on the structure of the subunit contact region, one would expect, and it is indeed observed (Table I), that the hexameric structure of the H136Q variant is more stable than that of the H140Q variant. His-136 is more exposed to solvent than His-140 so the Glu-136 side chain in the H136Q variant can swing out of the way to allow solvation both of Asp-143' and of Glu-136. Conversely, in the H140Q variant, the completely buried Glu140 will have unsatisfied hydrogen bond donors and acceptors that cannot be solvated (at least one His on the ɛ-amido group and lone pairs on the ɛ-carbonyl group). In addition, there is a loss of hydrophobic interaction between His-140 and His-140' in the H140Q variant. Finally, because 140 is much more likely to be His. Consequently, replacing either His will either weaken or destroy the ion pair depicted in Fig. 7B. In both cases, the replacement Glu can orient to allow its ɛ-amido group to replace the lost His-Asp hydrogen bond, thus partially mitigating the effect of the substitution. The helix-forming tendency of glutamine is slightly higher than that of histidine (O’Neil and DeGrado, 1990), thus making unlikely any destabilization of the α-helix A in the variant proteins.

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Another clear difference between the two variants is in their pH-stability profiles: the H136Q variant shows a pH optimum at about pH 7.2. This is consistent with two Asp-His' ion pairs stabilizing subunit association, with the loss of stability at lower and higher pH being due to protonation of Asp-143 and deprotonation of His-140, respectively. Here we note the report of Borschchik et al. (1986) that even WT-PPase will dissociate into trimers on incubation at pH 5. At present we have no cogent rationale for the pH stability profile of the H140Q variant.

**Catalytic Properties of the Hexameric Forms**—The kinetic parameters of WT and variant PPases are very similar (Table III); neither substitution greatly affects the active site. The pH profiles of $k_{off}$ are very similar to each other, and the differences are chiefly due to changes in the pH dependence of the rate constant for product release ($k_1$) between wild-type and the two variant PPases (Table V). In contrast, an active site D97E variant principally reduces the rate of the chemical catalysis step ($k_4$) (Käpylä et al., 1995).

Measured at pH 8.0, close to the optimum for both enzymes, $k_4$ and $k_{off}$ are 2–3-fold greater for the H136Q variant than for wild-type PPase, as are most of the microscopic rate constants (Table V). At least three possible explanations can be suggested for why this is so. First, the mutation may somehow optimize active site conformation. Second, weakening intersubunit interactions may increase conformational flexibility of the protein molecule, which may be important provided that some reaction steps involve rate-limiting conformational changes. Finally, the trimers in WT-PPase may interact such that the perfect hexameric symmetry breaks down and one trimer is always inactive, while in the H136Q-PPase the trimers are...
equivalent and active. Alternatively, the trimers could cycle: one “on” and one “off” as, for example, proposed for ATP synthase (Boyer, 1993; Abrahams et al., 1994). Hexameric H136Q-PPase would thus be more active than (hexameric) WT-PPase because its trimers would be more independent. We cannot, at present, select between the above possibilities. It is, however, intriguing that E. coli PPase crystallizes in two different forms. One has obligate perfect identity between all six monomers because the space group is R32 and there is only one monomer in the asymmetric unit (Heikinheimo et al., 1995). The other has two monomers in the asymmetric unit of a double-sized R32 unit cell and so the trimers, related by a noncrystallographic 2-fold axis (Kankare et al., 1994), are not constrained to be identical and, indeed, appear not to be.

Catalytic Properties of the Trimeric Forms—As the variant PPases dissociated easily, we could determine some of their catalytic properties as trimers. Although the substrate and Mg$^{2+}$ binding parameters for trimeric H136Q- and H140Q-PPases are somewhat different at pH 7.2 (Table III), the same trend is evident: the affinities for both ligands are drastically reduced on dissociation, while $k_h$ values remain essentially unchanged.

In terms of the three-dimensional structure, the binding

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Changes are explained by the fact that the interactions discussed above (Fig. 7B) not only stabilize the monomer-monomer interface but also define a specific conformation for the 141–143 loop that immediately follows helix A. That loop contains one of the more important active site residues. Mutation of Lys-142 to Arg-142 results in a large increase in the $K_{m,h}$ for Mg$_2$PP$_i$ (Salminen et al., 1995) and in weakened binding for both MgP$_i$ and a competitive inhibitor of Mg$_2$PP$_i$. Consequently, any destabilization of the 141–143 loop on hexamer dissociation might be expected to affect the active site and, in particular, the binding of PP$_i$ and P$_i$ (Salminen et al., 1995). The greater affinity of hexameric PPase for Mg$^{2+}$ and Mg$_2$PP$_i$ explains why these ligands stabilize it versus trimeric PPase.

We demonstrated (data not shown) that the increased $K_{m,h}$ is an intrinsic property of the trimers, as opposed to arising from an indirect effect of each mutation on the active site, by showing that WT-PPase trimer, formed by prolonged incubation at low pH following Borshchik et al. (1986), resembles the variants studied in this paper in having a $K_{m,h}$ value similar to that of the hexamer but much higher $K_{m,h}$ values. This latter result differs from that reported by Borshchik et al. (1986), who claimed that the $K_{m,h}$ values of trimer and hexamer were similar. It is, however, consistent with reports of activity loss following dissociation of the structurally related PPases of the thermophilic bacteria PS-3 (Hachimori et al., 1979) and Bacillus stearothermophilus (Schreier, 1980) into trimers. Our work suggests that this loss of activity is due to an effect on $K_{m,h}$. Finally, by analogy with our present results, we speculate that the loss of E. coli PPase activity found by Samejima et al. (1988) on chemical modification of His residues arises from dissociation of hexamer into trimers.

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Dissociation of Hexameric *Escherichia coli* Inorganic Pyrophosphatase into Trimers on His-136 → Gln or His-140 → Gln Substitution and Its Effect on Enzyme Catalytic Properties

Alexander A. Baykov, Valerij Yu. Dudarenkov, Jarmo Käpylä, Tiina Salminen, Teppo Hyytiä, Vladimir N. Kasho, Sari Husgafvel, Barry S. Cooperman, Adrian Goldman and Reijo Lahti

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