A Trimetal Site and Substrate Distortion in a Family II
Inorganic Pyrophosphatase*

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We report the first crystal structures of a family II pyrophosphatase complexed with a substrate analogue, imidodiphosphate (PNP). These provide new insights into the catalytic reaction mechanism of this enzyme family. We were able to capture the substrate complex both by fluoride inhibition and by site-directed mutagenesis providing complementary snapshots of the Michaelis complex. Structures of both the fluoride-inhibited wild type and the H98Q variant of the PNP-Bacillus subtilis pyrophosphatase complex show a unique trinuclear metal center. Each metal ion coordinates a terminal oxygen on the electrophilic phosphate and a lone pair on the putative nucleophile, thus placing it in line with the scissile bond without any coordination by protein. The nucleophile moves further away from the electrophilic phosphorus site, to the opposite side of the trimetal plane, upon binding of substrate. In comparison with earlier product complexes, the side chain of Lys296 has swung in and so three positively charged side chains, His98, Lys205 and Lys296, now surround the bridging nitrogen in PNP. Finally, one of the active sites in the wild-type structure appears to show evidence of substrate distortion. Binding to the enzyme may thus strain the substrate and thus enhance the catalytic rate.

The universally present inorganic pyrophosphatase (PPase, EC 3.6.1.1)4 is a central enzyme of phosphorus metabolism.

PPases are essential enzymes, because they hydrolyze the inorganic pyrophosphate (PPi) generated during a number of ATP-dependent cellular processes and thus provide the necessary thermodynamic pull for them (1). PPases require divalent metal cations for catalysis.

Soluble PPases comprise two families, which differ completely in both sequence (3, 4) and structure (4, 5). Family I PPases (reviewed in Ref. 6) occur in all types of cells from bacteria to man, whereas family II PPases occur almost exclusively in bacteria. Of the 57 known family II PPases, 53 occur in eubacteria, 3 in archaebacteria, and I in a unicellular eukaryote (Giardia lamblia). Three Vibrio species, including Vibrio cholerae, have genes for both family I and family II PPases. The frequent occurrence of family II PPase in human pathogens (e.g. Streptococcus agalactiae causes neonatal pneumonia, sepsis, and meningitis; Streptococcus mutans, dental caries; and V. cholerae, cholera) makes studies of this enzyme medically important. In addition, family II PPases belong to the “DHH” family of phosphoesterases, named after the characteristic DHH amino acid signature (7). All of these enzymes have similar structures (5, 8) and presumably related catalytic mechanisms.

In contrast to family I PPases, which have a simple cup-like single-domain structure, family II PPases have two domains, with the active site at the domain interface (4, 5) (Fig. 1A). The C-terminal domain of family II PPase contains the high affinity substrate-binding site, whereas the catalytic site that binds the nucleophile-coordinating metal cations is located in the N-terminal domain. Closure of the C-terminal domain onto the N-terminal one creates the catalytically competent conformation by bringing the electrophilic phosphate of substrate into the catalytic site (9). The trigger for domain closure is substrate binding to the C-terminal domain in the open conformation (9).

The two PPase families catalyze the hydrolysis of PPi, in what initially appeared to be similar active sites (4, 5) (Fig. 1B), but their functional properties are significantly different. The natural metal cofactor of family I PPases is Mg2+, binding to the enzyme with micromolar affinity, whereas family II enzymes are best activated by Mn2+ or Co2+, which bind with nanomolar affinity. With these metal ions, family II PPases are 10-fold more active than family I PPases (kcat of 1700–3300 s−1 versus 110–330 s−1) (10–12). Interestingly, Mg2+ confers lower activity but greater substrate-binding affinity on family II PPases.
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A. Schematic models of family I PPase (1E6A (33)) and family II PPase (this work). The structures are color-ramped from blue at the N terminus to red at the C terminus. Substrates are shown as sticks: PP, for family I and PNP for family II. B. Stereoview of the superposition of the active sites of WT-Bs-PPase (yellow) on family I yeast PPase (blue). Metals are shown as spheres, the $\text{Mn}^{2+}$ (in family I) is green, $\text{Mg}^{2+}$ (in family II) is gray. Labels for the metals are included according to the protein coloring. PP and PNP are shown as sticks (red, oxygen; blue, nitrogen; and brown, phosphorus). Colored by protein are the active site residues, labeled by amino acid code and sequence number, and the fluorides, shown as spheres.

than $\text{Mn}^{2+}$ ($K_d$ of 60 $\mu$m versus 180 $\mu$m), whereas, in family I enzymes, the reverse is true (13).

Several structures of family II PPases are available: *S. mutans* (Sm-PPase) complexed with $\text{Mn}/\text{Fe}^{3+}$ and two sulfates (4); *Streptococcus gordonii* (Sg-PPase) complexed with high affinity sulfate and $\text{Mn}^{2+}$ (5) or $\text{Zn}$ (9); *Bacillus subtilis* (Bs-PPase) complexed with with only $\text{Mn}^{2+}$ (5); *B. subtilis* metal-free complexed with the high affinity sulfate (9); and the *B. subtilis* N-terminal enzyme core complexed with $\text{Mn}^{2+}$ (9). The crystal structures showed that the preference for $\text{Mn}^{2+}$ over $\text{Mg}^{2+}$ in family II PPases is at least partly due to the histidine ligands that bind the metal ions and bidentate carboxylate coordination of metal ion in the binding site (4). However, our recent structural data revealed a more subtle reason. Substrate binding to family II PPase appears to require a change in the coordination number of the high affinity metal site from five to six (9). Varied five/six-coordinated geometry is typical for transition metals, such as $\text{Mn}^{2+}$ or $\text{Co}^{2+}$, but not for $\text{Mg}^{2+}$, which is almost always six-coordinated (14). Consequently $\text{Mn}^{2+}$ and $\text{Co}^{2+}$ fit the catalytic machinery better than $\text{Mg}^{2+}$. Furthermore, $\text{Zn}^{2+}$, which does not form six coordination easily (14), is a very poor activator of family II PPase (9, 15). However, the structural data described above do not explain the 10-fold difference in $k_{\text{cat}}$ between the two PPase families.

Our goal here was to solve the first structures of a family II PPase with a PP analogue, imidodiphosphate (PNP). We therefore crystallized the fluoride-inhibited WT-Bs-PPase-Mg$_4$-F-PNP complex, and the virtually inactive H98Q variant with PNP and the water nucleophile: H98Q-Bs-PPase-M$_2$H$_2$O-PNP.

**EXPERIMENTAL PROCEDURES**

Protein Preparation and Crystallization—Native Bs-PPase and its H98Q variant were expressed and purified as described previously (16). The protein stock solutions, 35–40 mg/ml, were dialyzed against 83 mM TES/K$^+$ buffer (pH 7.2) containing 17 mM KCl and 0.05 mM EGTA to remove metal ions (16). Reagents were analytical grade from Sigma.

To prepare the WT-Bs-PPase-Mg$_4$-F-PNP complex, the protein stocks were supplemented with 5 mM MgCl$_2$ and 10 mM NaF and incubated for 5–10 min at room temperature followed by addition of 1 mM PNP and a further 5-min incubation. The protein was then crystallized in sitting-drop vapor diffusion setups at 4 °C in a 3:2-ml mixture of protein samplewell solution (100 mM HEPES/K$^+$ (pH 7.5), 2.3–2.5 mM ammonium sulfate, 3–4% polyethylene glycol 400). The ammonium sulfate was ultra-pure, to make the concentration of transition metal cations as low as possible. Large single rhombic crystals (1.0 mm $\times$ 0.8 mm $\times$ 0.5 mm), space group $P2_12_12_1$, appeared within 2–3 days and diffracted to 1.75-Å resolution at the ESRF (Table 1). The same method and similar reagents were used to crystallize the H98Q variant. These crystals diffracted to 2.15-Å resolution on our home source (Table 1). The complex we obtained, however, contains $\text{H}_2\text{O}$ instead of $F^-$, and $\text{Fe}^{3+}$ and $\text{Mn}^{2+}$ in addition to $\text{Mg}^{2+}$ (see below). As a control, we also solved the structure without NaF, and the position of the density in the active site was unchanged (Table 2). This shows that the atom between M1, M2, and M4 is water, not $F^-$, in the H98Q crystals.

Data Collection and Processing—Crystals were briefly transferred to mother liquor supplemented with 18% glycerol and flash-frozen in a boil-off liquid nitrogen stream. Synchrotron data were collected for WT-Bs-PPase on beamline ID14-EH1 ($\lambda = 0.934$ Å) at the ESRF, Grenoble, France. Data for H98Q-Bs-PPase were collected at home using CuK$_\alpha$ radiation from a RU300 Rigaku rotating anode generator fitted with confocal
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mirrors and a Raxis IV+ image plate detector. Data were processed with XDS (17) and the HKL package (18) (Table 1).

Structure Solution and Refinement—The structures were solved by molecular replacement with CNS (19) using the metal-free Bs-PPase structure (PDB code 1WPM (9)) as a search model. We refined the structures with CNS using the MLF target and checked and rebuilt the models using O (20). Water molecules were built with CNS or ARP/wARP (21). The final rounds of refinement (Table 1), including TLS (translation, libration, and screw rotation displacement) refinement, were done with Refmac (22) from the CCP4 Program Suite (23). The occupancies of the active site ligands were verified by refinement in CNS (19). Both structures contain residues 2–308 in monomer A and residues 1–308 in monomer B out of the total 309 residues. The stereochemistry of the resulting models was good: PROCHECK (24) classified all the residues in the most favored and additional allowed regions in the Ramachandran plot (Table 1). Structures were overlaid and displayed in O. The electron density maps were calculated with CNS and Refmac, and the figures were generated with PyMOL.5

Identification of the Metals—To differentiate between enzyme-bound Mg2+ and transition metal cations, we used anomalous difference Fourier maps. Two 5.5–6.0 Å anomalous peaks observed at the positions of both M1 and M2 metal binding sites in the H98Q-Bs-PPase structure, collected at 1.54 Å wavelength, indicated the presence of atoms heavier than Mg2+. In agreement with this, the (2Fo – Fc) electron-density peaks associated with the M1 and M2 sites were too large (7–9σ) to be Mg2+. Fluorescence scans carried out at the ESRF around the manganese, iron, cobalt, and zinc K-edges indicated that only manganese and iron (presumably Mn2+ and Fe3+) were present (data not shown). We therefore collected anomalous diffraction data just above and below the manganese and iron absorption edges in order to be able to calculate the occupancies of both metal ions in the active sites (Table 2). Data above and below the iron K-edge were collected on ESRF beamline BM14, and data from the same crystal were collected above and below the manganese K-edge at ESRF beamline ID23-EH1 (Table 2). We also collected data from H98Q-Bs-PPase crystals grown in the presence and in the absence of fluoride to see if there was any difference between them (Table 2).

The geometry for Mn2+ and Mg2+ bound to PPase metal-binding sites was evaluated using angular distortion as previously described (9, 26). The r.m.s.d. between the ideal geometry angles and those observed in the structures, δ, gives a measure of the correspondence to the tested coordination type.

Kinetic Measurements—Imidodiphosphate hydrolysis was assayed at 25 °C by measuring phosphate accumulation with a sensitive Malachite green procedure (27). The reaction medium contained 1 mM free Mg2+, 1 mM PNP, 83 mM TES/KOH buffer, pH 7.2, 50 μM EGTA, and 17 mM KCl. In calculating the rate of the enzymatic reaction, we took into account that the second product (phosphoramidate) is rapidly converted into phosphate in strongly acidic medium in the course of phosphate determination (28). The amount of substrate consumed was thus assumed to equal half of the phosphate released.

5 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
addition, we only discuss monomer A in the WT and H98Q asymmetric units, unless there are significant differences between monomer A and monomer B.

Metal Binding—In contrast to all earlier structures of family II PPase, four metal ions are bound to the active site of each monomer in both WT-Bs-PPase and H98Q variant structures when crystallized together with PNP (Fig. 3). For WT, all four cations are clearly Mg\(^{2+}\), based on three lines of evidence. First, in an anomalous map calculated from data collected at 0.934 Å (Table 1), the peak heights at M1 and M2 are below the noise level of the map (data not shown). Second, the B-factors for the metal ions refined as Mg\(^{2+}\) are ~34 Å\(^2\), similar to the surrounding residues. Finally, WT-BsPPase clearly contains PNP (Fig. 3C), which means that fluoride is very likely to be present as an inhibitor, and F\(^-\) binds to Mg\(^{2+}\)-containing, not Mn\(^{2+}\)-containing, BsPPase (10, 12).

Conversely, anomalous difference Fourier calculations on the H98Q-BsPPase structure using data collected at the CuK\(_\alpha\) wavelength (Table 1) clearly indicated the presence of transition metal ions. X-ray fluorescence scans (see “Experimental Procedures”) indicated that the ions present were Fe\(^{3+}\) and Mn\(^{2+}\). By collecting data sets above and below the absorption edges, we could identify both the positions and the amounts of the transition metal ions in the active site; they are in sites M1 and M2, consistent with earlier data (4, 5). The Fe\(^{3+}\): Mn\(^{2+}\) ratio is about 6:1 in site M1 and about 2:1 in site M2, based on the peak heights (Table 2 and Fig. 4A). Apparently, traces of Mn\(^{2+}\) and Fe\(^{3+}\) were present in amounts sufficient to fill the M1 and M2 sites even in the presence of 5 mM Mg\(^{2+}\); this is consistent with the non-nomolar affinity of family II PPase for transition cations (10). In SmPPase, Mn\(^{2+}\) and Fe\(^{3+}\) do not exchange for Mg\(^{2+}\) even in the presence of a large excess of Mg\(^{2+}\) (4).

We could thus trap the OH\(^-\) on the enzyme. Because transition metal cations occupy the M1 and M2 sites, water/OH\(^-\), not fluoride, must be bound. This can be seen in two ways. First, the position of the nucleophile is the same when the H98Q variant crystals are grown in the absence of inhibitor, and F\(^-\) binds to Mg\(^{2+}\)-containing, not Mn\(^{2+}\)-containing, BsPPase (10, 12).
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of fluoride; i.e. when the site must be occupied by water/hydroxide ion (data not shown) and, second, this position is slightly different than it is in the WT-BsPPase-Mg$_4$F-PNP complex (Fig. 4A).

In both structures, the metal ions M1 and M2 are bound to six conserved residues from the N-terminal domain. M2, which binds tightest (9, 15), has six ligands: Asp$^{15}$O$_1$, Asp$^{75}$O$_2$, His$^{97}$Ne$_2$, Asp$^{149}$O$_6$, the P1 phosphate O$_2$, and nucleophilic water W$_n$ (in the H98Q variant) or the fluoride anion F$^-$ (in WT) (Fig. 3A) (referred to below as W$_n$/F). In both structures the M2 site has almost perfect octahedral geometry, with a low value of angular distortion ($\delta$ of 7.9–8.9 (26); see “Experimental Procedures”). This is in contrast to earlier structures (5, 9). When substrate analogue is not bound, the Mn$^{2+}$ at site M2 is five-coordinate.

The six-coordinate M1 site is formed by W$_n$/F, P1 phosphate O$_1$, His$^{97}$Ne$_2$, and the metal-ion bridge Asp$^{75}$O$_1$ and bidentately by Asp$^{13}$ (3A). Consistent with weaker binding to the M1 site (9, 15) the M1 geometry is clearly more distorted than that of M2 (6 of 15.2–19.1). Of the six ligands, the Asp$^{13}$O$_6$-M1 distance in monomer A of both structures is somewhat longer than the rest (2.5–2.6 Å in both WT and H98Q-Bs-PPase). This is similar to earlier structures in which M1 was also six-coordinate (5, 9) but usually with one long M1-ligand distance (9).

The M3 metal ion clearly binds with substrate: it bridges the two phosphate groups in PNP and has no direct protein ligands, the other four coordination sites being filled by water molecules. An analogous Mg$^{2+}$ ion was observed in the bridging position between the two sulfates bound to the active site of Sm-PPase (4).

However, a fourth metal ion, M4, is also present in both WT- and H98Q-Bs-PPase PNP-bound structures (Fig. 4B). This was seen neither in the empty nor in the sulfate-containing product mimic PPN complex structures (4, 9). Metal ion binding to the M4 site thus appears to correlate specifically with substrate binding. For instance, the occupancy of the M4 metal ion is the same as that of PNP in WT (see below). The Mg$^{2+}$ ion in M4 is bound to P1 phosphate O$_3$, W$_n$/F, Asp$^{149}$O$_6$, and Asp$^{13}$O$_6$ and two water molecules, one conserved in all available family II PPA structures and the other shared with the coordination sphere of M3 (Fig. 3A). Consequently, both Asp$^{149}$ and Asp$^{13}$

### TABLE 1

| Data collection and refinement statistics | Values for the highest resolution shell are shown in parentheses. |
|-----------------------------------------|---------------------------------------------------------------|
| **WT-Bs-PPase**                         | **H98Q-Bs-PPase**                                             |
| Wavelength (Å)                          | 0.934                                                        |
| Space group                             | P2$_2$P$_2$                                                 |
| Cell dimensions, a, b, c (Å)            | 60.0, 115.9, 147.9                                           |
| Resolution (Å)                          | 1.75 (1.80-1.75)                                             |
| $R_{	ext{sym}}$                         | 0.032 (0.452)                                                |
| I/σ(I)                                  | 14.9 (2.8)                                                   |
| Completeness (%)                        | 99.9 (100)                                                   |
| Redundancy                              | 4.2 (3.2)                                                    |
| **Reefinement**                         |                                                               |
| Resolution (Å)                          | 20-1.75                                                      |
| No. reflections                         | 99,323                                                       |
| $R_{	ext{work}}/R_{	ext{free}}$        | 0.166/0.186                                                  |
| Residues included (total 309)           | A: 2-308; B: 1-308; B: 1-308; B: 1-308                      |
| No. atoms                               | 4,940                                                        |
| Protein                                 | 43.4                                                         |
| Water                                   | 47.6                                                         |
| B-factors (Å$^2$)                        | 44.4                                                         |
| Protein                                 | 44.4                                                         |
| Water                                   | 47.6                                                         |
| r.m.s.d.                                | 0.016                                                        |
| Bond lengths (Å)                        | 1.52                                                         |
| Bond angles (°)                          | 91.8                                                         |
| Ramachandran plot (%)                   | 8.2                                                          |
| Most favored regions                    |                                                              |
| Additionally allowed regions            |                                                              |

### TABLE 2

| Data collection near the absorption edges of Mn$^{2+}$ and Fe$^{3+}$ | Values for the highest resolution shell are shown in parentheses. |
|---------------------------------------------------------------------|---------------------------------------------------------------|
| **H98Q-Bs-PPase above iron K-edge**                                 | **H98Q-Bs-PPase below iron K-edge**                          |
| Wavelength (Å)                                                      | 1.722                                                        |
| Space group                                                         | P2$_2$P$_2$                                                 |
| With fluoride                                                       |                                                               |
| Cell dimensions, a, b, c (Å)                                       | 59.9, 115.2, 146.8                                           |
| Resolution (Å)                                                      | 2.5 (2.65-2.5)                                               |
| $R_{	ext{sym}}$                                                     | 0.057 (0.164)                                                |
| I/σ(I)                                                              | 14.2 (5.2)                                                   |
| Completeness (%)                                                     | 98.6 (97.8)                                                  |
| Redundancy                                                          | 2.5 (2.5)                                                    |
| Peak heights (σ)                                                    | Monomer A: M1 and M2                                         |
|                                                                     | 23 and 24                                                    |
|                                                                     | Monomer B: M1 and M2                                         |
|                                                                     | 24 and 26                                                    |
| Without fluoride                                                    |                                                               |
| Cell dimensions, a, b, c (Å)                                       | 59.7, 115.4, 147.0                                           |
| Resolution (Å)                                                      | 2.5 (2.65-2.5)                                               |
| $R_{	ext{sym}}$                                                     | 0.072 (0.267)                                                |
| I/σ(I)                                                              | 11.1 (3.2)                                                   |
| Completeness (%)                                                     | 99.9 (98.6)                                                  |
| Redundancy                                                          | 2.5 (2.5)                                                    |
| Peak heights (σ)                                                    | Monomer A: M1 and M2                                         |
|                                                                     | 7 and 12                                                     |
|                                                                     | Monomer B: M1 and M2                                         |
|                                                                     | 10 and 13                                                    |

$^a$ $R_{	ext{sym}} = \Sigma |I_i| - |\bar{I}|/\Sigma |I|$, where $I$ is an individual intensity measurement and $\bar{I}$ is the average intensity for this reflection with summation over all data.
Figure 4. Trinuclear plane, identification of Fe$^{3+}$ and Mn$^{2+}$ in H98Q-Bs-PPase structure, and the fourth metal ion, A, the structures of the N-terminal domain of Bs-PPase (Bs-PPase-NT) (9) (PDB 1WPN), WT-Bs-PPase monomer A and H98Q-Bs-PPase monomer A were superimposed using the carboxyl group of Asp$^{75}$ and metals M1 and M2. Shown are part of the PNP molecule, nucleophilic water, Asp$^{149}$, and metal plane formed by M1, M2, and M4 for H98Q-Bs-PPase, colored as in Fig. 3. For clarity, we show only the nucleophile water (blue) in Bs-PPase-NT, which lacks PNP, and the F$^-$ (magenta) in WT-Bs-PPase monomer A, which contains PNP. Anomalous electron density maps calculated from the data collected at the iron and manganese edges (Table 2) are shown centered on the H98Q-Bs-PPase structure. The map from data collected at the iron edge is contoured at 2.5–2.6 Å and shown in brown; the manganese map is contoured at 6 Å and shown in green. The trinuclear plane is in black to emphasize the difference in the position of the nucleophile in the presence and absence of substrate/product at P1 (see text for details). The interactions from Wn to the metal ions are shown as dashed lines, and distances are shown in Ångströms. B, an omit map showing the presence of M4 magnesium in the WT-Bs-PPase monomer A. The map was calculated after removal of the M4 as well as coordinating water molecules and fluoride, and the structure was re-refined with Refmac. Electron density is contoured at 4 Å. Coloring of the atoms is identical to previous figures. Distances for the coordinating ligands of M4 and fluoride are shown in Ångströms.

FIGURE 4. Trinuclear plane, identification of Fe$^{3+}$ and Mn$^{2+}$ in H98Q-Bs-PPase structure, and the fourth metal ion. A, the structures of the N-terminal domain of Bs-PPase (Bs-PPase-NT) (9) (PDB 1WPN), WT-Bs-PPase monomer A and H98Q-Bs-PPase monomer A were superimposed using the carboxyl group of Asp$^{75}$ and metals M1 and M2. Shown are part of the PNP molecule, nucleophilic water, Asp$^{149}$, and metal plane formed by M1, M2, and M4 for H98Q-Bs-PPase, colored as in Fig. 3. For clarity, we show only the nucleophile water (blue) in Bs-PPase-NT, which lacks PNP, and the F$^-$ (magenta) in WT-Bs-PPase monomer A, which contains PNP. Anomalous electron density maps calculated from the data collected at the iron and manganese edges (Table 2) are shown centered on the H98Q-Bs-PPase structure. The map from data collected at the iron edge is contoured at 2.5–2.6 Å and shown in brown; the manganese map is contoured at 6 Å and shown in green. The trinuclear plane is in black to emphasize the difference in the position of the nucleophile in the presence and absence of substrate/product at P1 (see text for details). The interactions from Wn to the metal ions are shown as dashed lines, and distances are shown in Ångströms. B, an omit map showing the presence of M4 magnesium in the WT-Bs-PPase monomer A. The map was calculated after removal of the M4 as well as coordinating water molecules and fluoride, and the structure was re-refined with Refmac. Electron density is contoured at 4 Å. Coloring of the atoms is identical to previous figures. Distances for the coordinating ligands of M4 and fluoride are shown in Ångströms.

The nucleophile from the electrophilic phosphate at the P1 site (Fig. 4). In turn, the P1 phosphate binds each of the three metals through one of its three O atoms, thus placing the nucleophile in front of the phosphorous atom in a position ideal for the in-line catalytic attack (see below). When the nucleophilic water is replaced by F$^-$ in the WT-Bs-PPase structure, its binding to the metal ions becomes less symmetrical, with short bonds to M1 and M2 (~2.0 Å) and longer ones to M4 (~2.3–2.4 Å).

**PNP Binding and Geometry**—PNP is bound to the active site of each monomer in WT- and H98Q-Bs-PPase structures (Fig. 3, B and C). Intriguingly, though, the geometry of PNP in the two structures is not identical. In the variant PPase structure, the PNP molecule fits the $F^- - F$ electron density omit map without significant adjustments of its ideal bond lengths and angles in both monomer A and monomer B (Fig. 3B). The P–N–P angle of ~130° is within experimental error of the 127° determined by small molecule x-ray crystallography (30). The P–N–P distances are ~1.7 Å, similar to the small molecule value of 1.68 Å, indicating that there is significant double bond character in both P–N bonds (30). The electron density peaks for the PNP phosphate groups at P2 and P1 substrate-binding sites are of equal size, in agreement with the similar temperature factor values for the atoms of both P1 and P2 phosphates. This is also true in WT monomer B, where the P–N–P angle is 134° and P–N distances are 1.7 Å.

In contrast, the electron density peaks for the PNP phosphates in monomer A of the WT-Bs-PPase structure are more separated. To fit PNP properly into the electron density (Fig. 3C), we had to release three geometrical restraints on the PNP during refinement: for the lengths of the P–N bonds and for the P–N–P bend angle. Upon refinement, the PNP molecule in monomer A became more open, with a P–N–P angle of 143° (10° larger than in H98Q-Bs-PPase), and the PNP linkage appears no longer to be symmetrical. The N–P1 bond lengthens to 1.8 Å, whereas the N–P2 bond shortens to 1.5 Å (see “Discussion”). Determining the meaning and validity of these changes is difficult given the resolution of the data (1.7 Å), but they were consistently seen in different data sets collected on the WT-Bs-PPase-PNP complexes (see “Discussion”).
In both the WT monomers, the size of the peak corresponding to P1 phosphate is smaller than that of P2 (18 Å² versus 25 Å²) in the Fo/Fc electron density omit map (Fig. 3C). To account for this, we modeled a sulfate molecule in the P2 site at 15% occupancy in addition to PNP at 85% occupancy. The electron density corresponding to the M4 Mg²⁺ ion in WT-Bs-PPase also fitted 85% better than 100% occupancy (see above). In this model the temperature factors of the P1 and P2 phosphates were similar (33 Å² and 34 Å²), and the temperature factor of M4 Mg²⁺ (42 Å²) was similar to those of its protein ligands (37 Å² and 42 Å²). The occupancies were fitted in an iterative manner by inspecting the resulting electron density maps. Although we prefer fitting occupancies manually at this resolution, refining the occupancies in CNS (19) gave similar results (83–84% for PNP and M4). We believe that, during enzyme crystallization and/or preincubation, some of the PNP was hydrolyzed even in the presence of fluoride. Upon hydrolysis, exchange to sulfate at the high affinity P2 site is likely because of the similar affinity of the enzymes for phosphate and sulfate (Kₐ of 13 and 8 mM, respectively (10, 29), and because of the high concentration of sulfate in the crystallization mixture. Indeed, the two previously solved structures of PPase in the closed conformation both contain one sulfate bound to the Sul2 site (5, 9).

P1 and P2 Binding Sites—The P2 phosphate-binding site in the C-terminal domain (analogous to the earlier observed Sul2 site (4, 5, 9)) is formed by the side chain of Arg²⁹⁵, the main-chain nitrogen 296, and by the side chains of Lys²⁰⁵ and Lys²⁹⁶, whose N⁻ atoms bridge the P2 and P1 phosphates of PNP (Fig. 5A). The P1 phosphate (analogous to Sul1 in the two-sulfate structure (4)) is bound to the trimetal site in the N-terminal domain. In addition, the two phosphates are bridged by the M3 Mg²⁺ cation. The electrophilic phosphorus of the P1 phosphate is optimally placed with respect to the catalytic nucleophile so that the P–N bridge bond is almost in line with the nucleophile Wn/F (the Wn/F–P–N virtual angle is 170–178°).

The Wn/F nucleophile overlaps well only with the nucleophilic water molecule in the Sm-PPase structure containing sulfate at the metal-bound Sul1 site (4) (Fig. 5A). As described earlier (9) and shown here in Fig. 4A, the nucleophile can occupy two different positions: one for the substrate-free state with five-coordinate M2 and another for the occupied Sul1/P1 site and octahedral M2 (9). The nucleophilic water thus appears to move 1 Å away from the P1 site upon substrate binding.

The conserved and essential His⁹⁸ in WT-Bs-PPase is perfectly positioned (see below) so that its Nε forms a 2.8-Å hydrogen bond with the bridging NH of PNP (Fig. 5B). This arrangement is similar to that seen when only the Sul2 sulfate is present (1K20 and 1WPM in the Protein Data Bank (PDB) (5, 9)). In those cases, because of the similar closed conformation of the C-terminal domain (9), His⁹⁸ binds the Sul2 oxygen atom corresponding to the PNP bridging atom. In contrast, when two sulfates are present in Sm-PPase (PDB 1I74 (4)) His⁹⁸ binds the Sul1 sulfate alone because the protein is in a more open conformation.
The binding pattern is different, however, in the H98Q variant; here Gln$^{98}$ Oe2 contacts the NH bridge while Gln$^{98}$ Ne2 is bound to P1 O2 and to the side chain of Asp$^{77}$ (Fig. 5B). There is no interaction with Asp$^{96}$, unlike in wild type (see below).

The biggest conformational change in the active site compared with the product analogue sulfate complexes occurs at the conserved Lys$^{296}$ residue from the 294SRKKQ$^{298}$ C-terminal fingerprint sequence. In the sulfate complexes, only the Lys$^{296}$ backbone N participates in sulfate binding; the side chain is directed out of the active site. In the PNP complexes, the Lys$^{296}$ side chain turns so that N$_{c}$ is 2.9–3.1 Å from the bridging N. The Lys$^{296}$ N$_{c}$ is thus as close to the PNP bridging N as Lys$^{205}$ N$_{c}$ (3.0–3.2 Å). As a result, the bridging atom of PNP is surrounded by two positively charged residues: Lys$^{205}$ and Lys$^{296}$, as well as His$^{98}$, which can either donate or accept a proton depending on its protonation state. Because the His$^{98}$ Ne2 forms a hydrogen bond with the bridging N, and because it lies close to the plane defined by the P–N–P bonds, it appears that N$_{bridge}$ is sp$^{2}$-hybridized. This is in agreement with the structure of PNP (30). The two lysines are placed almost symmetrically on either side of this plane (Fig. 5).

Lys$^{205}$ and Lys$^{296}$ also form hydrogen bonds to the terminal oxygen atoms of the P1 and P2 phosphates (Fig. 5B). The eclipsed conformation in which the PNP molecule binds to the active site is thus stabilized by three interactions that bridge oxygens on the two phosphates: from Lys$^{205}$, from Lys$^{296}$, and from M3.

The DHH Motif—Until now, the role of the Asp in the DHH signature motif of the “DHH” enzymes (7) was unclear, although Ahn and coworkers (5) suggested that Asp$^{96}$ was important in positioning His$^{98}$. Our new structures show that this motif plays a critical role in arranging the entire active site for catalysis. Asp$^{96}$ positions both key catalytic residues His$^{97}$ and Asp$^{96}$, and links them to the binmetal binding Asp$^{75}$ (Fig. 5B). Asp$^{96}$ Oe2 forms two tight hydrogen bonds to the His$^{97}$ backbone N and to His$^{98}$ N$_{δ1}$. The bond to His$^{98}$ may also be part of a charge-relay system (see below). In addition, Asp$^{96}$ O$_{δ1}$ hydrogen bonds to Asn$^{77}$ N$_{δ2}$, thus connecting the His residues to Asp$^{77}$. Asn$^{77}$ completes the loop by donating a hydrogen bond to the backbone O of Arg$^{99}$.

**DISCUSSION**

**Metal Content and Inhibition by Fluoride Ion**—Even though both WT and the H98Q variant were treated the same, we were able to trap the substrate analogue in two different ways. Fe$^{3+}$ and Mn$^{2+}$, but not fluoride, were present in the H98Q structure, whereas Mg$^{2+}$ and fluoride were present in the WT structure. Crystallization of the H98Q-PNP complex without F$^{-}$ was possible because of the very low intrinsic activity of the variant (Ref. 16; see also above). In addition, Fe$^{3+}$ inhibits the family II PPass from *Methanococcus jannaschii* (12); were Fe$^{3+}$ to have a similar effect, its serendipitous inhibition might also have helped stabilize the substrate analogue complex in the present study. On the other hand, fluoride is a hard anion, explaining why it is not found in the coordination sphere of the bound Fe$^{3+}$/Mn$^{2+}$ ions in H98Q-PPass.

In WT-PPass, substrate analog stabilization is achieved due to fluoride replacing the nucleophilic water coordinated to the Mg$^{2+}$ cluster. In principle, this strategy should have also worked in H98Q-PPase. However, because of subtle differences in active site structure caused by the mutation, the Fe$^{3+}$/Mn$^{2+}$-water-PNP complex in the variant seems to have greater thermodynamic stability than the Mg$^{2+}$-P-PNP complex.

Although equilibrium dialysis shows that the M2 site has nanomolar affinity for transition metal cations, the affinity at the M1 site is only millimolar (11). Why, then, did the H98Q structure have Fe$^{3+}$/Mn$^{2+}$ at both M1 and M2? We suspect that binding substrate or product significantly increases the affinity of M1 for transition metal cations, explaining why they were also observed in our original structure of Sm-PPase complexed with sulfate (4). The excess of Fe$^{3+}$ in the M1 site is consistent with the Fe$^{3+}$ preference for His and bidentate carboxyl ligands (14).

**Trimetal Nucleophile-binding Site**—Solution equilibrium-dialysis data show that, in the absence of substrate, family II PPases have only one high affinity metal binding site ($K_{d}$ of 0.5 nm for Mn$^{2+}$ and 10–90 μm for Mg$^{2+}$) but can bind at least two more metal ions with lower affinity ($K_{d}$ in the millimolar range for both Mn$^{2+}$ and Mg$^{2+}$) (11). Earlier structures of family II PPases, however, were not consistent with these data. In these structures, only M1 and M2 had protein ligands. M3 did not interact directly with protein and therefore would be a poor choice for the third metal ion seen in dialysis experiments (4, 5, 9).

Our new structures of PPass PP$^{3}$analogue complexes reveal a third protein-coordinated metal ion, M4. Its ligands are Asp$^{143}$, Asp$^{149}$, W$_{n}$/F, one oxygen from substrate, and two water molecules (Fig. 3A). It appears that -PO$_{3}$ in the P1 site forms an essential part of the ligand environment for M4. Moreover, M4 is clearly not fully occupied and has a higher temperature factor than M1 and M2 even when PNP is bound (see “Results”). Together, M1, M2, and M4 form a unique trimetal center coordinating the nucleophile W$_{n}$/F and the three terminal oxygen atoms of P1. The geometry of the catalytic unit, in which the nucleophile is located below the center of a metal ion triangle (Fig. 4A) at the center of an approximate tetrahedron (M–W$_{n}$–M angles of 101°, 112°, and 121°) would accommodate the three lone pairs on an hydroxide anion but is clearly not suitable for a water molecule, which has only two lone pairs. It thus seems even clearer here than in family I PPases (31–33) that the nucleophile is actually a hydroxide anion. The trimetal catalytic center also precisely directs substrate binding. Because each of the three oxygen atoms of the P1 phosphate symmetrically binds a corresponding metal, no additional coordination by protein ligands is required for the P1 phosphate to be located directly above the nucleophile in the most efficient way for catalysis (Fig. 4) with an N-P1-Nuc angle of 178°.

The most remarkable feature of the nucleophile site, however, is the correlation of the nucleophile position with occupancy of the P1 site (Fig. 4A). If this happens during catalysis, the nucleophile must move away as substrate binds. The nucleophilic water (W) is above the trimetal plane in the absence of PNP (blue, Fig. 4A) but below the trimetal plane in the structures reported here (F$^{-}$ and W$_{n}$, Fig. 4A).

**Metal Cofactor Specificity**—Family II PPases are much less active with Mg$^{2+}$ than with Mn$^{2+}$ (10). Mn$^{2+}$ accelerates both
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hydrolysis and product release. Our results suggest a possible explanation. If, as described earlier (9), the Mn$^{2+}$ ion at M2 does not readily adopt six-coordinate geometry in the substrate-bound state, the expulsion of nucleophile from the tri-metal site may be driven by Mn$^{2+}$ aiming to return to its preferred five-coordinate state. Consistent with this, the nucleophile in substrate-free Mg$^{2+}$-Bs-PPase$^6$ is in the same place as in the PNP-containing structures reported here, and Mg$^{2+}$ at M2 adopts six-coordinate octahedral geometry. This agrees with the relatively high rate constant for substrate release from the Mn$^{2+}$-activated enzyme (10). A slowing of nucleophile motion during catalysis could easily account for the 10-fold decrease in reaction rate in Mg$^{2+}$-containing BsPPase versus Mn$^{2+}$-containing BsPPase.

Why Is the H98Q Variant Inactive?—In both WT and H98Q structures, there is a hydrogen bond between the His$^{98}$ or Gln$^{98}$ and the bridging N in PNP. The bridging N is probably the hydrogen bond donor, because His$^{98}$ N61 donates a hydrogen bond to Asp$^{96}$, whereas Gln$^{98}$ N62 donates a hydrogen bond to Asp$^{75}$ (Fig. 5B). However, with the true substrate, PP$, His^{98}$ may act as a general acid, protonating the leaving group, consistent with the large reduction in $k_{cat}$ seen in the variant (16). If so, one possibility is that the deprotonated Asp$^{96}$ orients the protonated His$^{98}$ for catalysis. Alternatively, a protonated Asp$^{96}$ could be part of a charge-relay system, becoming deprotonated when neutral His$^{98}$ transfers its proton to the bridging oxygen. This is not possible for the Gln$^{98}$ – Asp$^{75}$ pair, both because of the nature of Gln$^{98}$ and because Asp$^{75}$ cannot be protonated, because it binds both M1 and M2 (Fig. 3A). Although the pK$^a$ of the acidic group responsible for the decline in $k_{cat}$ changes very little in the H98Q variant (16), the lack of effect of mutation on the pK$^a$ may be because the effects of the mutation are pleiotropic, as we have observed in yeast PPase.

Substrate Distortion—Bs-PPase appears to contain a clear example of enzyme-based substrate distortion (34–36). In the inactive H98Q variant, the geometry of the PNP was undistorted. However, to fit the electron density in WT monomer A, we had to release the restraints on the P–N bonds and the P–N–P angle (see above), leading to the N–P1 bond lengthening to 1.8 Å, while the N–P2 bond shortened to 1.5 Å. Together with the 10° increase in the P–N–P angle to 143°, the P–P distance lengths by 0.1 Å. It is clear that such changes are at the limit of what is resolvable at 1.75-Å resolution. However, this change was consistently seen in all four data sets we collected on WT-PPase and was stable as to refinement procedure. In addition, the same effect did not occur upon releasing the same restraints in monomer B in WT nor in the H98Q variant. Consequently, we believe these surprising changes are likely to be real, although we cannot explain why they are seen only in monomer A. The major difference observed between monomers A and B of WT-Bs-PPase is a small change in the orientation of the domains: in monomer B the C-terminal domain is more closed (∼1°) and less twisted (∼2°) with respect to the N-terminal domain (as defined in Ref. 9). The P–N distances of distorted PNP, 1.8 Å and 1.5 Å, agree remarkably well with single- and double-bond P–N distances of 1.44–1.49 and 1.77 Å, respectively (37, 38). Consequently, we speculate that a P–N double bond tends to localize at the leaving group P2, with a P–N single bond at the electrophilic P1. The positively charged Lys$^{205}$–Lys$^{296}$–His$^{98}$ cluster located around the bridging N, along with M3, may be part of the mechanism by which this happens. Enzyme-induced substrate strain (34) would be consistent with a ground-state distortion mechanism of rate enhancement. The enzyme might increase the transmission coefficient for the reaction rather than specifically binding transition state tighter (35, 36).

Dissociative Versus Associative Mechanism—In phosphate chemistry, two possible extreme mechanisms for hydrolysis or exchange conceptually exist. They differ in the timing of the bond-making and bond-breaking events (39, 40). In a dissociative (S$_{2}$,1-type) mechanism, a metaphospho PO$_{4}$ intermediate forms, whereas in an associative one, there is a formally bonded, five-coordinate PO$_{4}$ phosphorane intermediate. (The S$_{2}$,2-type mechanism, where bond-making and bond-breaking are concurrent, is intermediate between the two.) Linear free-energy relationships in nonenzymatic phosphoryl transfer reactions demonstrate that P–O–P bond hydrolysis in solution proceeds through a dissociative mechanism (40). For enzymes, however, a fully dissociative mechanism is usually impossible, because the reacting water is also bound in the active site in the transition state. It is thus more relevant with enzymes to discuss the extent of fractional associativity/dissociativity of the transition state (25).

The overall structure of the substrate or product analogue complexes of family II PPases is clearly similar to that of family I PPases (Fig. 1B) (4). This remarkable example of convergent evolution, where similar active sites occur in completely different folds, led us to propose that the catalytic mechanisms might also be similar and that family II PPases would proceed through an associative-type mechanism, as do family I PPases (4, 32, 33). Some aspects of the structures reported here, however, suggest that this might not be so.

In family I enzymes, the nucleophile remains fixed in place in all the structures seen so far (32, 33). However, the motions of the nucleophile in Bs-PPase are unusual; upon binding substrate (Fig. 4), the nucleophile moves away from the incoming substrate by ∼1 Å. This motion is correlated with formation of octahedral geometry at site M2 (see above). It also occurs in the Sm-PPase structure containing sulfate at the metal-bound Sul site (4) (Fig. 5A) and in substrate-free Bs-PPase crystallized with Mg$^{2+}$ alone.$^6$

Coupled to this, the presence of M4 (the third metal ion binding the nucleophile) means that all three lone pairs on a hydroxide anion nucleophile (Fig. 4) are bound. This means that the proton on the OH$^-$ (or lone pair on an O$^{2-}$) must point directly away from the PNP substrate, given the close to tetrahedral arrangement of the nucleophile below the three metal ions (Fig. 4A). For catalysis to occur, therefore, one of the metal ions must release the nucleophile so that a lone pair is free to attack the electrophilic phosphorus. There are thus two possi-

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$^6$ I. P. Fabrichniy, unpublished observation.

$^7$ E. Oksanen, manuscript in preparation.
bilities: either a very strong electrophile is required to persuade the hydroxide ion to attack, such as metaphosphate, in which case the reaction would proceed via a dissociative “pull-you” mechanism. Alternatively, catalysis starts by repositioning the nucleophile to the same side of the trimetal ion plane as PNP, in which case the reaction proceeds via an associative “push-me” mechanism. A possible help in such a push would be the hydrophobic Cβ of Asp^{15}, because it is the nearest neighbor to W_{o/F} on the side opposite the trimetal plane. Finally, the His^{98}–Lys^{205}–Lys^{296} cluster around the bridging atom presumably also reflects a mechanistic difference between family I and family II PPases.

In either case, this His-Lys-Lys cluster, the trimetal site, and ground-state distortion of PNP represent features of family II PPase catalysis that have not been observed before and that mark significant structural and, therefore, presumably mechanistic differences between family I and family II PPases. A full understanding of the mechanism of family II PPases requires further kinetic and structural studies aimed at distinguishing between the models presented above. The proposed role of His^{98} as a general acid would of course be common to all members of the DHH family of phosphoesterases (7, 8).

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REFERENCES
1. Kornberg, A. (1962) in Horizons in Biochemistry (Kasha, M., and Pullman, B., eds) pp. 251, Academic Press, New York
2. Shintani, T., Uchiumi, T., Yonezawa, T., Salminen, A., Baykov, A. A., Lahti, R., and Hachimori, A. (1998) FEBS Lett. 439, 263–266
3. Young, T. W., Kuhn, N. J., Wadeson, A., Ward, S., Burges, D., and Cooke, G. D. (1998) Microbiology 144, 2563–2571
4. Merckel, M. C., Fabrichniy, I. P., Salminen, A., Kalkkinen, N., Baykov, A. A., Lahti, R., and Goldman, A. (2001) Structure (Camb.) 9, 289–297
5. Ahn, S., Milner, A. J., Futterer, K., Konopka, M., Ilias, M., Young, T. W., and White, S. A. (2001) J. Mol. Biol. 313, 797–811
6. Baykov, A. A., Cooperman, B. S., Goldman, A., and Lahti, R. (1999) Prog. Mol. Subcell. Biol. 23, 127–150
7. Aravind, L., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 17–19
8. Yamagata, A., Kakuta, Y., Masui, R., and Fukuyama, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5908–5912
9. Fabrichniy, I. P., Lehtio, L., Salminen, A., Zyryanov, A. B., Baykov, A. A., Lahti, R., and Goldman, A. (2004) Biochemistry 43, 14403–14411
10. Zyrryanov, A. B., Vener, A. V., Salminen, A., Goldman, A., Lahti, R., and Baykov, A. A. (2004) Biochemistry 43, 1065–1074
11. Parfenyev, A. N., Salminen, A., Halonen, P., Hachimori, A., Baykov, A. A., and Lahti, R. (2001) J. Biol. Chem. 276, 24511–245118
12. Kuhn, N. J., Wadeson, A., Ward, S., and Young, T. W. (2000) Arch. Biochem. Biophys. 379, 292–298
13. Zyrryanov, A. B., Shestakov, A. S., Lahti, R., and Baykov, A. A. (2002) Biochemistry 41, 901–906
14. Harding, M. M. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 401–411
15. Zyrryanov, A. B., Tammenkoski, M., Salminen, A., Kolomiytseva, G. Y., Fabrichniy, I. P., Goldman, A., Lahti, R., and Baykov, A. A. (2004) Biochemistry 43, 14395–14402
16. Halonen, P., Tammenkoski, M., Niiranen, L., Hupalahti, S., Parfenyev, A. N., Goldman, A., Baykov, A., and Lahti, R. (2005) Biochemistry 44, 4004–4010
17. Kabash, W. (1993) J. Appl. Crystallogr. 26, 795–800
18. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
19. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nîlges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
20. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
21. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463
22. Murshudov, G. N. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
23. C CCP4. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
24. Laskowski, R. A., MacArthur, M. V., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
25. Mildvan, A. S. (1997) Proteins 29, 401–416
26. Harding, M. M. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 857–867
27. Smirnova, I. N., Baykov, A. A., and Avaeva, S. M. (1986) FEBS Lett. 206, 121–124
28. Chanley, I. D., and Feageson, E. (1963) J. Am. Chem. Soc. 85, 1181–1190
29. Zyrryanov, A. B., Lahti, R., and Baykov, A. A. (2005) Biochemistry (Moscow) 70, 908–912
30. Larsen, M., Willett, R., and Yount, R. G. (1969) Science 166, 1510–1511
31. Salminen, T., Käpylä, J., Heikinheimo, P., Goldman, A., Heinonen, J., Baykov, A. A., Cooperman, B. S., and Lahti, R. (1995) Biochemistry 34, 782–791
32. Heikinheimo, P., Lehtonen, J., Baykov, A., Cooperman, B. S., Lahti, R., and Goldman, A. (1996) Structure 4, 1491–1508
33. Heikinheimo, P., Tuomolin, Y., Ahonen, A. K., Tepljakov, A., Cooperman, B. S., Baykov, A. A., Lahti, R., and Goldman, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3121–3126
34. Pauling, L. (1946) Chem. Engng. News 24, 1375–1377
35. Cannan, W. R., Singleton, S. F., and Benkovic, S. J. (1996) Nat. Struct. Biol. 3, 821–833
36. Rajagopalan, P. T., and Benkovic, S. J. (2002) Chem. Rev. 102, 24–36
37. Chatani, Y., and Yatsuyanagi, K. (1987) Macromolecules 20, 1042–1045
38. Cruickshank, D. W. J. (1964) Acta Crystallogr. Sect. D Biol. Crystallogr. 17, 671–672
39. Allen, K. N., and Dunaway-Mariano, D. (2004) Trends Biochem. Sci. 29, 495–503
40. Admirai, S. I., and Herschlag, D. (1995) Chem. Biol. 2, 729–739