Molecular Basis for the Local Conformational Rearrangement of Human Phosphoserine Phosphatase*

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Human phosphoserine phosphatase (HPSP) regulates the levels of glycine and L-serine, the putative co-agonists for the glycine site of the NMDA receptor in the brain. Here, we describe the first crystal structures of the HPSP in complexes with the competitive inhibitor 2-amino-3-phosphonopropionic acid (AP3) at 2.5 Å, and the phosphate ion (Pi) and the product uncompetitive inhibitor L-serine (HPSP-L-Ser-Pi) at 2.8 Å. The complex structures reveal that the open-closed environmental change of the active site, generated by local rearrangement of the α-helical bundle domain, is important to substrate recognition and hydrolysis. The maximal extent of this structural rearrangement is shown to be about 13 Å at the L4 loop and about 25° at the helix α3. Both the structural change and mutagenesis data suggest that Arg-65 and Glu-29 play an important role in the binding of the substrate. Interestingly, the AP3 binding mode turns out to be significantly different from that of the natural substrate, phospho-L-serine, and the HPSP-L-Ser-Pi structure provides a structural basis for the feedback control mechanism of serine. These analyses allow us to provide a clear model for the mechanism of HPSP and a framework for structure-based drug development.

Phosphoserine phosphatase (PSP) is an important enzyme in the phosphorylated pathway of serine biosynthesis, which contributes a major portion of the endogenous L-serine (1, 2). In the mammalian nervous system, L-serine is converted from L-serine by serine racemase (3, 4) and acts as a co-agonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, a major neurotransmitter receptor family (5). NMDA receptors require coactivation at a glycine site where D-serine, present in high levels in the mammalian brain, is three times more potent than glycine (6). Recently, a subset of NMDA receptors has been found to be activated by glycine or L-serine in the absence of glutamate (7). The enzymatic reaction of PSP is Mg2+-dependent and results in the dephosphorylation of phospho-L-serine with the formation of a phosphoenzyme intermediate, which is subsequently autodephosphorylated. The resulting product, L-serine, is not only a precursor for the biosynthesis of glycine but also an uncompetitive inhibitor for the enzymatic reaction of PSP (8). It may be possible to regulate NMDA activity by using selective inhibitors against serine racemase and/or PSP.

The PSP family and P-type ATPases are members of the haloacid dehalogenase-like hydrolase family (9–11). The crystal structures have been elucidated for several members of the haloacid dehalogenase superfamily: the L-2-haloacid dehalogenases, CheY, P-type ATPase and, recently, Methanococcus jannaschii (MJ) PSP (12–16). Three conserved motifs have been observed in amino acid sequences in members of the haloacid dehalogenase superfamily, and these residues are located in the active pocket. The significance of these motifs was confirmed by an assay of mutants for substrate hydrolysis (17). Other phosphoesters, including phosphoethanolamine and phosphoproteins such as phosphotyrosine and casein, were found to be poor substrates (2). In the crystal structure of the P-type ATPase (14), the active site is in the open environment, exposing the active site to the solvent. Recently, in the case of MJ PSP (15, 16, 24), the active site has been shown to be in the open-closed environment. Structural studies of the PSP family indicate that the conformational rearrangement of the active site is required to catalyze the reaction. Until recently, there has been limited structural evidence supporting the existence of such a rearrangement in the PSP family.

Human phosphoserine phosphatase (HPSP), an attractive target for structure-based drug design, contains 225 amino acids with a relative molecular mass of 25 kDa (18) and forms a dimer under physiological conditions. To gain a precise understanding of the molecular details of HPSP-specific substrate recognition, we have determined the first crystal structures of HPSP in complexes with the competitive inhibitor 2-amino-3-phosphonopropionic acid (AP3) (19) and with phosphate ion and L-serine, the product uncompetitive inhibitor, by the multi-wavelength anomalous dispersion (MAD) method using selenomethionine (Se-Met) crystals (Table I). In this paper, we present movement of the small helical domain in HPSP, and the complex structures provide a great deal of information about how AP3 interacts with HPSP at 2.5 Å resolution and the...
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TABLE I
Statistics from the crystallographic analysis

| Data set          | Peak     | Edge     | Remote   | PLS      |
|-------------------|----------|----------|----------|----------|
| Wavelength (Å)    | 0.9792   | 0.9794   | 0.9724   | 1.100    |
| Space group       | 14       |          |          |          |
| Resolution (Å)    | 2.8      | 2.7      | 2.8      | 2.8      |
| Observations      | 82983    | 82810    | 80678    | 52545    |
| Unique reflections| 15855    | 12989    | 11754    | 11843    |
| Data coverage (%) | 95.2     | 96.8     | 97.8     | 98.4     |
| R_{sym} (%)       | 3.4      | 4.6      | 6.1      | 5.9      |
| Mean FOM\(^b\) (20.0–2.8 Å) | 0.53    |          |          |          |

Refinement statistics\(^c\)

| R.m.s.d.\(^d\) | 0.010    | 0.011    |          |          |
| Bonds (Å)      |          |          |          |          |
| Angles (°)     | 1.580    | 1.563    |          |          |
| Reflections (|F| > 2|σ|F|) | 15098    | 10343    |          |          |
| Total atoms    | 3495     | 3506     |          |          |
| R factor (%)   | 21.3     | 20.4     |          |          |
| R_{free} (%)   | 28.4     | 27.5     |          |          |

\(^a\) R_{sym} = \sum_{h,k,l} I_{h,k,l} - (\langle I_{h,k,l} \rangle) / \sum_{h,k,l} for the intensity (I) of i observations of reflection h.

\(^b\) FOM, figure of merit.

\(^c\) AP3 and PLS correspond to AP3·HPSP and t-serine·phosphate·HPSP, respectively.

\(^d\) Root mean square deviations from ideal geometry.

\(^{\ast}\) R factor = \|F_{o,b} - F_{c,a}\|/\|F_{c,a}\|, where F_{o,b} and F_{c,a} are the observed and calculated factors, respectively.

\(^{\ast\ast}\) R_{free} = R factor calculated using 7% of the reflections data chosen randomly and omitted start of refinement.

RESULTS AND DISCUSSION

Overall Structure—The monomer of HPSP consists of two domains, the core domain and the subdomain, containing a four-helix bundle and dimer interface regions with overall dimensions of about 32 × 37 × 55 Å\(^3\) (Fig. 1a). The refined HPSP structure comprises two molecules in the asymmetric unit. The crystal structure of the biologically active HPSP dimer is illustrated in Fig. 2. The larger core domain resembles a Rossmann fold (29) and comprises amino acid residues 4–29, 81–135, and 147–225, which account for about 70% of all the residues of the enzyme. It has an α/β structure consisting of a central six-stranded parallel β-strand (order of β8, β7, β6, β1, β2, and β3) sandwiched between four short α-helices (α8–α11) on one side and α6 and α7, the N-terminal helix, and the C-terminal helix on the other side of the sheets. The subdomain is formed by amino acid residues 30–80 and 136–146 and account for about 30% of the enzyme. It consists of a four-helix bundle region (α2–α5) and dimer interface region (β4–β5). The four-helix bundle is packed in an approximately antiparallel manner, with the two β-sheets of the dimer being antiparallel. The L4 loop, which connects α3 and α4, is the hinge region that allows for the conformational adjustment between the open to the closed structures. The N- and C-terminal helix face each other and are not required for in vitro enzyme activity but stabilize the protein (data not shown). There is a large cleft between the core domain and subdomain. This cleft includes the active site and contains residues invariant across the 19 PSP classes. These conserved residues come together in the active site to bind the competitive and the uncompetitive inhibitor.

Dimer Interface—The crystallographic asymmetric unit of HPSP contains a dimer (molecules A and B, Fig. 2). Moreover, gel filtration chromatography and dynamic light scattering data show that HPSP is a dimer in solution. Mutant residues such as Y138K, F139K, and Y143K, which could interfere with dimer interfaces, exist in an aggregated and insoluble form in various buffer solutions (data not shown). Therefore, the dimerization of HPSP may be increased by the stability and solubility of the subunits. The dimer interface forms between the amino acid residues 130–140 (strand β4–β5). As shown in Fig. 2, it is

Structural Basis of the Feedback Control Mechanism of Serine at 2.8 Å. These structural studies on the HPSP protein may provide a clear mechanism and a framework for inhibitor design.

MATERIALS AND METHODS

Protein Expression and Purification—The human phosphoserine phosphatase gene was cloned from a human lymphocyte cDNA library into the pET15b vector, overexpressed in Escherichia coli, as a histidine-tagged fused protein, and purified by nickel affinity chromatography. The histidine tag fusion protein was cleaved with thrombin, further purified by gel filtration chromatography (Hi-Load Superdex 200) and the final protein was concentrated by ultrafiltration. Preparation of the selenomethionyl protein for MAD experiments was performed as described (20).

Crystallization, Data Collection, and Processing—Crystals of human phosphoserine phosphatase were grown at 22 °C by the hanging drop vapor diffusion method. The crystals were obtained by mixing the protein solution (20 mg/ml) with the same volume of the well buffer containing 2.4 M sodium/potassium phosphate (pH 5.0), 0.1 M LiCl, 2% polyethylene glycol 4000, and 10 mM dithiothreitol. The crystals belong to space group I4 with a = b = 106.05 Å, c = 87.34 Å, and a = b = c = 90° and contain two molecules in the asymmetric unit. The MAD and complex data sets were collected using a synchrotron radiation source at beamline 6B at Pohang Light Source (PLS, Pohang, South Korea). Data were processed with the programs DENZO and SCALEPACK (21). The inhibitor-complexed co-crystals were obtained by incubation for 1 h in the presence of 5 mM Mg\(^{2+}\) ion and 10 mM AP3 or 5 mM Mg\(^{2+}\) ion, 10 mM phosphoserine, and 10 mM t-serine.

Site-directed Mutagenesis and Enzyme Assay—Site-directed variant proteins were generated using QuikChange (Stratagene) and HPSP in the pET15b plasmid as the template. All constructs were verified by DNA sequencing, and all the mutant proteins were purified using the same method that was employed for the isolation of wild type HPSP. The phosphatase activity was measured using a coupled enzyme assay method as described (8).

Structure Determination and Refinement—The structure of the complex of HPSP and the inhibitor AF60 was determined by the multilength anomalous-diffraction method using data collected with selenomethionyl protein crystals. The phases, calculated with the program SOLVE and RESOLVE (T. Terwilliger, www.solve.tau.ac.il), had a mean figure of merit of 0.52 at 2.8 Å resolution. The MAD phasing statistics and refinement statistics are given in Table I. The model was completed by iterative cycles of model building with QUANTA (Accelrys) and refinement with CNS (22). Initial models for the complex with L-serine, phosphate ion (P\(^i\)), and the apo protein (HPSP·t-Ser·P\(^i\)) were obtained by molecular replacement with the program CNS using a model of complex AP3·HPSP as a search model. The final model contains 452 amino acids and two AP3 molecules for AP3·HPSP and 450 amino acids, two phosphate ions, and two t-serines for the HPSP·t-Ser·P\(^i\) complex.

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Fig. 1. *a*, schematic ribbon diagram of the overall structure of a human phosphoserine phosphatase protomer (closed conformation; MolA). The N- and C termini of the protein are labeled, and certain helices and sheets are numbered. Each domain is shown in a different color, blue and cyan. The regions of structural adjustment between the two conformations are shown in magenta. AP3 is shown in yellow. *b*, a schematic ribbon diagram of the overall structure of a human phosphoserine phosphatase monomer (open conformation; MolB). *c*, a stereodiagram of a Ca trace of a human phosphoserine phosphatase structure in the same orientation as in panel *a*. Every twentieth residue is labeled. *d*, representative MAD electron density calculated at a resolution of 20–2.8 Å and contoured at 1.0 σ surrounding the small subdomain of HPSP with the model superimposed is shown.
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**Fig. 2. A schematic ribbon diagram showing the human phosphoserine phosphatase dimer.** The detailed interaction residues are indicated as ball and stick representations in the inset (right), which show a close-up view of the dimeric interface region. The four antiparallel β-sheets are clearly shown in cyan. Residues (blue) from one protomer (MolA) make hydrophobic and hydrophilic interactions with residues (orange) from the other protomer (MolB).

a relatively narrow region that is 12% of the total accessible surface of the dimer and buries with a surface area of 2443.3 Å². There are non-polar (63.6%), polar (18.2%), and polar-charged (18.2%) residue fractions in the dimer interface. Strand β4 (residues 135 to 138) forms a hairpin together with strand β5 and pairs with strand β4 of the opposite protomer in an antiparallel way, resulting in four antiparallel β-strands. There are three ionic contacts across the dimer interface. First, the amide nitrogen atom of Phe-139 (MolA) in one protomer forms a hydrogen bond with the carbonyl oxygen atom of Leu-135 (MolB) in the other protomer. Second, the NZ atom of Lys-136 (MolB) interacts with the carbonyl oxygen atom of Ala-144 (MolA) and the hydroxy group of Tyr-138 (MolA). Additionally, a salt bridge between the side chains Arg-134 and Glu-142 of the opposite protomer is formed (2.5 Å). On the other hand, hydrophobic residues such as Leu-135, Phe-137, Phe-139, Tyr-143, and Phe-146 point into the hydrophobic core of one protomer and contribute to form the hydrophobic cluster on both sides of the central six β strands. Consequently, the hydrogen bonds and ionic interactions between the two protomer subunits and the hydrophobic interactions in each protomer stabilize the dimer state of the HPSP protein.

The Open-Closed Environment of the Active Site; Implications for Enzyme Mechanism—The competitive inhibitor, AP3, binds in the active site cleft between the large core domain and the small four-helix bundle domain. The MAD electron density map of the bound AP3 reveals that the two inhibitor molecules are well ordered, and the electron density is consistent with full occupancy of the catalytic site (Fig. 4). The structures show an inhibitor bound to each protomer with the same overall molecular geometry. Interestingly, although the active site of one is buried under the molecular surface in MolA, the inhibitor is more exposed to solution in MolB (Fig. 3b). The overall root mean square deviation of all Ca atoms (residues 4–225) is 0.77 Å; leaving out the N terminus, C terminus, and parts of the four-helical bundle region (residues 4–12, 38–70, and 217–225) reduces the root mean square deviation to 0.37 Å. The detailed difference in interaction for each protomer is as follows (Fig. 3a): whereas the carbonyl oxygen of AP3 is 2.9 Å and 3.2 Å away from the O61 atom of Asp-179 and the Oγ1 atom of Thr-182 in open-state (MolB), respectively, it is 3.4 Å and 3.1 Å away from the NH1 atom of Arg-202 and the O61 atom of Asp-183 in closed state (MolA), respectively. Additionally, whereas the amine group of AP3 is 3.7 Å away from the Oγ1 atom of Thr-182 in MolB, it is 3.4 Å away in MolA. In the case of MolB, the oxygen atoms of the phosphate group in AP3 interact with the main chain amide of Asp-22 (3.3 Å), Gly-110 (2.9 Å), and the side chain atoms of Glu-29 (3.0 Å) and Lys-158 (2.9 Å). Whereas, in case of MolA, it interacts with the side chain atoms of Asp-20 (2.3 Å), Asp-22 (2.4 Å), Lys-158 (3.2 Å), and the main chain amide of Gly-110 (2.3 Å). Overall, AP3 is positioned closer to the helix α3 in the closed state than in the open state. The side chain amino groups of Arg-202 are clearly positioned to interact with the carboxyl atoms of AP3. It is possible that substrate binding is responsible for this conformational change in the active site region, leading to the high affinity binding of the inhibitor. Furthermore, the orientation of the P–C bond in AP3, corresponding to the scissile P–O bond in phosphoserine, is not in a suitable position for nucleophilic attack by Asp-20. In contrast, the BeF₃⁻–PSP structure (Protein Data Bank number 1J97) has the scissile P–O bond in a more suitable position for catalysis than in our structure (Fig. 6a). The strictly conserved glycine-rich pocket (residues 53–54, 110–111, and 178–180) in the vicinity of the active site may contribute to the local flexibility in this region and may result in the tighter binding of the substrate. Local conformational rearrangements, which are generated by movement of the four-helical bundle domain, could explain the access of its substrate and the release of its products. The maximal extent of this structural rearrangement is shown to be ~13 Å at the L4 loop and about 25° at helix α3 (Fig. 3a). In the closed conformation, helix α3 is about two helical turns longer than in the open conformation. In fact, in the open conformation of the Ca²⁺–ATPase, the phosphorylation site is more than 25 Å away from...
the ATP binding site (14). Recently, the structures of MJ PSP were also reported in the open and closed state (15, 16, 24). Therefore, the open and closed conformations of the PSP family help to explain the molecular mechanism of the P-type ATPase and the other PSP families.

L-Serine Binding Site—L-serine is a negative feedback inhibitor, and this type of allosteric inhibition has been characterized as uncompetitive (8). An omit map showed strong electron density for a phosphate ion and L-serine in the active site (Fig. 4b). Previous studies showed that analogs of phosphoserine lacking the β-hydroxy group or a derivatized β-hydroxy group were inactive. Furthermore, substitutions of serine with an elongated side chain, the hydroxyamino regioisomer or D-serine, were also shown to be inactive (8). The binding mode of L-phosphoserine appears to be highly selective, which explains why several types of analog are inactive. The detailed interaction for each protomer is as follows (Fig. 4b). First, the carbonyl oxygen atoms of L-serine interact with the NH atoms of Arg-202 (2.2 Å and 3.2 Å), and the amide nitrogen atom of Ala-181 (2.5 Å). Both R202K and R202A mutant proteins have very low enzyme activity (Fig. 5). Therefore, the residue Arg-202 may be important to the enzyme reaction and the allosteric feedback mechanism. Second, the amino group of L-serine forms a hydrogen bond with the side chain of Thr-182 (2.3 Å). Finally, the hydroxyl oxygen atom of L-serine is 2.2 Å away from the oxygen atom (O3') of the phosphate ion group, indicating that the phosphate ion group may be necessary for negative feedback regulation. The N7 atom of Lys-158 forms a hydrogen bond with the side chain of Thr-182 (2.3 Å). Finally, the hydroxyl oxygen atom of L-serine is 2.2 Å away from the oxygen atom (O3') of the phosphate ion group, indicating that the phosphate ion group may be necessary for negative feedback regulation.
Asp-22. Overall, the side chain of Asp-20, Asp-22, Lys-158, and the hydroxy group of Ser-109 interact with the oxygen atoms of Pi, and these interactions have been shown to be similar to those of the MJ PSP structure (15).

In summary, both AP3 and L-SerPi adopted a very similar orientation upon binding to the active site. However, this type of orientation is significantly different from that adopted by phospho-L-serine in the MJ PSP complex structure (24). In the phospho-L-serine-MJ PSP structure, interactions between phospho-L-serine and Arg-52 (Arg-65 of HPSP), and Glu-20 (Glu-29 of HPSP) seemed to be responsible for the orientation and specificity for serine. The phosphonic group of AP3 bound to the phosphate binding site and adopted an orientation that can accommodate the inhibitor binding. This orientation seems to be the unproductive in terms of catalysis. Fig. 4a suggests that the unproductive orientation of the phosphonic group could be the reason why Mg2+ is absent in the active site.

**Mutational Analysis**—Previous mechanism studies, based on mutation experiments on the active site in HPSP (17), have shown that Asp-20, Asp-22, Ser-109, Lys-158, Glu-178, Asp-179, Gly-180, and Asp-183 residues are very important for enzymatic hydrolysis. Our structures clearly explain how these residues play a key role in hydrolysis. Furthermore, to understand the key feature of substrate binding and rearrangement, we have isolated several mutants by site-directed mutagenesis and characterized their enzymatic activity in vitro. The assay data are summarized in Fig. 5. Although the S23T mutation caused a moderate decrease in activity, other mutations activated the enzyme by >50% (S23A) or even >99% (T182S, R202A, and R202K). The side chains of Ser-23, Thr-182 and Arg-202 are very close to the binding sites of the inhibitors (AP3 and L-serine) and the phosphate ion. Additionally, the Glu-29, Arg-65, and Asn-133 residues are completely conserved in 19 members of the PSP family. Whereas the N133A mutation shows a lower activity of 22% compared with the wild type, the N133D mutation shows enhanced activity. The N2 atom of Asn-133 is 2.3 and 3.2 Å away from the carbonyl oxygen atoms of Ala-133 and Asp-147, respectively. Above all, the O61 atom of Asn-133 is 3.5 Å away from the backbone amide nitrogen of Ala-153. These residues are located at the base of the

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Fig. 4. The detailed interactions of inhibitors in the active site cleft of the HPSP complex structures. The orientation of each inhibitor is the same. *a*, stereo view of the AP3 binding site as a ball and stick representation in closed state. An omit Fo - Fc electron density map, contoured at 2.0 σ, shows a well ordered AP3 molecule (carbon in yellow, oxygen in red, nitrogen in blue and phosphorus in green). *b*, stereo view of the L-serine and phosphate ion binding site as a ball and stick representation in closed state. Omit Fo - Fc electron density map, contoured at 1.7 σ, shows a ordered L-serine and phosphate ion (carbon in yellow, oxygen in red, nitrogen in blue, and phosphorus in green).

Fig. 5. Phosphoserine phosphatase activities of the human phosphoserine phosphatase point mutants. Reactions contained wild type or mutant HPSP, l-phosphoserine, and malachite green dye. Assays were performed in triplicate, and error bars denote the standard deviation.
dimerization region in the HPSP crystals and may play an important role in structural integrity of the four-helix bundle domain during the rearrangement from the open to the closed conformations. Mutations E29D and E29N inactivated the enzyme by >99%, indicating that this residue may play a key role in the reaction mechanism of HPSP. Residue Arg-65, located on one of the four-helical bundles and the α-helix, has shown a very flexible region. Interestingly, the R65K and R65A mutations completely abolished the activity of hydrolysis. Therefore, it is reasonable to suppose that the Glu-29 and Arg-65 residues are essential for binding the carboxyl and amide groups of the substrate through hydrogen bonds. On the basis of the conformational rearrangement, mutagenesis, and previous biochemical studies, we have built a theoretical model of the phosphoserine-enzyme complex and propose a plausible reaction mechanism (Fig. 6).

In summary, establishing the mechanism of phosphoserine phosphatase as a distinct mode of conformational rearrangement will have a great impact both on understanding fundamental issues such as the protein structure-function relationship and on practical applications such as structure-based drug development.

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