Structural Units Important for Activity of a Novel-type Phosphoserine Phosphatase from *Hydrogenobacter thermophilus* TK-6 Revealed by Crystal Structure Analysis*

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**Background:** Sequence-based annotation of novel-type phosphoserine phosphatases (iPSPs) is difficult because of their diverse substrate preferences.

**Results:** Crystal structure and mutation analyses of iPSP1 from *Hydrogenobacter thermophilus* identified the residues involved in substrate recognition.

**Conclusion:** The His85 side chain and C-terminal region of iPSP are important for its phosphoserine phosphatase activity.

**Significance:** These findings will help to identify iPSPs from sequence databases.

Novel-type serine-synthesizing enzymes, termed metal-independent phosphoserine phosphatases (iPSPs), were recently identified and characterized from *Hydrogenobacter thermophilus*, a chemolithoautotrophic bacterium belonging to the order Aquificales. iPSPs are cofactor-dependent phosphoglycerate mutase (dPGM)-like phosphatases that have significant amino acid sequence similarity to dPGMs but lack phosphoglycerate mutase activity. Genes coding dPGM-like phosphatases have been identified in a broad range of organisms; however, predicting the function of the corresponding proteins based on sequence information alone is difficult due to their diverse substrate preferences. Here, we determined the crystal structure of iPSP1 from *H. thermophilus* in the apo-form and in complex with its substrate L-phosphoserine to find structural units important for its phosphatase activity toward L-phosphoserine. Structural and biochemical characterization of iPSP1 revealed that the side chains of His85 and C-terminal region characteristic of iPSP1 are responsible for the PSP activity. The importance of these structural units for PSP activity was confirmed by high PSP activity observed in two novel dPGM-like proteins from *Cyanobacteria* and *Chloroflexus* in which the two structural units were conserved. We anticipate that our present findings will facilitate understanding of the serine biosynthesis pathways of organisms that lack gene(s) encoding conventional PSPs, as the structural information revealed here will help to identify iPSP from sequence databases.

Novel-type serine-synthesizing enzymes, termed metal-independent phosphoserine phosphatases (iPSPs); EC 3.1.3.3; L-O-phosphoserine + H2O → L-serine + phosphate), were recently identified and characterized from *Hydrogenobacter thermophilus* TK-6, an obligately chemolithoautotrophic, thermophilic bacterium belonging to the order Aquificales (1–3). Although the kinetic parameters of iPSPs for L-phosphoserine are similar to those of conventional Mg2+-dependent phosphoserine phosphatases (dPSPs), iPSPs do not require divalent cations for their activity. In addition, iPSPs show no amino acid sequence homology to dPSPs. Therefore, the identification and properties of iPSPs suggest that these enzymes connect the missing link between phosphoserine and serine in the serine biosynthetic pathway of *H. thermophilus*, which lacks a gene encoding a conventional dPSP.

To date, two types of iPSPs have been identified in *H. thermophilus*, iPSP1 and iPSP2. The former is a homodimer of PspA subunits, and the latter is a heterodimer of PspA and PspB subunits. In contrast to dPSPs, which belong to the haloacid dehalogenase-like hydrolase superfamily, PspA and PspB belong to the histidine phosphatase superfamily, which is composed of a functionally diverse group of phosphatases and mutases, including cofactor-dependent phosphoglycerate mutases (dPGMs; EC 5.4.2.1), fructose-2,6-biphosphatase (EC 3.1.3.46), and phytases (EC 3.1.3.3) (4). Members of the histidine phosphatase superfamily share four conserved catalytic core residues consisting of two histidine and two arginine residues (e.g. Arg(9), His(10), Arg(27), and His(51) in *Bacillus stearothermophilus*).

The abbreviations used are: iPSP, metal-independent phosphoserine phosphatase; PSP, phosphoserine phosphatase; dPSP, metal-dependent PSP; dPGM, dependent phosphoglycerate mutase; PDB, Protein Data Bank.

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mophilus PhoE). In PhoE, His\textsuperscript{10} acts as the base to extract the phosphate group from the substrate, whereas the other three core residues interact electrostatically with the phosphate group during the reaction. In addition to the four conserved residues, an aspartate or glutamate residue (e.g. Glu\textsuperscript{83} in PhoE), which acts as a proton donor, is also required for the dephosphorylation reaction (4—6).

When compared with proteins with known function, PspA and PspB, which share 35% sequence identity, have the highest amino acid sequence similarity with dPGM (22 and 18% identities, respectively, to E. coli dPGM). In addition, the five catalytic core residues (four conserved residues and an additional aspartate/glutamate) that are characteristic of the histidine phosphatase superfamily are completely conserved in both iPSP subunits and dPGMs. For this reason, iPSPs were originally annotated as dPGMs; however, iPSNs lack the residues proposed to be important for mutase activity and show no phosphoglycerate mutase activity (1,7,8). As iPSPs exhibit substantial phosphatase activity specific to \( \lambda \)-phosphoserine, these enzymes are now thought to function as PSPs in vivo.

Proteins with homology to dPGM, but without mutase activity, have been found in a broad range of organisms and are predicted to act as phosphatases, although their physiological roles remain unknown (8—10). For example, PhoE from B. stearothermophilus is a homodimeric phosphatase with broad substrate specificity, and it has the highest specificity toward 3-phosphoglyceric acid and \( \alpha \)-naphthyl phosphate (8). Our previous study revealed that the PspA and PspB subunits of iPSPs exhibit completely different substrate specificities from that of PhoE (1). Namely, PspA has strict substrate specificity toward \( \lambda \)-phosphoserine, whereas PspB appears to have much lower phosphatase activity toward \( \lambda \)-phosphoserine and higher activity toward \( \eta \)-nitrophenyl phosphate. We also found that PspA homologs from Thermus thermophilus and Arabidopsis thaliana had slight PSP activity. However, the T. thermophilus PspA homolog had higher phosphatase activity toward \( \eta \)-phosphothreonine and 3-phosphoglycerate than toward \( \lambda \)-phosphoserine (1). Taken together, these results indicate that dPGM-like phosphatases have diverse substrate preferences, and their function cannot be predicted based on the whole amino acid sequence similarity alone. Therefore, identification of the structural units in the PspA subunit of H. thermophilus iPSP1 that is important for the PSP activity are expected to enable the identification of iPSPs among dPGM-like proteins with increased accuracy.

Here, we report the crystal structures of the apo- and the substrate-bound forms of iPSP1. Based on these crystal structures and mutagenesis data, we determined the structural units that would aid in the identification of iPSPs among dPGM-like proteins.

**EXPERIMENTAL PROCEDURES**

**Gene Cloning and Protein Expression and Purification**—The genes encoding the PspA and PspB subunits of H. thermophilus TK-6 (IAM 12695, DSM 6534), pspA (YP_003431771, HTTH0103), and pspB (YP_003431851, HTTH0183) were cloned into the expression vectors pCDFDuet-1 and pET21c (Novagen, Darmstadt, Germany), respectively, as described previously (1). The constructed plasmids were then mutated for expressing H9A mutants of the PspA and PspB subunits in which His\textsuperscript{9} was converted to Ala, and H85A, \( \Delta \)C1, and \( \Delta \)C7 mutants of PspA, in which His\textsuperscript{85} and His\textsuperscript{85} were converted to Ala, respectively, and Ile\textsuperscript{211} was deleted (iPSP1 (\( \Delta \)C1)), and Vaj\textsuperscript{250} and Ile\textsuperscript{211} were deleted (iPSP1 (\( \Delta \)C7)), using PrimeSTAR mutagenesis basal kit (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. The genes encoding iPSP homologs from Roseiflexus castenholzii DSM 13941 (YP_001432425, RCAS2324; YP_001430323, RCAS0171; and YP_001433513, RCAS3445) and Thermosynechococcus elongatus BP-1 (NP_682322, TRL1532) were amplified by PCR from the genomic DNAs of respective species. The amplified fragments were inserted into the Ndel/Xhol (R. castenholzii ones) or Ndel/EcoRI (T. elongatus) sites of pET-21c. The nucleotide sequences of the above-mentioned genes were verified by sequencing. The primers used in this study are listed in Table 1.

**Escherichia coli** BL21-Codon Plus (DE3)-RIL was used for the individual and co-expression of the PspA and PspB subunits, and their mutants. E. coli BL21 (DE3) was used for the expression of iPSP homologs from R. castenholzii and T. elongatus. The transformed E. coli cells harboring the expression plasmids were inoculated into Luria-Bertani medium containing the appropriate antibiotic(s) as follows: 50 \( \mu \)g ml\(^{-1}\) streptomycin, 50 \( \mu \)g ml\(^{-1}\) ampicillin, and/or 34 \( \mu \)g ml\(^{-1}\) chloramphenicol. After cultivating the cells aerobically at 37 °C until the absorbance at 600 nm reached \( \sim 0.6 \), the expressions of iPSP1, iPS2, and their mutants were induced by the addition of 1 mM (final concentration) isopropyl thio-\( \beta \)-D-galactopyranoside to the medium, followed by further cultivation for 3 h at 37 °C. The expressions of the R. castenholzii and T. elongatus iPSP homologs were induced with 0.5 mM isopropyl thio-\( \beta \)-D-galactopyranoside for 4 h at 37 °C. The cells were then harvested by centrifugation, resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA (4 ml/g of wet cells), sonicated, and subjected to centrifugation (100,000 \( \times \) g for 1 h) to obtain supernatants. iPSPs from H. thermophilus and its mutants were purified from the supernatants using butyl-Toyopearl (Tosoh, Tokyo, Japan) and Mono Q columns (GE Healthcare) after heat treatment at 80 °C for 10 min, as described previously (1). For the purification of iPSP homologs from R. castenholzii

### Table 1

| Name  | DNA sequence                                                                 |
|-------|-----------------------------------------------------------------------------|
| PspA H9A F | 5′-GTGCGAAGCGTCGAGGACGCGATGGA-3′            |
| PspA H9A R | 5′-CCCGAAGCCCGAATCCGATATAGCTT-3′             |
| PspB H9A F | 5′-GCTGCGAAGCGTCGAGGACGCGATGGA-3′            |
| PspB H9A R | 5′-CTCGGACGCCCTCGAGAAAATACACGAC-3′           |
| PspA H85A F | 5′-ATAGACCGCGGTATGTTGTCGCGGCT-3′             |
| PspA H85A R | 5′-CAACCACGGCTATCCGATATAGCTT-3′             |
| PspA ΔC1 F | 5′-AGGACGTGTAGTGCGCAATCAATAGC-3′         |
| PspA ΔC1 R | 5′-AACGCTATGGCTACCTGATACCC-3′                |
| PspA ΔC7 F | 5′-GGTTTTATGATGCTTCCGACATGAC-3′             |
| PspA ΔC7 R | 5′-GCAAGCTATAAAAAACCTGCAAGATG-3′             |
| TLR1532 F | 5′-ACAGAAATCCCTCTATAGCCCTCC-3′               |
| TLR1532 R | 5′-TCCCGAAGCGTCGAGGACGCGATGGA-3′             |
| RCAS2324 F | 5′-AGTCTGATTGCCGATGATGAC-3′                  |
| RCAS2324 R | 5′-CTGCTGATGATGAC-3′                        |
| RCAS0171 F | 5′-TATCCGATTGACGACCCCGCTTC-3′                |
| RCAS0171 R | 5′-GCCGACCGTGATGACGACCCCGCTTC-3′             |
| RCAS0171 R | 5′-GCCGACCGTGATGACGACCCCGCTTC-3′             |
| RCAS3445 F | 5′-CCCGAAGCGAGGATGACGACCCCGCTTC-3′           |
| RCAS3445 R | 5′-TCCCGAAGCGAGGATGACGACCCCGCTTC-3′           |
and *T. elongatus*, nonheat-treated supernatants were loaded onto a Q-Sepharose fast-flow column (GE Healthcare) and partially purified, as described previously (11).

**Enzyme Assays**—PSP activity was assayed by measuring the production of inorganic phosphate, as described previously with minor modifications (1). Briefly, the reaction mixture contained 10 μl of 1.0 mM HEPES-NaOH (pH 8.0 at room temperature), 5 μl of 50 mM L-phosphoserine, 0.5 μl of 100 mM EDTA (pH 8.0), 5 μl of enzyme solution in 20 mM Tris-HCl (pH 8.0), and 29.5 μl of water (total volume = 50 μl). For the determination of kinetic parameters, reaction mixtures containing 0.5, 1.0, 3.0, 5.0, 10.0, and 20.0 mM L-phosphoserine were used. Note that in our previous study, the reaction mixture contained 0.5–10 mM L-phosphoserine for the determination of kinetic parameters (1) because of the limited amount of purified native enzymes. Here, we used up to 20 mM L-phosphoserine to more accurately determine the kinetic parameters. Therefore, the final buffer concentration of the reaction mixture was increased from 20 to 200 mM to maintain a constant pH. The reaction mixture was incubated for 5–20 min at 70 °C for *H. thermophilus* proteins and 55 °C for *R. castenholzii* and *T. elongatus* proteins, which were close to the optimal growth temperatures of the host organisms. One unit of PSP activity was defined as the amount of enzyme producing 1 μmol of inorganic phosphate/min.

**Protein Assay**—Protein concentrations were measured using Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard.

**Crystallization and Data Collection for iPSP1 and iPSP1 (H9A)-L-Phosphoserine**—The crystallization and x-ray diffraction data collection of wild-type iPSP1 (iPSP1 (WT)) were performed as described previously (12). In brief, iPSP1 (WT) was crystallized by the sitting-drop vapor diffusion method. A 1.5-μl drop was first prepared by mixing 0.75 μl of an iPSP1 (WT) solution (10 mg ml⁻¹ in 5 mM Tris-HCl (pH 8.0)) and a 0.75-μl reservoir solution (100 mM HEPES-NaOH (pH 7.5), 10% (v/v) 2-propanol, and 20% (w/v) polyethylene glycol 4000). Obtained crystals were cryoprotected by soaking in a 3:1 (v/v) mixture of reservoir solution and ethylene glycol. Two x-ray diffraction data sets from a single crystal of iPSP1 (WT) were collected as described previously (12). The obtained data sets were indexed, integrated, scaled, and merged with the XDS program (13).

iPSP1 (H9A) complexed with L-phosphoserine was crystallized by the sitting-drop vapor diffusion method. A 1.5-μl drop was first prepared by mixing 0.75 μl of an iPSP1 (H9A) solution (10 mg/ml in 5 mM Tris-HCl (pH 8.0) containing 12-molar excess of L-phosphoserine) and 0.75 μl of reservoir solution (100 mM sodium acetate trihydrate (pH 4.6), 20 mM calcium chloride dihydrate, and 30% (v/v) (±)-2-methyl-2,4-pentanediol). Obtained crystals were cryoprotected by soaking in a 3:1 (v/v) mixture of reservoir solution and ethylene glycol. X-ray diffraction experiments for iPSP1 (H9A)-L-phosphoserine crystals were performed at beamline NE3A at Photon Factory-Advanced Ring (Ibaraki, Japan). A data set for the iPSP1 (H9A)-L-phosphoserine crystal was collected using an ADSC Quantum 270 detector under the following conditions: wavelength, 1.0000 Å; distance between the crystal and the detector, 213.9 mm; oscillation angle, 1.0°; and exposure time, 1 s per image. The collected dataset was indexed, integrated, scaled, and merged with the programs XDS (13).
exhibited PSP activity (39 ± 0 units mg⁻¹; Table 2). These results indicate that the PspB subunit has no or undetectable PSP activity.

**Structural Determination of iPSP1 (WT) without a Ligand and iPSP1 (H9A) Complexed with L-Phosphoserine**—The crystal structure of iPSP1 (WT) with no ligand was solved at 1.5-Å resolution by molecular replacement using the atomic coordinates of PhoE from *B. stearothermophilus* as a search model. The iPSP1 crystal belonged to the orthorhombic space group P2₁2₁2₁ with unit cell parameters a = 49.8 Å, b = 73.6 Å, and c = 124.3 Å, and it contained two PspA subunits in an asymmetric unit. The Ramachandran plot indicated that 98.6 and 1.4% of the residues were in the most favored and allowed regions, respectively. The data collection and refinement statistics are shown in Table 3.

The crystal structure of the iPSP1 (H9A)-L-phosphoserine complex was solved at 1.8-Å resolution by molecular replacement using the atomic coordinates of iPSP1 (WT) as a template. The iPSP1 (H9A)-L-phosphoserine complex crystal belonged to the orthorhombic space group P2₁2₁2₁ with unit cell parameters a = 49.3 Å, b = 68.9 Å, and c = 123.6 Å, and it consisted of two PspA (H9A) subunit L-phosphoserine complexes in an asymmetric unit. The Ramachandran plot indicated that 98.0 and 2.0% of the residues were in the most favored and allowed regions, respectively. The data collection and refinement statistics are presented in Table 3.

**FIGURE 1. Multiple sequence alignment of PspA and its homologs constructed using the MUSCLE program.** A, gaps after Gly201 (PspA subunit numbering) were manually removed. Amino acids surrounded by a black box and shaded indicate residues that can form hydrogen bonds with the phospho group and other parts of L-phosphoserine in PspA, respectively, and their corresponding amino acids conserved in other sequences. Residue numbers of PspA are shown above the sequences. B, alignment used for structure modeling. The alignments shown here, other than that of the C terminus, are the same as those shown in A.
Crystal Structures and Functional Sites of Novel Phosphatase

Overall Structures of iPSP1s—The final model of iPSP1 (WT) contained all of the amino acid residues (residues 1–211) for the two PspA subunits (chains A and B) in an asymmetric unit, and it consisted of 3481 protein atoms, one chloride ion, four ethylene glycol molecules, and 242 water molecules. The final model of the iPSP1 (H9A)-l-phosphoserine complex contained all of the amino acid residues, with the exception of several C-terminal residues (residues 1–207 and 1–203 for chains A and B, respectively), and it consisted of 3365 protein atoms, two chloride ions, seven ethylene glycol molecules, and 147 water molecules.

The PspA subunit adopted a dPGM-like fold containing six α-helices and six β-strands that formed an α/β/α sandwich of the central mixed β-sheet flanked by α-helices on both sides (Fig. 3, A and B). An additional smaller domain was predominantly composed of two α-helices (α3 and α4). Approximately 11% of the surface area of PspA (1,082 Å² per monomer) was buried in the dimer interface, 40% of which was occupied by hydrophobic residues. At the dimer interfaces that were mainly formed by the β6 strand and a loop connecting α6 and β5, 24 hydrogen bonds, eight salt bridges, and a disulfide bond were predicted to exist as well as hydrophobic interactions (Fig. 3A and Table 4). These findings were consistent with the gel filtration data of iPSP1 indicating that the protein behaves as a homodimer of the PspA subunit in solution (1). The dimerization behavior of the PspA subunit in iPSP1 was similar to that of the dPGM-like protein Rv3124 from Mycobacterium tuberculosis (PDB code 2A6P) (27).

The root mean square deviations between PspA (WT), PspA (H9A), and PhoE ranged from 0.2 to 1.6 Å, suggesting that their overall structures were nearly identical. The spatial positions and orientations were highly conserved between the essential residues of the PspA subunit (Arg9, His10, Asn15, Gln21, Gly22, Arg58, Glu83, and His150) and those of PhoE (Arg9, His10, Asn16, Gln22, Gly23, Arg59, Glu83, and His151) (Fig. 3C).

Binding Manner of l-Phosphoserine to iPSP1—Electron densities of l-phosphoserine were observed at the catalytic pocket

![FIGURE 2. 12% SDS-PAGE analysis of partially purified iPSPs used for the enzymatic activity measurements. Lane 1, Q-Sepharose fraction of RCAS2324; lane 2, Q-Sepharose fraction of TLR1532; lanes 3–5, supernatant of iPSP2-expressed and heat-treated CFE. Lane 3, PspA (WT); PspB (H9A); lane 4, PspA (H9A);PspB (WT); lane 5, PspA (H9A);PspB (H9A). M, molecular mass markers. The arrowheads indicate the bands corresponding to the expressed proteins.](http://www.jbc.org/)

![TABLE 3 Crystallographic data collection and refinement statistics](http://www.jbc.org/)

| Protein name          | iPSP1 (WT) | iPSP1 (H9A)/l-phosphoserine |
|-----------------------|------------|----------------------------|
| X-ray data collection |            |                            |
| Beamline              | SPring-8 BL-32XU | PF-AR NE3A                |
| Wavelength            | 1.0000 Å   | 1.0000 Å                   |
| Resolution*           | 45.0 to 1.50 Å (1.54 to 1.50 Å) | 38.2 to 1.80 Å (1.85 to 1.80 Å) |
| Space group           | P2₁,2,2    | P2₁,2,2                    |
| Unit cell parameter   | a 49.3 Å, b 73.6 Å, c 124.3 Å | a 49.3 Å, b 68.9 Å, c 123.6 Å |
| Total reflections*    | 630,969 (32,454) | 277,086 (16,180) |
| Unique reflections*   | 73,612 (5300) | 39,566 (2740) |
| Redundancy*           | 8.6 (6.1)  | 7.0 (5.9)                  |
| Completeness*         | 99.9% (99.0%) | 99.3% (94.1%)             |
| R_{model}/R_{free}    | 9.1% (58.7%) | 4.2% (16.6%)              |
| χ²/(ν)                | 14.8 (3.3)  | 33.6 (10.4)                |

Refinement

| Protein Data Bank code | 4IJ5       | 4IJ6                   |
|-----------------------|------------|------------------------|
| Resolution            | 36.1 to 1.50 Å | 38.2 to 1.80 Å |
| R_{model}/R_{free}    | 18.4/19.7% | 19.0/21.8% |
| Root mean square deviations | 0.0084 Å | 0.0053 Å |
| Bonds                 | 1.2716°    | 1.0986°                |
| Angles                | 3481 (16.0) | 3365 (17.3) |
| Protein               | 242 (23.2) | 22 (11.5)              |
| Water                 | 16 (22.3)  | 147 (23.9)             |
| Ethylene glycol       | 1 (13.0)   | 28 (23.1)              |
| Chloride              |            | 2 (19.8)               |

Observed residue range(s)

| Chain A | 1 to 211 | 1 to 207 |
| Chain B | 1 to 211 | 1 to 203 |

Ramachandran plot

| Most favored region | 98.6% | 98.0% |
| Additional favored regions | 1.4% | 2.0% |

* Values for the highest resolution shells are shown in parentheses for x-ray data collection.

b R_{work} = Σ(|Fo|−|Fc|)/Σ|Fo|, R_{free} = Σ(|Fo|−|Fc|)/Σ|Fo|.

c R_{work} = Σ(|Fo|−|Fc|)/Σ|Fo|, R_{free} was calculated with 95% of the data used for refinement, and R_{work} was calculated with the remaining 5% of the data.
when the inactive iPSP1 (H9A) mutant was crystallized in the presence of this substrate (Fig. 3D). In the crystal structure of the enzyme-substrate complex, oxygen atoms of the carboxyl group of L-phosphoserine formed hydrogen bonds with main-chain amides of Glu82, and N2 of Glu82, and partly formed a hydrogen or an ionic bond with N2 of His85. Nitrogen atom of amino group of L-phosphoserine formed a hydrogen or an ionic bond with oxygen atoms of the side-chain carboxyl group of Glu82, and formed hydrogen bonds with N2 of His85 and Oγ of Thr151. As the phosphate group of L-phosphoserine was considered to be negatively charged, the crystal structure of iPSP1 (H9A)/L-phosphoserine complex indicated that this group interacted with iPSP1 residues as follows: (i) O1P of the phosphate group formed an ionic bond with Nε of Arg8, and hydrogen bonds with side chains Nε2 of Asn15 and Nε2 of Glu82; (ii) O2P of the phosphate group formed an ionic bond with Nε and Nε1 of Arg8, and formed a hydrogen or an ionic bond with Nε2 of Glu82 and Nδ1 of His150; and (iii) O3P of the phosphate group formed an ionic bond with Nε of Arg8, and a hydrogen bond with main-chain amide of Thr151 (Fig. 3E and Table 5).

To predict the substrate-binding manner of iPSP1 (WT), we modeled the structure of iPSP (WT) in complex with L-phosphoserine by superposing the crystal structure of iPSP1 (WT) onto that of iPSP1 (H9A) (Fig. 3E). The modeled structure of the protein/ligand complex suggested that hydrogen bonds could be formed by oxygen atom of the phosphate group of L-phosphoserine and Nε2 of His85, and a hydrogen bond with main-chain amide of Thr151 (Fig. 3E and Table 5).

FIGURE 3. Structure of iPSP1 and its homologs. Overall structure of homodimeric iPSP1 (WT; A) and its protomer (B). The conserved catalytic core residues (Arg8, His9, Arg58, Glu82, and His150) are shown as sticks. C, superimposition of active-site residues of iPSP1 (WT) (gray) and PhoE (PDB code 1H2E) (cyan). The PspA subunit numberings are shown. D, Fobs – Fcalc, omit map of iPSP1 (H9A)/L-phosphoserine. E, superimposition of iPSP1 (WT) (gray) and iPSP1 (H9A)/L-phosphoserine (pink). F, 2Fobs – Fcalc, electron density map contoured at 1.0 (blue mesh) of the C-terminal residues region (residues 205–211) of iPSP1 (WT) (shown as sticks). L-Phosphoserine, also shown as sticks, is modeled by superposing iPSP1 (WT) and iPSP1 (H9A) complexed with L-phosphoserine. G, superimposition of L-phosphoserine onto the structure of PspA (H9A)/L-phosphoserine. Carbon atoms of iPSP1 (WT), iPSP1 (H9A), and o- and L-phosphoserine are shown in gray, pink, cyan, and light green, respectively. Phosphorus, oxygen, and nitrogen atoms are shown in orange, red, and blue, respectively. Images were generated with PyMOL.
Multiple amino acid sequence alignment indicated that the PspA subunit residues that interact with L-phosphoserine were highly conserved in the PspB subunit and PhoE (Fig. 1A). Among the PspA residues involved in forming hydrogen and/or ionic bonds with the phosphate group of L-phosphoserine (Arg$^8$, His$^9$, Asn$^{15}$, Gln$^{21}$, Arg$^{58}$, His$^{50}$, and Thr$^{151}$), only Thr$^{151}$ was replaced by Gln in both the PspB subunit and PhoE. These findings suggest that the phosphate group binding and dephosphorylation mechanisms of the PspA subunit in iPSP1 are similar to those of the PspB subunit, PhoE, and other proteins in the histidine phosphatase superfamily. The alignment analysis also indicated that the residues forming hydrogen and/or ionic bonds with the L-serine moiety of the substrate were also conserved in PspB and PhoE, with the exception of His$^{85}$, which was replaced by Phe$^{85}$ and Leu$^{86}$ in PspB and PhoE, respectively. Thus, His$^{85}$ was suggested to be important for the substrate specificity of iPSP1.

The crystal structure-based modeling also revealed that several C-terminal residues in iPSP1 (WT) formed a lid covering the active site cleft. In the iPSP1 (WT)-L-phosphoserine model, the C-terminal Ile$^{211}$ residue in iPSP1 (WT) was found to be close enough to form a hydrophobic interaction with L-phosphoserine (Fig. 3F), whereas the orientation of the side chain of Ile$^{211}$ in iPSP1 (H9A) complexed with L-phosphoserine was unclear because no electron density was observed for Ile$^{211}$ in iPSP1 (H9A). The residue corresponding to Ile$^{211}$ was absent in the PspB subunit and PhoE, whose C termini were seven and five residues shorter, respectively, than that of PspA (Fig. 1A), suggesting that the C-terminal residues of PspA, PspB, and PhoE interact differently with the bound substrate. Thus, the C-terminal residues of the PspA subunit were also suggested to be important for the PSP activity of iPSP1.

**Kinetic Analyses of iPSP1 Mutants**—The kinetic parameters of several iPSP1 mutants were determined and compared with those of iPSP1 (WT) to confirm the validity of the residues suggested to be important for the PSP activity based on the crystal structure analyses. The iPSP1 mutants, iPSP1 (H85A), iPSP1 (ΔC1), and iPSP1 (ΔC7), were purified by the same method as used for iPSP1 (WT), and their activities were then assayed using 0.5–20 mM L-phosphoserine (Table 6 and Fig. 4). iPSP1 (H85A) exhibited a markedly decreased affinity to L-phosphoserine ($K_m > 20$ mM) when compared with iPSP1 (WT) ($K_m = 1.5$ mM). In contrast, iPSP1 (ΔC1) displayed a drastically decreased $V_{\text{max}}$ (9.5 units mg protein$^{-1}$) when compared with iPSP1 (WT) (217 units mg protein$^{-1}$), whereas its $K_m$ was almost the same level as that of iPSP1 (WT). iPSP1 (ΔC7) showed the lowest PSP activity of the three examined mutants, displaying higher $K_m$ and lower $V_{\text{max}}$ values than iPSP1 (WT). The PSP activities of iPSP1 (H85A), iPSP1 (ΔC1), and iPSP1

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**Table 4**

| Interface of iPSP1 (WT) homodimer |
|----------------------------------|
|                                  |
| Chain A  | Chain B  | Distance |
|----------|----------|----------|
| Disulfide bond                     |
| Cys$^{188}$S$^\gamma$  | Cys$^{188}$S$^\gamma$  | 2.07     |
| Hydrogen bonds                     |
| Val$^{188}$/O                    | Arg$^{188}$/N$^{\gamma1}$ | 2.85 |
| Glu$^{188}$/O                    | Arg$^{188}$/N$^{\gamma2}$ | 3.82 |
| Leu$^{188}$/O                    | Lys$^{188}$/N$^\gamma$   | 2.75 |
| Ser$^{187}$/O                    | Ile$^{188}$/N$^\gamma$   | 2.81 |
| Asp$^{187}$/O$^{12}$            | Lys$^{189}$/N$^\gamma$   | 2.93 |
| Asn$^{189}$/O                    | Ser$^{173}$/O$^\gamma$   | 2.80 |
| Ile$^{173}$/O                    | Gly$^{189}$/N$^\gamma$   | 2.87 |
| Leu$^{194}$/O                    | Asn$^{189}$/N$^\gamma$   | 2.79 |
| Leu$^{194}$/O                    | Leu$^{194}$/N$^\gamma$   | 3.12 |
| Asn$^{195}$/O                    | Lys$^{189}$/N$^\gamma$   | 2.82 |
| Thr$^{197}$/O$^{11}$            | Lys$^{189}$/N$^\gamma$   | 2.87 |
| Thr$^{197}$/O$^{11}$            | Val$^{189}$/O$^\gamma$   | 2.74 |
| Arg$^{188}$/N$^{\gamma2}$       | Glu$^{189}$/O$^\gamma$   | 3.08 |
| Arg$^{189}$/N$^{\gamma2}$       | Glu$^{189}$/O$^\gamma$   | 3.71 |
| Lys$^{189}$/N$^\gamma$          | Leu$^{190}$/O$^\gamma$   | 2.79 |
| Ile$^{191}$/N$^\gamma$          | Ser$^{173}$/O$^\gamma$   | 2.82 |
| Lys$^{195}$/N$^\gamma$          | Asp$^{173}$/O$^\gamma$   | 2.90 |
| Salt bridges                     |
| Glu$^{189}$/O$^\gamma$          | Arg$^{187}$/N$^{\gamma1}$ | 3.74 |
| Glu$^{189}$/O$^\gamma$          | Arg$^{187}$/N$^{\gamma1}$ | 3.81 |
| Glu$^{190}$/O$^{\gamma2}$       | Arg$^{187}$/N$^\gamma$   | 3.64 |
| Glu$^{190}$/O$^{\gamma2}$       | Asp$^{173}$/O$^\gamma$   | 3.98 |
| Asp$^{173}$/O$^\gamma$          | Lys$^{189}$/N$^\gamma$   | 2.93 |
| Arg$^{187}$/N$^\gamma$          | Glu$^{189}$/O$^\gamma$   | 3.08 |
| Lys$^{193}$/N$^\gamma$          | Asp$^{173}$/O$^\gamma$   | 2.90 |

**Table 5**

| Hydrogen bonds formed between iPSP1 (H9A) and L-phosphoserine |
|---------------------------------------------------------------|
| Molecule/atom name                          | Molecule/residue/ Atom/ Distance |
|---------------------------------------------------------------|
| l-Phosphoserine/O1P                                | PspA/Arg$^8$/N$^{\gamma2}$ | 2.99 |
| l-Phosphoserine/O1P                                | PspA/Asn$^{15}$/O$^\gamma$ | 2.88 |
| l-Phosphoserine/O1P                                | PspA/Gln$^{21}$/N$^{\gamma2}$ | 2.99 |
| l-Phosphoserine/O2P                                | PspA/Gln$^{21}$/N$^{\gamma2}$ | 3.44 |
| l-Phosphoserine/O2P                                | PspA/Arg$^8$/N$^{\gamma1}$ | 3.01 |
| l-Phosphoserine/O2P                                | PspA/Arg$^8$/N$^\gamma$   | 3.40 |
| l-Phosphoserine/O2P                                | PspA/His$^{50}$/O$^\gamma$ | 3.70 |
| l-Phosphoserine/O3P                                | PspA/His$^{50}$/O$^\gamma$ | 3.12 |
| l-Phosphoserine/O3P                                | PspA/Thr$^{151}$/N$^\gamma$ | 2.97 |
| l-Phosphoserine/O$^\gamma$                       | PspA/Thr$^{151}$/O$^\gamma$ | 2.84 |
| l-Phosphoserine/N                                  | PspA/His$^{50}$/N$^\gamma$ | 3.09 |
| l-Phosphoserine/N                                  | PspA/Thr$^{151}$/N$^\gamma$ | 3.23 |
| l-Phosphoserine/O                                 | PspA/Gln$^{21}$/N$^\gamma$ | 2.92 |
| l-Phosphoserine/O                                 | PspA/Gln$^{21}$/N$^{\gamma2}$ | 2.83 |
| l-Phosphoserine/O                                 | PspA/Gln$^{21}$/N$^{\gamma2}$ | 2.96 |
| l-Phosphoserine/O                                 | PspA/Arg$^8$/N$^{\gamma2}$ | 3.40 |
| l-Phosphoserine/O                                 | PspA/Arg$^8$/N$^{\gamma2}$ | 3.95 |

**Table 6**

| Kinetic parameters for wild-type and mutant iPSP1 |
|--------------------------------------------------|
| iPSP1                                           |
| $K_m$  | $V_{\text{max}}$  | $k_{\text{cat}}/K_m$ |
| WT     | 1.5 ± 0.1  | units mg$^{-1}$ s$^{-1}$ | 98 |
| H85A   | ≥20  | 173 ± 5  | 2.0 |
| ΔC1    | 3.3 ± 0.1 | 9.5 ± 0.1  | 2.0 |
| ΔC7    | 15.2 ± 1.4  | 5.2 ± 0.3  | 0.24 |
(ΔC7) in the presence of 5 mM L-phosphoserine (15 ± 0.1, 5.7 ± 0.1, and 1.3 ± 0.1 units mg protein⁻¹, respectively) were markedly lower than that of iPSP1 (WT) (173 ± 9 units mg protein⁻¹). These results confirmed that both His<sup>85</sup> and the C-terminal residues were critical for the PSP activity of iPSP1.

Detection of PSP Activity in iPSP Homologs—Proteins with high amino acid sequence similarity to the PspA subunit of iPSP1, namely TLR1532 from *T. elongatus* and RCAS0170, RCAS2324, and RCAS3445 from *R. castenholzii* (Fig. 1A), were expressed in *E. coli* BL21 (DE3) and subjected to PSP activity assays. TLR1532, RCAS2324, and RCAS3445 were obtained in the soluble fraction, whereas RCAS0170 formed inclusion bodies. PSP activity was detected in the cell-free extracts of TLR1532- and RCAS2324-expressing *E. coli* but was not observed in that of RCAS3445-expressing *E. coli*. Partially purified TLR1532 and RCAS2324 (8.9- and 2.3-fold purified, respectively, from *E. coli* cell-free extract; Fig. 2) showed PSP activities of 63 ± 1 and 9.8 ± 1 units mg protein⁻¹, respectively, suggesting that these proteins possessed substantial PSP activities.

**DISCUSSION**

In this study, we solved the crystal structures of the substrate-free and substrate-bound forms of iPSP1 from *H. thermophilus*. An inactive mutant iPSP1 (H9A) was used for the structural analysis of the bound form. Moreover, we identified that two structural units are necessary for the PSP activity of iPSP1 based on both structural and functional data.

His<sup>85</sup> in the PspA subunit was not conserved in the PspB subunit nor in PhoE and was found to be critical for the high PSP activity of iPS1, because iPSP1 (H85A) possesses only 8.7% of the PSP activity of iPSP1 (WT) in the presence of 5 mM L-phosphoserine. His<sup>85</sup> very likely contributes to substrate affinity, as iPSP1 (H85A) has a markedly increased *K<sub>m</sub>* value compared with that of iPSP1 (WT). The successful determination of the crystal structure of iPSP1 (H9A)-L-phosphoserine has demonstrated that the *N*<sup>ε2</sup> atom of His<sup>85</sup> forms a hydrogen bond with the nitrogen of L-phosphoserine and allows us to speculate on the molecular mechanism of L-phosphoserine recognition by iPSP1. The imidazole group of His<sup>85</sup> is perpendicular to the aromatic ring of Phe<sup>116</sup>, which is suggested to form an aromatic hydrogen bond with hydrogenated *N*<sup>ε1</sup> of His<sup>85</sup> (28). In addition, as the amino group of L-phosphoserine was positively charged (–NH<sub>3</sub><sup>+</sup>) around physiological pH values (29, 30), the nonhydrogenated *N*<sup>ε2</sup> of His<sup>85</sup> may be appropriately oriented for recognizing the amino group of L-phosphoserine by the interaction between the hydrogenated *N*<sup>ε1</sup> of His<sup>85</sup> and the side chain of Phe<sup>116</sup>. It is possible that the PspB subunit in iPSP2 lacks PSP activity due to the replacement of His<sup>85</sup> with Phe<sup>85</sup> in this subunit, because the side chain of Phe<sup>85</sup> cannot form a hydrogen bond with the amino group of L-phosphoserine.

The importance of His<sup>85</sup> for substrate affinity also sheds light on the stereo selectivity of iPSP1. Our previous study revealed that iPSP1 has extremely low activity toward D-phosphoserine (1). This property can be explained by the formation of hydrogen bonds between the amino group of L-phosphoserine and the side chain of His<sup>85</sup>, and the carboxyl group of L-phosphoserine and the main-chain amides in Gln<sup>21</sup> and Gly<sup>22</sup>, which are considered to contribute to substrate binding. When D-phosphoserine is superimposed onto the PspA (H9A)-L-phosphoserine structure by fitting the α-carbon and carboxyl groups of L- and D-phosphoserine, the amino group of D-phosphoserine cannot form a hydrogen bond with the side chain of His<sup>85</sup> or any other residue (Fig. 3G). Therefore, the affinity of PspA to D-phosphoserine is expected to be lower than that to L-phosphoserine, resulting in a lower phosphatase activity to D-phosphoserine. This explanation is supported by the fact that iPSP1 also shows no activity toward 3-phosphoglyceric acid, in which the amino group of D-phosphoserine is substituted by a hydroxyl group (1).

The C-terminal region of the PspA subunit was also revealed to affect the PSP activity of iPSP1, because ΔC1 and ΔC7 mutants of iPSP1 have drastically reduced activities toward L-phosphoserine as a target substrate. The deletion of the C-terminal Ile<sup>211</sup> residue in iPSP1 (ΔC1), which could interact with L-phosphoserine in the active cleft, clearly reduced *V*<sub>max</sub> but did not significantly affect *K<sub>m</sub>* values. These results suggest that Ile<sup>211</sup> contributes to the PSP activity of iPSP1 by stabilizing the transition state or increasing the pre-exponential factor of the reaction. However, it remains unclear whether the deletion of a single C-terminal residue at position 211 or the substitution of it by a residue without a hydrophobic side chain can cause the drastic decrease of PSP activity. Although further study is needed, we speculate that not only the length of the C terminus but the hydrophobicity of the side chain may influence the activity of iPSPs because the C-terminal residues of all proteins from which substantial levels of PSP activity have been detected and iPSP1 orthologs from *Aquificae* (1) are Ile or Leu.

The C-terminal regions of histidine phosphatase superfamily proteins, including dPGM and fructose-2,6-bisphosphatase, have been studied because these regions protrude over the active-site cleft and are presumed to affect substrate selectivity (8, 31–33). Several C-terminal residues in iPSP1 also form a lid that covers the active site cleft, as shown in the wild-type structure (Fig. 5). This lid is expected to have a certain degree of flexibility, as in the case of dPGM (34), because the entrance to the active site of iPSP1 (WT) is too small for L-phosphoserine (Fig. 5) and the B-factor of the C terminus is relatively high. The C-terminal lid of iPSP1 likely functions to hold L-phosphoserine in the active site, as the *K<sub>m</sub>* value of iPSP1 (ΔC7), in which the lid is completely lost and the active site cleft is fully exposed to the solvent (Fig. 5), was increased ~10-fold when compared with that of iPSP1 (WT). It has been reported that deletion of the C-terminal region (residues 235–246) of dPGM from *Saccharomyces cerevisiae* reduces the mutase activity but has no effect on phosphatase activity (31, 32). However, the C-terminal length of yeast dPGM was found to be 12 residues longer than that of PspA (data not shown). In addition, the phosphatase activity of dPGM is only a side reaction and is considerably lower than that of iPSP. Therefore, there is no conflict between the contribution of the C termini of dPGMs and iPSPs on their phosphatase activities.

The active site of the PspB subunit in iPSP2 is predicted to be wide open and to lack a lid based on the homology model constructed here using the crystal structure of the PspA subunit in...
iPSP1 (WT) as the template, because the C terminus of the PspB subunit is seven residues shorter than that of the PspA subunit (Fig. 5). The molecular model of the PspB subunit helps explain why PspB lacks PSP activity and has stronger phosphatase activity toward $p$-nitrophenyl phosphate than PspA (1); the wider entrance of the active-site pocket of PspB enables access to larger substrates, as in the case of PhoE (Fig. 5) (8).

Interestingly, dPGM-like proteins from both Cyanobacteria and Chloroflexi (TLR1532 and RCAS2324, respectively, which contain conserved histidine residues corresponding to His$^{85}$ of iPSP1) exhibit substantial PSP activity. In contrast, dPGM-like proteins that lack this conserved histidine residue (PspB from H. thermophilus, RCAS3445, TTHA0368, At5g04120, and At3g05170 (1)) possess no activity or very weak PSP activity. Moreover, the substrate selectivity of PhoE, in which His$^{85}$ is not conserved, markedly differs from that of iPSP1, although the PSP activity of PhoE has not been tested (1, 8). Although PhoE and dPGM-like proteins with low PSP activity (TTHA0368 and At5g04120) have a histidine residue at the position corresponding to Asp$^{84}$ (adjacent to His$^{85}$) of iPSP1, the crystal structure of PhoE (PDB code 1H2E) shows that the imidazole ring of this histidine residue is oriented in the opposite direction of the active core. Based on this finding, it is unlikely that the histidine residue at position 84 of PhoE, or other similar dPGM-like proteins, can form hydrogen bonds with the substrate.

We also determined that the C-terminal regions of TLR1532 and RCAS2324, which have high PSP activities, are similar to that of iPSP1 (Fig. 1B). These proteins contain conserved “$^{199}\text{HLG}^{201}$” and “$^{210}\text{A(I/L)211-COOH}$” (PspA subunit numbering) sequences that are connected by a loop consisting of 8–10 residues that are not conserved (Fig. 1). Homology models of TLR1532 and RCAS2324 suggest that the interactions between these conserved sequences and neighboring residues are similar to those that occur in iPSP1 (Fig. 6). In addition, the connecting loops are bound to the entrance region of the active
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sites by a comparable number of interactions as those identified in iPSP1, even though the sequences of the loops are not conserved. Therefore, the C-terminal Leu residue of TLR1532 and RCAS2324 will also be able to thrust into the active-site cleft in the same manner as the Ile residue of iPSP1. These observations strongly suggest that the C termini of dPGM-like proteins from Cyanobacteria and Chloroflexi also interact with the substrate, product, and/or the reaction intermediates in the active site and are important for PSP activity.

The amino acid sequence identities of TLR1532 and RCAS2324 with the PspA subunit in iPSP are 32 and 36%, respectively, although that between the PspA and PspB subunits is 35%. The similar levels of sequence identity despite the differences in substrate specificity highlight the difficulty in predicting high PSP activity from the primary structure of a dPGM-like protein. However, our present findings indicate that the presence of His85 and the characteristic C-terminal sequence and/or length are useful indicators of high PSP activity of a dPGM-like protein. Using these indicators, we can predict that several Firmicutes species, in addition to most Cyanobacteria and Chloroflexi species, which lack conventional dPSP gene(s) (1), possess iPSP gene(s).

The identification of iPSP gene(s) is important for understanding the serine and glycine anabolic pathways that function in organisms lacking dPSP gene(s) (1). For example, nearly all Cyanobacteria species have an iPSP gene instead of a dPSP gene and synthesize serine from L-phosphoserine as indicated by 14C-labeling analysis (35). Therefore, iPSP may actually function as a serine-synthesizing enzyme in Cyanobacteria.

In conclusion, the crystal structures of iPSSP1 (WT) and (H9A)·L-phosphoserine from H. thermophilus determined in this study and the kinetic analyses of mutated proteins have revealed that the presence of a His residue at position 85 and the sequence and/or length of the C-terminal region are both necessary for the PSP activity of iPSSP1. These two structural features can be used as indicators to identify dPGM-like proteins with high PSP activity.

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