The **thrH** Gene Product of *Pseudomonas aeruginosa* Is a Dual Activity Enzyme with a Novel Phosphoserine:Homoserine Phosphotransferase Activity*

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The **thrH** gene product of *Pseudomonas aeruginosa* has been shown to complement both homoserine kinase (**thrB** gene product) and phosphoserine phosphatase (**serB** gene product) activities **in vivo**. Sequence comparison has revealed that ThrH is related to phosphoserine phosphatases (PSP, EC 3.1.3.3) and belongs to the L-2-haloacid dehalogenase-like protein superfamily. We have solved the crystal structures of ThrH in the apoform and in complex with a bound product phosphate. The structure confirms an overall fold similar to that of PSP. Most of the catalytic residues of PSP are also conserved in ThrH, suggesting that similar catalytic mechanisms are used by both enzymes. Spectrophotometry-based **in vitro** assays show that ThrH is indeed a phosphoserine phosphatase with a **K_m** of 0.207 mM and **k_cat** of 13.4 min^{-1}, comparable with those of other PSPs.

More interestingly, using high pressure liquid chromatography-based assays, we have demonstrated that ThrH is able to further transfer the phosphoryl group to homoserine using phosphoserine as the phosphoryl group donor, indicating that ThrH has a novel phosphoserine:homoserine phosphotransferase activity.

In most bacteria, a major portion of endogenous threonine is generated from homoserine (Hse)1 in a two-step pathway: 1) phosphorylation of homoserine to phosphohomoserine (P-Hse), a reaction catalyzed by homoserine kinase (**thrB** gene product), followed by 2) isomerization and dephosphorylation of P-Hse to produce L-threonine (1). On the other hand, serine is synthesized from phosphoglycerate through sequential reactions catalyzed by 3-phosphoglycerate dehydrogenase (SerA), 3-phosphoserine aminotransferase (SerC), and phosphoserine phosphatase (SerB) (2). **ThrB** and SerB thus function in two distinct pathways of amino acid biosynthesis in *Escherichia coli* and many other species, inactivation of **thrB** gene results in threonine auxotrophy (3). However, in *Pseudomonas aeruginosa*, **thrB** mutants do not lead to threonine auxotrophy (4). A new gene named **thrH** has been identified that complements homoserine kinase activity **in vivo** (5). Furthermore, overexpression of ThrH also rescues serine auxotrophy caused by **serB** mutant in both *P. aeruginosa* and *E. coli* (5). **In vitro** assays have confirmed the phosphoserine phosphotase (PSP) activity of ThrH, but no homoserine kinase (HSK) activity of ThrH has been detected by the standard coupled kinase assays (5).

Sequence comparison reveals that ThrH protein is homologous to the PSP encoded by **serB** gene, which belongs to the HAD (L-2-haloacid dehalogenase)-like hydrolase superfamily (5, 6). Several members of this superfamily including phosphoserine phosphatase, phosphoglycerate phosphatase, phosphomannomutase, and β-phosphoglucomutase use a aspartyl-phosphate phosphoenzyme intermediate during catalysis (7, 8). Recent structural studies on these enzymes in complex with a series of substrates and transition state intermediates provided a wealth of structural data delineating the catalytic mechanism of this class of aspartyl-utilizing enzymes (9–12). ThrH is expected to use a similar phospho-aspartyl intermediate during its catalytic cycle.

It is intriguing though how ThrH, homologous to PSP, would perform the function of HSK in the cell. A recent survey of all of the kinases has shown that the majority of HSKs belong to the GHMP family, and its structure representatives have been characterized (13–15). However, homoserine kinases found in certain bacteria including *P. aeruginosa*, *Neisseria meningitides*, *Zymomonas mobilis*, and *Methylobacillus flagellatus* are different from the GHMP-type kinases, instead they are distantly related to protein kinases (13, 16). Additionally, ThrH protein in *P. aeruginosa* that compensates HSK activity **in vivo** belongs to yet another different protein fold, namely the HAD-like fold (5). To elucidate the structural and the chemical mechanisms of catalysis performed by ThrH, we have undertaken a crystallographic and functional analysis of this enzyme.

Here we report the crystal structures of ThrH protein in its apoform and in complex with one of the products (phosphate). These structures delineate the active site architecture of ThrH, which displays significant similarity to PSP. The PSP activity of ThrH is characterized with the continuous spectrophotometric assays. Moreover, using HPLC-based assays, we have found that ThrH is able to phosphorylate homoserine in the presence of phosphoserine in an ATP-independent manner, indicating that ThrH has a novel phosphotransferase activity and can transfer phosphate from phosphoserine to homoserine directly and generate phosphohomoserine.
Cloning, Expression, and Purification of ThrH—The thrH gene encodes a 205-residue polypeptide with a molecular mass of 23.5 kDa. It was amplified from P. aeruginosa (ATCC 17833) genomic DNA by the polymerase chain reaction and was cloned into a pProEX (Invitrogen) vector. It was expressed in the minimum medium supplemented with selenomethionine and other nutrients following standard protocols (17) and purified as described above. The overexpressed protein was first purified with a nickel-nitrilotriacetic acid (Ni-NTA) column and subsequently completed manually using program O (25). Refinement was performed using REFMAC5 (26). The final model contains a total of 412 amino acids for the 2 ThrH monomers in the asymmetric unit, 391 solvent atoms, 4 Mg$^{2+}$ ions, and 6 ethylene glycol molecules. The structure of the phosphate bound form of the enzyme was solved by molecular replacement using the native structure as the starting model. The refinement statistics of both structures are listed in Table I.

### EXPERIMENTAL PROCEDURES

#### Cloning, Expression, and Purification of ThrH

The thrH gene was codon-optimized for expression in E. coli and cloned into a pProEX (Invitrogen) vector. It was expressed in the minimum medium supplemented with selenomethionine and other nutrients following standard protocols (17) and purified as described above. The overexpressed protein was first purified with a nickel-nitrilotriacetic acid (Ni-NTA) column and subsequently completed manually using program O (25). Refinement was performed using REFMAC5 (26). The final model contains a total of 412 amino acids for the 2 ThrH monomers in the asymmetric unit, 391 solvent atoms, 4 Mg$^{2+}$ ions, and 6 ethylene glycol molecules. The structure of the phosphate bound form of the enzyme was solved by molecular replacement using the native structure as the starting model. The refinement statistics of both structures are listed in Table I.

#### Crystallization and Data Collection

ThrH crystals were grown at 20 °C using hanging drop vapor diffusion method. ThrH protein of concentration 25 mg/ml in 50 mM HEPES, pH 7.2, and 0.3 mM NaCl was used for crystallization. 2 μl of this protein solution was mixed with an equal volume of the reservoir solution containing 100 mM sodium cacodylate, pH 6.5, 200 mM MgCl$_2$, and 22.5% polyethylene glycol 1000 and equilibrated against the reservoir. Crystals of ~0.5–1.0 mm appeared typically within 2 days. The crystals belong to the orthorhombic space group C222, with cell dimensions of a = 71.75 Å, b = 97.24 Å, c = 131.77 Å, and α = β = γ = 90°. With two molecules per asymmetric unit, the Matthews coefficient was calculated to be 2.39 Å$^3$/Da with a solvent content of 48.6%. The selenomethionine crystals were grown under similar conditions and were of similar diffraction quality as the native crystals. Crystals of the phosphate complex were grown in the native crystal. For data collection at 100 K, crystals were transferred sequentially into the cryoprotectant solutions containing the reservoir solution and additional 10, 20, and 30% ethylene glycol. A 2.0 Å resolution data set at selenium absorption peak wavelength (0.979 Å) and a 1.5 Å resolution native data set were collected at beamline 8.2.2 at the Advanced Light Source (Berkeley, CA). Data for the phosphate bound complex was collected in-house on a RAXIS-IV image plate detector. All of the diffraction data were processed and scaled with the HKL2000 package (18), and the statistics of the data sets are listed in Table I.

#### Structure Determination and Refinement

The initial phases were obtained by the single wavelength anomalous dispersion (SAD) phasing method with data collected to 2.0 Å at the selenium peak wavelength (0.979 Å) using the program SOLVE (19). Eight selenium sites were located, and the resulting phases had a figure of merit of 0.83 after density modification procedures using RESOLVE (20). The phases were further improved and extended for native data set to 1.5 Å using the program DM as implemented in the CCP4 package (21, 22). This resulted in a clearly interpretable electron density map. The polypeptide chain was first traced automatically using ARWpARP (23, 24) and subsequently completed manually using program O (25). Refinement was performed using REFMAC5 (26). The final model contains a total of 412 amino acids for the 2 ThrH monomers in the asymmetric unit, 391 solvent atoms, 4 Mg$^{2+}$ ions, and 6 ethylene glycol molecules. The structure of the phosphate bound form of the enzyme was solved by molecular replacement using the native structure as the starting model. The refinement statistics of both structures are listed in Table I.

### Table I

| Crystal data and refinement statistics of ThrH | Se-MET peak λ | Native | PO$^4-$ complex |
|-----------------------------------------------|----------------|--------|-----------------|
| Wavelength (Å)                                | 0.97926        | 0.97679| 1.5418          |
| Resolution (Å)                                | 2.00–0.80      | 50–1.47 Å | 50–1.9 Å       |
| Total observations                            | 232,992        | 1,412,636| 344,730         |
| Unique reflections                            | 75,972         | 75,288 | 35,227          |
| Completeness (outer shell)                    | 58.1% (92.6%)  | 95.6% (71.9%) | 94.9 (99.1%) |
| $R_{crys}$ (outer shell)$^a$                   | 0.047 (0.158)  | 0.034 (0.445) | 0.035 (0.104) |
| llo (outer shell)                             | 42.21 (9.19)   | 48.36 (2.5) | 41.98 (12.7)   |
| Figure of merit                               | 0.63           |         |                 |
| Refinement                                    | 18.9           | 18.6   |                 |
| $R_{work}$                                    | 22.1           | 23.0   |                 |
| Protein atoms (Avg. B factor)                  | 3359 (24.2)    | 3333 (29.3) |             |
| Hetero atoms (Avg. B factor)                   | 22 (27.9)      | 30 (37.9) |             |
| Solvent atoms (Avg. B factor)                  | 389 (36.5)     | 326 (38.4) |             |
| R.m.s.d. bond length                          | 0.014 Å        | 0.012 Å |                 |
| Ramachandran Plot                             | 91.4           | 90.3   |                 |
| Most favored region (%)                        | 8.6            | 9.7    |                 |
| Additional allowed region (%)                  |                |        |                 |

$^a$ $R_{crys}$ = $\sum_{i} |F_o| - |F_c|$/$\sum_{i} |F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively.

$^b$ 5% of the reflections were used in the calculation of $R_{work}$. 

#### Continuous Kinase and Phosphatase Assays—Homoserine kinase activity of ThrH was assayed by the method described by Hsu and Viola (27). The activity was monitored by following the production of ADP using the pyruvate kinase-lactate dehydrogenase-coupled assay. The assay mixture (typically 500 μl) contained 100 mM Tris, pH 8.0, 10 mM KCl, 10 mM Mg acetate, 2 mM ATP, 0.7 mM phosphoenolpyruvate, 0.1 mM NADH, and 20 units each of pyruvate kinase and lactate dehydrogenase. 2–10 μM ThrH was added in the mixture. The reaction was monitored following the disappearance of NADH at 340 nm using a Beckman Coulter DU640 spectrophotometer. The temperature of the assay was maintained at 30 ± 1 °C by an Isotemp circulating water bath. As a control, the same assay was also carried out with a known HSK from *Methanococcus jannaschii* (14, 28).

Phosphoserine phosphatase assay of ThrH was performed using EnzChek® phosphatase assay kit (Molecular Probes, Eugene, OR) following manufacturer’s instructions. The assay uses the method of Webb (29) that monitors the release of free phosphate by coupling the phosphatase reaction with the enzymatic conversion of 2-amino-6-mercaptop-7-methyl-purine riboside to 2-amino-6-mercaptop-7-methyl-purine and ribose-1-phosphate by purine nucleoside phosphorylase. The substrate 2-amino-6-mercaptop-7-methyl-purine riboside has an absorbance maximum of 330 nm, whereas the maximum absorption of the product is 360 nm. Each 500-μl reaction mixture contains 50 mM Tris, pH 7.5, 1 mM MgCl$_2$, 0.1 mM sodium azide, 200 μM 2-amino-6-mercaptop-7-methyl-purine riboside, 1 unit of purine nucleoside phosphorylase, and 2.7 μM of the enzyme ThrH. Reactions were started by the addition of various phosphorylated amino acid substrates (5 μl). To test whether ThrH can dephosphorylate P-Hse, the HSK-catalyzed homoserine phosphorylation was coupled to the phosphatase assay. The following components were added in place of the substrate: 1.3 μM *M. jannaschii* HSK, 100 μM ATP, and 100 μM homoserine.

### HPLC Assays—HPLC assays were performed on a Beckman Coulter HPLC GOLD® system equipped with a UV detector and a Rainin Dynamax fluorescence detector (model FL1). Amino acids were analyzed using the AccQTag® method of Waters Corporation (Milford, MA) by labeling the reaction contents with the AccQ-Fluor® reagent (6-aminquinolyl-N-hydroxysuccinimidyl carbamate) prior to loading on a C18 reverse-phase column. Compositions of the two elution buffers were: (A) 140 mM sodium acetate, 17 mM triethylamine, pH 4.95, and (B) 60% acetonitrile in H$_2$O and 0.01% acetic acid. For fluorescence detection, excitation wavelength was $\lambda_{ex}$ = 250 nm and emission wavelength was $\lambda_{em}$ = 395 nm. Labeling of reaction contents was done by mixing 5 μl of the reaction mixture with 15 μl of 20 mM HCl and 60 μl of borate buffer supplied by the manufacturer. 20 μl of the Fluor
reagent was then added. The mixture was vortexed for 10 s and incubated at room temperature for 1 min. This was followed by incubation in 55 °C water bath for exactly 10 min. Labeled samples were stored on ice until prior to injection. Following injection, linear gradients of 0–7% Buffer B over 17 min, 7–10% B over 4 min, and 10–34% B over 13 min were applied. The retention times of individual amino acid standards phosphoserine (P-Ser), Ser, and Hse were first determined by individually labeling freshly dissolved 10 mM amino acid using the above protocol. The retention time of P-Hse was determined using P-Hse generated from homoserine and ATP in the presence of M. jannaschii HSK (28). The phosphotransferase reaction was done by incubating 100 μM each of Hse and P-Ser along with ThrH enzyme for 3 h in the presence of 20 mM each of KCl and MgCl2 in 50 mM Tris buffer, pH 7.5. The reaction was quenched by the addition of 20 mM HCl, and the reaction contents were labeled with the Fluor reagent as noted above. 5 μL of the labeled reaction mixture was loaded onto the C18 column and monitored by both fluorescence and UV absorbance.

RESULTS

Structure Description of ThrH—The crystal structure of P. aeruginosa ThrH contains two monomers in the asymmetric unit. Each ThrH monomer folds into a kidney bean-shaped structure with two lobes or domains (Fig. 1A). Domain I adopts a classical Rossmann-like fold consisting of a central six-stranded parallel β-sheet with helices on either side. Domain II comprises a four-helix bundle (residues 14–68) inserted between the first strand (a) and the first helix (E) of domain I (Fig. 1A) and a β-hairpin (residues 111–127) inserted between the third strand c and the third helix G of the Rossmann domain. The second helix (B) in the four-helix bundle is shorter than the other three and is structurally more flexible (see below). Superposition of the two monomers in the asymmetric unit resulted in an r.m.s. deviation of 0.875 Å for 196 Cα positions. There appears to be a hinge motion between the four-helix bundle and the rest of the structure (Fig. 1B). The largest difference is in the positions of the two helices (B and C) along with their connecting loop. In one of the monomers, this region moves toward domain I by ~4.2Å compared with the other monomer. Residues in this loop region in both monomers have higher temperature factors than the rest of the structure, indicating a higher degree of flexibility (Fig. 1C).

The two ThrH monomers observed in the asymmetric unit are organized in such a manner that the six-stranded β sheet of the Rossmann-like domain in each monomer are packed side-by-side to form a nearly continuous twelve-stranded β-sheet (Fig. 1C). The strand e at the edge of each monomer makes extensive interactions with the other monomer across the in-
The active site of each monomer, as marked by the bound phosphate and Mg$^{2+}$ ions, is facing away from the interface and is located on opposite sides of the dimer. The dimer interface is predominantly hydrophobic and buries a surface area of 1344 Å$^2$/monomer (13% of the total surface area) upon dimerization. The B-factor distribution for the two monomers indicates that one of the monomers is more flexible in the four helical bundle region when compared with the other (Fig. 1C).

**Structure Comparison with PSP and Other HAD Superfamily Proteins**—Sequence comparison has shown that ThrH is clearly related to phosphoserine phosphatases (5). The previously determined crystal structures of PSP have revealed that PSP belongs to the HAD-like hydrolase fold (30, 31). As expected, the overall structure of ThrH is similar to that of PSP. Superposition of ThrH with *M. jannaschii* PSP resulted in an r.m.s. deviation of 2.6 Å for 182 superimposable C$\alpha$ atoms (Fig. 2A). This similarity is not limited to the Rossmann-like domain or the conserved sequence motifs. It extends to the second domain as well (Fig. 2A and B). The domain II of both ThrH and PSP contains a four helical bundle and a β-hairpin, and...
these secondary structure elements are largely superimposable (Fig. 2A).

Even though all of the members of HAD superfamily are marked by three highly conserved sequence motifs that are mapped on the three loop regions in domain I (Figs. 1A and 2B), there are large differences in the structures of the second domains. Some members of the HAD superfamily such as phosphonoacetaldehyde hydrolase (PDB code 1fez) (12), phosphoglucomutase (PDB code 1vvh) (11), and l-2-haloacid dehalogenase (PDB code 1q5) (32) also contain a second four-helical bundle domain with the same topological connections as PSP/ThrH, although other HAD members such as the hypothetical protein Cof from *Thermoplasma acidophilum* (PDB code 1l6r) and the catalytical domain P of Ca^{2+}/ATPase (PDB code 1eul) (33) may contain a completely different second domain. In the case of the probable phosphatase Yrbl of *Haemophilus influenzae* (PDB code 1k1e) (34), the second domain is missing entirely.

**The Active Site of ThrH**—The closest protein neighbor of ThrH, PSP, has recently been well characterized structurally (7–9, 30, 31, 35, 36). Sequence and structure comparison of ThrH and PSP revealed a very similar active site configuration. The active site of ThrH is located at the junction of the two domains. Two Mg^{2+} ions are found in and near the active site (Fig. 3). Their coordination exhibits a nearly ideal octahedral geometry. The ligands for the first Mg^{2+} ion are the negatively charged side chains of Asp-7, Asp-152, the backbone carbonyl of Glu-9, and three water molecules. The second Mg^{2+} ion is coordinated to the side chain oxygen of Asp-152, Asp-152, the backbone carbonyl of Glu-9, and three water molecules. The second Mg^{2+} ion may help to neutralize the negative charges around the active site (Fig. 2A) and thus facilitate substrate binding. However, this second Mg^{2+} is not observed in the PSP structures. Because ThrH crystals were grown in the presence of 0.2 M MgCl₂, the possibility remains that this second Mg^{2+} may be a crystallization artifact.

In the phosphate product-bound structure, the phosphate interacts directly with the first Mg^{2+} ion (2.31 Å), replacing one of the Mg^{2+} ligand water molecules in the apostructure (Fig. 4A). It also interacts with side chains of Lys-133 (2.74 Å) and Ser-90 (3.04 Å) and with the backbone amide groups of Asp-91 (3.04 Å) and Glu-9 (2.90 Å). Additionally, the side chains of Asp-7 and Glu-9 are also within the contact distance to the phosphate (3.2–3.4 Å from the phosphorus). Asp-7 is the invariant Asp in the first conserved motif of HAD superfamily that is involved in the nucleophilic attack on the phosphorus of the phosphate product.
substrate and subsequent formation of the phospho-aspartyl intermediate. Glu-9 is also highly conserved, and it probably acts as a general base to extract a proton from the attacking water molecule during the later step of hydrolyzing the phosphoenzyme intermediate (9).

**Phosphatase Activity of ThrH**—Given the profound similarity between ThrH and PSP, it is not surprising that ThrH possesses PSP activity as has been shown previously (5). We have carried out steady state kinetic analysis of phosphoserine phosphatase activity of ThrH. (Fig. 5A). The measurements yielded a $K_m$ of 0.207 mM and $k_{cat}$ of 13.36 min$^{-1}$, comparable with what have been reported for *M. jannaschii* PSP ($K_m = 0.62$ mM, $k_{cat} = 20$ min$^{-1}$) (9). The ability of ThrH to dephosphorylate phospho-DL-Thr and phospho-D-Ser was also tested (Fig. 5B). Moreover, the ability of ThrH to dephosphorylate P-Hse was tested by coupling the phosphatase assay with the reaction catalyzed by homoserine kinase (for details, see “Experimental Procedures”). As is shown in Fig. 5B, ThrH exhibits various degrees of phosphatase activity using these alternative substrates and is in general not as effective as when the native substrate phospho-l-Ser is used.

**Phosphotransferase Activity of ThrH**—It has been established unequivocally that ThrH is able to compensate homoserine kinase activity in vivo and thus has been annotated as a homoserine kinase isoenzyme (5). However, the ATP-dependent kinase activity of ThrH has not been demonstrated in vitro. Using highly homogeneous ThrH protein purified, we also failed to detect any in vitro homoserine kinase activity for ThrH under various conditions with different divalent ions and buffers (data not shown). Patte *et al.* (5) have speculated that ThrH may be able to perform phosphate exchange between P-Ser and Hse via the phosphoryl enzyme intermediate. The phosphatase assays described in the previous section have shown that ThrH indeed is able to dephosphorylate both P-Ser and P-Hse, indicating that its active site can accommodate either substrate. We then set to investigate whether ThrH was able to transfer phosphate from P-Ser to Hse by HPLC assays (Fig. 6). First, the retention times for the three amino acid standards under the experiment condition were observed to be 21.8 min for P-Ser, 25.6 min for Ser, and 27.9 min for Hse (Fig. 6A). The retention time of P-Hse was determined to be 22.3 min using homoserine kinase-catalyzed reaction with *M. jannaschii* HSK enzyme, which generates P-Hse (Fig. 6B). Clearly, two peaks corresponding to the substrate Hse (27.9 min) and the
product P-Hse (22.3 min) were observed. To probe for phosphotransferase activity of ThrH, we incubated ThrH with P-Ser and Hse and labeled the reaction contents before column separation. A distinct peak with a retention time of 22.3 min was observed, indicating the formation of P-Hse (Fig. 6C).

DISCUSSION

Substrate Recognition by ThrH—The overwhelming similarity between mjPSP and ThrH allowed us to easily model a substrate P-Ser molecule in the active site of ThrH without any steric hindrance (Fig. 7). The interactions observed between the modeled substrate and the active site residues were very similar to those seen in mjPSP and are summarized in Fig. 7. In the model, the phosphate moiety of P-Ser occupied essentially identical position as the product phosphate and made the same interactions with ThrH protein atoms. However, the optimal interactions between the enzyme and the amino acid moiety of the substrate would require conformational changes on domain II of ThrH. Specifically, the side chain of Glu-15 was oriented to interact with the backbone amino group of the substrate P-Ser. This residue corresponds to Glu-20 in mjPSP where it has been shown to be involved directly in P-Ser binding and indirectly in hydrolyzing the phospho-aspartyl intermediate by helping to correctly orient the attacking water molecules (9). Asn-155 of ThrH was superimposed with Asn-170 of mjPSP, which, upon closure of domain II interacts with the phosphate oxygen of substrate P-Ser. In the current ThrH-phosphate complex structure, the protein adopted a relatively open conformation and Asn-155 is outside the hydrogen-bond range with the phosphate oxygens (3.86 Å). However, it can be expected that substrate binding-induced domain closure would easily bring Asn-155 into direct contact with the substrate in a manner similar to that observed in PSP. Similarly, Arg-56 side chain of mjPSP forms favorable hydrogen bonds with the carboxylate of P-Ser, whereas in the present “open” form of ThrH, Arg-46 (corresponding to Arg-56 of mjPSP on the third helix of domain II) is also outside hydrogen bond range (distance from Arg-46 side chain to the modeled P-Ser carboxylate is −4.0 Å). However, the same domain movement would bring this residue into the contact position as well. Another PSP active site residue, Phe-49, however, is replaced by Tyr-39 in ThrH, which could have been in position to interact with the carboxylate of P-Ser. Modeling studies suggest that ThrH active site would also be able to accommodate P-Hse and would require a slightly lesser degree of conformational changes on domain II because Hse side chain is one carbon longer than Ser (data not shown).

Substrate binding-induced conformational changes that results in a “closed” active site have been a common theme in several HAD superfamily enzymes (9, 31, 36). The conformational differences between the two independent ThrH monomers in the asymmetric unit demonstrated the structural flexibility of ThrH especially at the hinge region between domains I and II. It is reasonable to assume that substrate binding would induce similar conformational changes in ThrH as that observed in PSP. This structural flexibility should be important for ThrH to accommodate both P-Ser and P-Hse/Hse substrates.

Proposed Mechanisms of Phosphotransfer by ThrH—The demonstration that ThrH can generate P-Hse using P-Ser as the phosphoryl donor explains the ability of ThrH to compensate HSK activity in vivo. In particular, it emphasizes that although ThrH complements thrB⁻ mutant in vivo, it achieves this function by a completely different chemical mechanism from that of true homoserine kinases. Thus, it may not be appropriate to call ThrH a homoserine kinase isoenzyme.

Based on the conservation of the active sites of PSP and ThrH, it is likely that ThrH uses the same mechanisms as PSP for the initial P-Ser dephosphorylation step (9). The apoenzyme has an open conformation. The binding of P-Ser substrate induces a hinge motion between the two domains, resulting in a “closed” conformation that brings several additional residues into contact with the substrate. The critical Asp-7 is now close to the phosphate moiety of the substrate for the nucleophilic attack on the phosphorus. After the initial phosphate transfer from P-Ser to Asp-7 and the formation of the phosphoenzyme intermediate, the active site opens to release the dephosphorylated amino acid and allows water molecules to enter and hydrolyze the phospho-aspartyl intermediate. For ThrH, however, Hse molecule is able to bind in the active site after serine dissociates and before hydrolysis of the phospho-aspartate occurs. The phospho-enzyme intermediate subsequently transfers the phosphate to homoserine instead of water. The structural flexibility at the ThrH active site appears to enable the enzyme to recognize homoserine as an alternative phosphoryl acceptor.

Our HPLC assays clearly detected the presence of P-Hse as the product of this phosphotransfer reaction. Notably, this product was not abundant in the final reaction mixture. One of the reasons may be that there are three simultaneous competing reactions: the phosphoserine phosphatase, phosphohomo-
serine phosphatase, and P-Ser:Hse phosphotransferase reactions. The product serine from the first step of the reaction competes with homoserine for the binding site, the P-Hse phosphatase activity of the enzyme also depletes the P-Hse population. Therefore, there are complicated multiple kinetic/equilibrium parameters governing the overall rate of phosphoryl transfer from phosphoserine to homoserine. More detailed kinetic studies, especially the measurements of the rate for each step of the reaction (those of phosphotransfer to and from Asp 7 of the enzyme with respect to different substrates), and the relative affinity of various substrates will be critical in understanding the mechanism of the reaction. However, so far an effective continuous assay to monitor this phosphotransfer reaction is yet to be developed.

The discovery that, in *P. aeruginosa*, inactivation of *thrB* gene does not lead to the usual *thr* phenotype and that only inactivation of both *thrB* and *thrH* results in the Thr auxotroph suggested that ThrH compensates the function of ThrB in *vivo* (5). ThrH was thus annotated as a homoserine kinase isoenzyme despite lack of *in vitro* homoserine kinase activity. The results of the structural and biochemical investigations reported here illustrated the biochemical mechanisms of ThrH functions, which are different from that of HSK. Most importantly, we demonstrate that ThrH is not an ATP-dependent kinase but possesses a novel phosphoserine:homoserine phosphotransferase activity.

The phosphotransferase activity of ThrH is closely related to its phosphoserine phosphatase activity. We have shown that the kinetic property of ThrH regarding phosphoserine phosphatase is comparable with that of other PSPs, indicating that ThrH is essentially a PSP. However, different from other *bona fide* PSPs, ThrH is able to further transfer the phosphoryl group to homoserine. Although this phosphotransfer reaction may be considered a side reaction catalyzed by ThrH, it nevertheless has distinct physiological consequences in *P. aeruginosa*. Furthermore, such phosphotransferase activity has not been reported for any other PSPs. The fact that many PSPs do not compensate ThrB activity *in vitro* may indicate, although indirectly, that they do not have the phosphoserine:homoserine phosphotransferase activity. In this sense, ThrH is unique in that it has acquired an additional function of transferring the phosphoryl group to another phosphoryl acceptor instead of water. More structural and biochemical studies, especially the structures of the enzyme complexed with respective phosphoryl acceptor and donor, will be required to understand the structural determinants that distinguish ThrH from other PSPs.

ThrH protein appears to be the first enzyme identified that transfers a phosphoryl group from one non-nucleotide phosphorylated metabolite directly onto another with similar structure. The utilization of a phosphoenzyme intermediate during catalysis is well suited for such a phosphoryl transfer reaction. Such phosphoryl transfer reaction represents an efficient and economic way to reuse the phosphoryl groups in the cell. It is likely that there may be more phosphoryl transfer processes similar or analogous to that catalyzed by ThrH in the cell. A recent survey of all of the identified kinases in the Enzyme Commission data base showed that, of the 184 kinase activities listed, only 112 (less than two-thirds) have been characterized at the molecular level (13). Thus, there are still many missing genes coding for the known phosphorylation processes in the cell that are yet to be identified. As might be suggested by the results from present work, possibilities may arise that some of these assumed kinase-catalyzed phosphorylation reactions could actually be carried out by enzymes such as phosphotransferases using different phosphoryl group donors other than ATP.

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