Crystal Structures of Threonine Synthase from Thermus thermophilus HB8

CONFORMATIONAL CHANGE, SUBSTRATE RECOGNITION, AND MECHANISM*

Received for publication, July 24, 2003, and in revised form, August 29, 2003
Published, JBC Papers in Press, September 2, 2003, DOI 10.1074/jbc.M308065200

Rie Omi‡§, Masaru Goto‡§, Ikuko Miyahara‡§, Hiroyuki Mizuguchi, Hideyuki Hayashi, Hiroyuki Kagamiyama, and Ken Hirotsu‡§**

From the ‡Department of Chemistry, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan, the §Harima Institute/SPring-8, The Institute of Physical and Chemical Research (RIKEN), Sayo-gun, Hyogo, 679-5148, Japan, the ¶Department of Biochemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, and the ¶Department of Biochemistry, Osaka Medical College, Takatsuki, Osaka 569-8686

Threonine synthase, which is a PLP-dependent enzyme, catalyzes the β,γreplacement reaction of L-homoserine phosphate to yield threonine and inorganic phosphate. The three-dimensional structures of the enzyme from Thermus thermophilus HB8 in its unliganded form and complexed with the substrate analogue 2-amino-5-phosphonopentanoic acid have been determined at 2.15 and 2.0 Å resolution, respectively. The complexed form, assigned as an enamine, uncovered the interactions of the cofactor-analogue conjugate with the active site residues. The binding of the substrate analogue induces a large conformational change at the domain level. The small domain rotates by about 25° and approaches the large domain to close the active site. The complicated catalytic process of the enzyme has been elucidated based on the complex structure to reveal the stereocchemistry of the reaction and to present the released inorganic phosphate as a possible catalyst to carry a proton to the Cγ atom of the substrate.

Threonine synthase (ThrS),1 which is a member of the fold-type II of pyridoxal 5′-phosphate (PLP)-dependent enzymes (1–3), catalyzes the last step of the threonine biosynthetic pathway where L-homoserine phosphate (HSerP) is converted into threonine and inorganic phosphate (Scheme I). The reaction catalyzed by ThrS is one of the most sophisticated among the PLP-dependent enzymes, tryptophan synthase β-subunit (9), threonine deaminase (10), and O-acetylserine sulfhydrolase (OASS) (11). The active site structure including the cofactor PLP remains unsettled because of the lack of PLP, although the PLP binding site is assessed.

Recently, the structure of the unliganded yeast ThrS (yThrS) in the PLP form has been solved at 2.25 Å resolution (8). The enzyme is a homo dimer, and the folding of the subunit is similar to those of the fold-type II PLP-dependent enzymes, tryptophan synthase β-subunit (9), threonine deaminase (10), and O-acetylserine sulfhydrolase (OASS) (11). The active site structure including the cofactor PLP remains unsettled because of the lack of PLP, although the PLP binding site is assessed.

The closed form of the enzyme made it possible to investigate the catalytic mechanism so far proposed (4–6) from a stereochemical point of view. We now report the x-ray crystallographic study of the following two forms of tThrS: the unliganded tThrS, which has been overexpressed in Escherichia coli, has 351 residues per subunit, with a subunit molecular weight of 37,000. Sequence alignment showed that tThrS belongs to the class I subfamily, and the sequence identities of tThrS with respect to Arabidopsis thaliana ThrS (aThrS, class I subfamily) and yeast ThrS (yThrS, class II subfamily) are 32 and 14.2%, respectively. We have determined the structures of the unliganded tThrS in the PLP form and tThrS in the complex with the substrate analogue, 2-amino-5-phosphonopentanoic acid (AP5) where the ester oxygen of the terminal phosphate in HSerP is replaced by a methylene group. The enzyme exists as a dimer, and the subunit structure was shown to be similar to those of aThrS, yThrS, and other fold-type II enzymes. The unliganded structure of tThrS was compared with that of the complex to reveal the conformational change from the open to the closed form approximated as a rigid body rotation of 25° at the domain level upon the substrate binding. The active site closure plays important roles in substrate recognition and the catalytic process. The closed form of the enzyme made it possible to investigate the catalytic mechanism so far proposed (4–6) from a stereochemical point of view. We now report the x-ray crystallographic study of the following two forms of tThrS: the unligand-
ded form at 2.15 Å resolution and the complexed form with AP5 at 2.0 Å resolution.

EXPERIMENTAL PROCEDURES

Expression and Purification—Using T. thermophilus HB8 genomic DNA and a set of primers (5′-CATATGCGGCGCCCGCCCTAATA-3′ and 5′-CATGAGGCGAGGGATCCCGCCGAGTAGT-3′), the *tThrC* gene was amplified by PCR and cloned into the TA-vector pGEM (IN-VITROGEN). After confirmation of the nucleotide sequence, *tThrC* gene was ligated into the expression vector pET28b (+) at the *NdeI* BamHI sites. The resulting expression plasmid was used to transform *E. coli* BL21(DE3)pLysS. The cells were grown in LB containing 50 µg/ml ampicillin at 37 °C. When the OD₆₀₀ had reached ~0.6 (~5 h after the beginning of a large scale culture), 1 mM isopropyl β-D-thiogalactopyranoside was added, and culture was continued under the same conditions for 20 h.

The enzyme was purified by a two-step procedure of column chromatography. The cell lysate was centrifuged (15,000 rpm) for 50 min at 277 K and applied to a DEAE-Toyopearl column (TOSOH) equilibrated with 20 mM KH₂PO₄ buffer, pH 7.0. The flow-through fractions containing tThrS were pooled and centrifuged. Ammonium sulfate was added to the resultant supernatant to make a 20% saturated ammonium sulfate solution, which was applied to a Phenyl-Toyopearl column (TOSOH) equilibrated with 10 mM KH₂PO₄ buffer, pH 7.0, containing 5 mM mercaptoethanol. tThrS was eluted with a linear gradient of ammonium sulfate from 20 to 0% in the same buffer. The peak fractions were dialyzed against 10 mM Tris-HCl, concentrated to 7 mg ml⁻¹ and stored at 277 K.

**Enzyme Assay**—Enzymatic activity was measured by analyzing the phosphate released by the method of Bradford (16). The enzyme has a specific activity of 0.25 ± 0.06 µmol min⁻¹ mg⁻¹ at 290 K and 4.25 ± 0.09 µmol min⁻¹ mg⁻¹ at 343 K.

**Crystallization and Data Collection**—The unliganded tThrS was crystallized into trigonal or orthorhombic forms by the hanging drop vapor diffusion method at 293 K using sparse matrix screens (17). Trigonal crystals were obtained by equilibrating a mixture containing 2 µl of protein solution and 2 µl of reservoir solution (0.1 M Mes, pH 6.5 buffer containing 1.45 M ammonium sulfate) against 400 µl of reservoir solution. Yellow octahedral crystals appeared within 3 days of incubation and grew to maximum dimensions of 0.3 × 0.2 × 0.2 mm. The space group is *P₃₂₁* having cell dimensions of *a* = *b* = 113.2 Å and *c* = 150.3 Å with two subunits in the asymmetric unit. The 10% polyethylene glycol 8000 as a reservoir solution gave orthorhombic crystals. Yellow plate-like crystals had grown to dimensions of about 0.2 × 0.2 × 0.02 mm within 5 days. The space group is *P₂₂₂₁* having cell dimensions of *a* = 69.1 Å, *b* = 83.3 Å, and *c* = 123.5 Å with two subunits in the asymmetric unit. The orthorhombic crystals were obtained by equilibrating a hanging drop composed of 2 µl of protein solution, 2 µl of reservoir solution (0.1 M Tris-HCl, pH 8.5, 10% PEG 8000, and 0.2 M Ca(OAc)₂), and 1 µl of additive solution (reservoir solution containing 25 mM AP5). Within 1 week of incubation, yellow crystals had grown to dimensions of about 0.4 × 0.4 × 0.2 mm. The space group is *P₂₂₂₁* having cell dimensions of *a* = 116.6 Å, *b* = 119.5 Å, and *c* = 123.4 Å with four subunits in the asymmetric unit.

The data sets for the trigonal, orthorhombic, and tThrS-AP5 complex crystals were collected at 100 K on the BL40B2, BL44B2, and BL40B2 stations at Spring-8 (Hyogo, Japan), processed, and scaled by HKL2000 (18), MOSFLM (19), and HKL2000, respectively. The data sets for the trigonal crystals soaked in solutions containing heavy atom reagents were collected at 293 K with a Rigaku R-Axis IV++ and processed using CrystalClear (Molecular Structure Corporation, a Rigaku company) (Table I).

**Structure Determination and Refinement**—The structure of the unliganded tThrS was solved by the multiple isomorphous replacement method, using two isomorphous data sets for the crystal in the trigonal form. The scaling of all the data and the map calculations were performed with the CCP4 program suite (20). The difference Patterson map calculations for the ethylmercurithiosalicylic acid and KAu(CN)₄ data sets allowed the interpretation of two mercury sites and two gold sites, respectively. Refinement of heavy atom parameters was performed by the program SOLVE (21). The resulting MIR map has a mean figure-of-merit of 0.40 at a resolution of 20–3.1 Å. The map was significantly improved by the process of solvent flattening with the program RESOLVE (21) and the mean figure-of-merit reached 0.67 with the same resolution range. The map was of good quality, and the model of tThrS was gradually built into a 3.1 Å resolution map through several cycles of model building using the program O (22). The resultant model was composed of 556 residues out of 702 as a dimer with an *R*<sub>free</sub> of 40.1% and an *R*<sub>free</sub> of 44.3%. Using this model as the search model for the molecular replacement calculation, the initial structure of the unliganded TThrS in the orthorhombic form was determined with AMoRe (23). The structure of TThrS in the orthorhombic form was refined by simulated annealing and energy minimization with the program CNS (24), using the x-ray data from 50.0–2.8 Å resolution to give an *R*<sub>free</sub> of 28.2% and an *R*<sub>free</sub> of 34.5%. The resolution was progressively increased to 2.15 Å, and after several rounds of refinement and manual rebuilding, *R*<sub>free</sub> and *R*<sub>free</sub> were reduced to 24.0 and 26.5%, respectively. A difference map displayed one large peak per subunit, which was assigned to the cofactor, PLP. Water molecules were picked up from the 2F<sub>o</sub>–F<sub>c</sub> map on the basis of the peak heights and distance criteria, except for those whose thermal factors after refinement were above 50 Å<sup>2</sup> (corresponding to the maximum thermal factor of the main chain atoms). Further model building and refinement cycles resulted in an *R*<sub>free</sub> of 21.0% and an *R*<sub>free</sub> of 25.1%, calculated for 38,585 reflections (|F<sub>o</sub>| > 2σ|F<sub>c</sub>|) observed in a 10.0–2.15 Å resolution range (Table I).

The structure of TThrS in the complex with AP5 was determined with AMoRe (23), using the coordinates of the unliganded tThrS in the orthorhombic form except for those of PLP. The refinement procedure similar to that for the unliganded tThrS was applied to tThrS-AP5. When the *R*<sub>free</sub> value became less than 30%, the difference Fourier map clearly exhibited a residual electron density corresponding to the AP5 cofactor conjugate system. The residual electron density of AP5 is connected to the N4 atom of the cofactor, indicating that AP5 is covalently bonded to the cofactor. The AP5 cofactor bond structure was assigned to an enamine as a major component, which showed a good fit to the residual electron density (Fig. 1, see "Active Site of tThrS Complex with AP5 in the Closed Form") (25). Refinement cycles gave *R*<sub>free</sub> and *R*<sub>free</sub> values of 19.6 and 23.0%, respectively, calculated for 110,109 reflections (|F<sub>o</sub>| > 2σ|F<sub>c</sub>|) observed in a 10.0–2.0 Å resolution range (Table I).

**Spectroscopic Measurements**—The absorption spectra of the unligan-
 RESULTS AND DISCUSSION

Quality of the Structure—The final model of the unliganded tThrS as a dimeric enzyme comprises 2 × 350 residues (one C-terminal residue of each subunit is not visible), two PLPs, and 283 water molecules with an Rfactor of 21.0% at 2.15-Å resolution (Table I). The six residues of one subunit and the five residues of the other subunit, the side chains of which were disordered and not located on the electron density map, were replaced by alanine residues. The main chain atoms of the two subunits in the asymmetric unit were superposed by least-squares fitting with an r.m.s deviation of 0.50 Å and a maximum deviation of 5.96 Å (Met1) (20), indicating that the two independent subunits have the same structure. The average thermal factors of the main-chain atoms (N, Ca, C, and O) in the two subunits are 21.5 and 19.5 Å². The model was of good quality with all the residues falling in the most favorable (88.9%) and additionally allowed region (11.1%) of the Ramachandran plot, when the stereochemistry was assessed by PROCHECK (26).

The final model of the tThrS-AP5 complex contains 4 × 351 amino acid residues, four AP5-cofactor bond structures, and 885 water molecules with an Rfactor of 19.6% at 2.0 Å resolution (Table I). The main chain atoms of the four subunits in the asymmetric unit were superposed by least-squares fitting with an r.m.s deviation of 0.27 Å and a maximum deviation of 2.80 Å, indicating that the four independent subunits have the same structure. The average thermal factors of the main chain atoms in the four subunits are 13.8, 14.1, 17.4, and 17.7 Å². All the main chain atoms fall within the most favorable (90.4%) and the additionally allowed region (9.6%). Structure diagrams were drawn using the programs Molscript (27), Bobscript (25), PyMOL (28), and Raster3D (29)

Overall and Subunit Structure—The overall structure of the unliganded tThrS is shown in Fig. 3A. The subunit structure of tThrS is shown with secondary structure assignments by the program DSSP in Fig. 3B (30). The tThrS is folded into a dimeric form, and the Ca atoms of the two subunits are related by a non-crystallographic 2-fold axis. The surface area of the subunit interface was calculated to be 7223 Å², which amounts to about one-fourth of the subunit surface area. The enzyme consists of a large domain, a small domain, and a swap domain. The large domain is formed by two parts of the polypeptide chains from the N terminus to Gly7 and from Val134 to Glu149, the small domain from Ala177 to Ser185, and the swap domain from Leu333 to the C terminus. The large domain is an 8/6 domain with an open twisted β-sheet structure. The six β-strands designated b1, b2, b10, b7, b8, and b9 (all parallel except for the b1-strand) form a twisted β-sheet structure as a central core surrounded by four α-helices (a2, a12, a13, and a14) from one side of the β-sheet and four α-helices (a8, a9, a11, and a10) from the other side of the β-sheet (Fig. 3B). Many of the active site residues, which interact with the cofactor are situated at or near one end of the six-stranded β-sheet (the C terminus of b2, b7, and b10-strands), forming the base of the active site cavity. The N-terminal 29 residues and α-helices (a3 and a7) of the large domain form a cluster, which is located between the small domain and the α/β core of the large domain.

The small domain also assumes an α/β structure with a four-stranded parallel β-sheet (b6, b3, b4, and b5) surrounded by two α-helices (a4 and a5) from the protein side and one α-helix (a6) from the solvent side. The C-terminal swap domain extends its α-helix (a15) toward the small domain of the other subunit to interact with it and is involved in the formation of the tThrS molecule as a dimer. The a15 helix lies on the β-sheet of the small domain of the other subunit from the solvent side and behaves like a part of the α/β structure of the small domain (Fig. 3A).

Open-Closed Conformational Change—One of the striking features of tThrS is the overall enzymatic conformational change from the open to the closed form, depending on the binding of the substrate analogue (AP5). It is well known that aspartate aminotransferase from higher animals and Escherichia coli show the open-closed conformational change (31–33). The substrate binding to aspartate aminotransferase induces a large movement of a small domain as a rigid body to close the active site and shield the substrate from the solvent region. This domain movement plays important roles in the substrate recognition and catalytic actions of the enzyme. The Co atoms of 270 residues out of 351 residues in the unliganded tThrS can be superimposed onto the corresponding ones in tThrS-AP5 within 1.0 Å with an r.m.s deviation of 0.23 Å. The r.m.s deviation for all residues is 2.21 Å with a maximum displacement of 9.2 Å (20). Out of 270 residues used for least-squares fitting by Co atoms, 238, 32, and 0 residues are from the large, small, and swap domains, respectively, indicating that, upon binding of AP5, the small and swap domains change their orientations relative to the large domain and/or change their conformations (Fig. 4, A and B). Fig. 4C displays the deviations of Co atoms, which have one significant and two large peaks in the small domain region and one large peak in the swap domain region.

The corresponding Co positions of the small domains in the unliganded tThrS and the tThrS-AP5 are superimposed within 0.74 Å with a maximum displacement of 4.46 Å (Thr85) except for the α-helix a4. This α-helix faces the large domain and does not change its position upon binding of AP5. Because the secondary structures (α-helices and β-strands) of the small domain except for a4 show an excellent overlap (r.m.s deviation of 0.41 Å) between the unliganded and the complex forms, the overall conformational change in tThrS upon binding of AP5 is caused essentially by the rigid-body movement of the small domain except for a4 (Fig. 4B). The small domain of tThrS-AP5 rotates by 25° around an axis which approximately goes through Val89 (β-strand b3), Ala123 (b4), and Ala130(b5) with

![Absorption spectra of tThrS in the absence and the presence of tThrS-AP5 at pH 7.0 and 298 K with a light path of 1 cm. Concentrations were: tTS, 1 mg/ml; 10 mM tThrS-AP5, 150 mM KCl; 50 mM KPB, pH 7.0.](http://www.jbc.org/)

![Overall and Subunit Structure.](http://www.jbc.org/)
respect to that of the unliganded tThrS to close the active site. The overall rotation of the small domain accompanies local conformation changes in the loop regions connecting the secondary structures, because the Cα atoms of the loop regions of the small domain exhibit an r.m.s. deviation of 0.74 Å, significantly larger than 0.41 Å for the secondary structures. The local conformational change is related to the substrate recognition of the enzyme (see “Active-Site Structure of tThrS-AP5 in the Closed Form”). The swap domain also shows a large r.m.s. deviation of 2.48 Å when the Cα atoms of the large domains are superimposed between the unliganded and the complex tThrS. The swap domain, which interacts with the small domain of the other subunit, especially with the β5-strand of the small domain, behaves like a part of the small domain and moves as a rigid body in consort with the conformational change in the small domain of the other subunit.

**Active Site of tThrS in the Open Form**—The active site is located at the domain interface and is formed by the residues from one subunit of the dimeric molecule. A large crevice (20 Å in length, 6–8 Å in width and 7–11 Å in depth) filled with

**TABLE I**

| Orthorhombic | Trigonal | EMTS | KAu(CN)2 | AP5complex |
|--------------|----------|------|----------|------------|
| Diffraction data | | | | |
| Wavelength (Å) | 1.0 | 1.0 | 1.5418 | 1.5418 | 1.0 |
| Resolution (Å) | 50–2.15 | 20.0–2.25 | 50.0–3.1 | 50.0–3.4 | 20.0–2.0 |
| Total no. of reflections | 262628 | 486914 | 83654 | 69799 | 1141164 |
| Unique no. of reflections | 38595 | 53148 | 42652 | 32541 | 110109 |
| Completeness (%) | 97.9 (97.9) | 99.7 (99.4) | 99.0 (99.0) | 98.7 (98.7) | 95.0 (96.6) |
| Rmerge (%) | 10.5 (30.5) | 8.0 (32.3) | 10.5 (30.6) | 14.9 (31.4) | 9.4 (32.1) |
| MIR | | | | |
| Soaking time, hour | 2 | 2 | | |
| Rdiff (%) | 22.4 | 19.6 | | |
| Phasing power | 0.90 | 1.10 | | |
| No. of site | 22 | | | |
| Occupancy | 0.53 | 0.48 | | |
| Refinement | | | | |
| Resolution limits (Å) | 10.0–2.15 | 10.0–2.25 | 10–2.0 | | |
| Rfactor (%) | 21.0 (22.5) | 20.1 (21.1) | 19.6 (21.4) | | |
| Rfree (%) | 25.1 (23.4) | 21.9 (22.6) | 23.0 (27.5) | | |
| Deviations | | | | |
| Bond length (Å) | 0.003 | 0.006 | 0.006 | | |
| Bond angles (deg) | 1.0 | 1.3 | 1.4 | | |
| Mean B factor | | | | |
| Main chain atoms (Å²) | 20.5 | 24.8 | 15.7 | | |
| Side-chain atoms (Å²) | 21.6 | 25.7 | 16.9 | | |
| Water atoms (Å²) | 25.9 | 28.7 | 23.7 | | |
| Hetero atoms (Å²) | PLP 13.9 | PLP 19.7 | 13.1 | | |

* Ethylmercurithiosalicylic acid, sodium salt.

b The values in the parentheses are for the highest resolution shells.

c Rmerge = \frac{\sum |I_h| - \langle |I_h| \rangle}{\sum |I_h|} \langle |I_h| \rangle /

d Rdiff = \frac{|F_{\text{diff}}| - |F_{\text{PH}}|}{|F_{\text{PH}}| + |F_{\text{PH}}|}

\text{where } |I_h| = \text{observed intensity and } \langle |I_h| \rangle = \text{average intensity for multiple measurements.}

e Phasing power is the ratio of the r.m.s. of the heavy atom scattering amplitude and the lack of closure error.

![Fig. 3. Ribbon structural drawings of the unliganded tThrS](https://www.jbc.org/doi/fig/10.1074/jbc.M003011200)
water molecules is formed between the large and the small domain of one subunit with PLP bound to the bottom of the central region of this crevice. The stereo structure and hydrogen-bonding scheme of the active site are shown in Figs. 5 A and 6 A, respectively. The PLP forms a Schiff base bond with Lys61 and interacts with the large domain residues with its si-face directed toward the protein side.

The residues comprising the binding site for PLP are made up of four parts. The first part forms the bottom of the active site pocket, which consists of the residues, Thr317 (from the loop between b10 and a-helix a14), and Phe60 and Lys61 (from the loop between b2 and a3). The second part is Ala240 and Ile241 (from the long loop between a10 and a11), which resides on the re-face side of PLP. The pyridine ring of PLP is sandwiched by the first and second parts from the si- and re-face sides, respectively. The third part forms a semicircular loop to bind the phosphate group of PLP and consists of Gly187, Asn188, Ala189, Gly190, and Asn191 (from the loop between b7 and a9, and the two N-terminal residues of a9). The fourth part consisting of Ser290, Thr291, Gly292, Asn294, and Thr296 (from the loop between b3 and a4, and the three N-terminal residues of a4) resides on the O3’-side of PLP to form the binding site for the carboxylate of the substrate in the closed form. All the residues directly interacting with PLP in the open form are from the large domain residues except for Asn87 of the fourth part. The fourth part approaches PLP with a large conformational change upon binding of AP5 (see “Active Site of tThrS-AP5 in the Closed Form”). Phe60, Lys61, and Thr317 are conserved in other ThrSs (7). Ala240-Leu241 and the sequences for PLP-phosphate and substrate-carboxylate binding sites are the consensus ones in the class I subfamily of ThrS.

In most of the fold-type II enzymes, the hydroxyl group of threonine or serine is hydrogen-bonded to the N1 atom of PLP. This is in contrast to the fold-type I or IV enzymes where the negatively charged carboxylate of aspartate or glutamate forms a salt bridge with the positively charged protonated N1 atom of PLP (34). In tThrS, the nitrogen atom (N1) of the pyridine ring of PLP is hydrogen-bonded by the hydroxyl group of Thr317. The N1 atom is reasonably assumed not to act as a hydrogen bond donor (N1–H → O) but as a hydrogen bond acceptor (N1–H-O) because Thr317 is further hydrogen-bonded by the hydroxyl group of Ser290, which forms a hydrogen bond with the main chain NH group of Gly291 (Fig. 6 A). The replacement of the salt bridge interaction between the N1–H and −OOC in the fold-type I or IV enzyme by the neutral hydrogen bond in tThrS may reduce the electron-withdrawing effect of the pyridine ring of the cofactor as an electron sink. The same type of neutral hydrogen bond was observed for the fold-type II enzyme, OASS (11).

The main chain of the semicircular loop starting from Gly187

FIG. 4. Open-closed conformational change upon binding of AP5. A, the open form of the subunit is displayed by a ribbon model with a molecular surface. Blue and reddish purple ribbons correspond to the large and small domains, respectively. The PLP-Lys61 Schiff base (yellow) is drawn by the CPK model. The molecular surface shows that a large cavity is formed on the cofactor PLP. B, upon binding of AP5, the small domain (reddish purple) rotates by about 25° toward the large domain (blue) to close the active site. The cofactor-AP5 conjugate (yellow) is drawn by the CPK model. The conjugate is enclosed in the protein inside with AP5 interacting not only with the large domain but also with the small domain residues. C, the graph shows the deviations in the corresponding Ca atoms between the open and closed form when the large domains of both forms are superimposed. The horizontal axis is the residue number. The shaded, open, and full frames correspond to the large, small, and swap domain. There are three peaks observed in the small domain region, exhibiting large movements of the Ca atoms amounting to 9 Å. The large peak at the C-terminal region is caused by the correlated motion of the swap domain with the small domain of the other subunit.

Downloaded from http://www.jbc.org/ by guest on July 18, 2018
and leading to Asn 191 forms a binding site for the phosphate group of PLP. The phosphate group is involved in five hydrogen bonds with the main chain NH groups of the loop and three hydrogen bonds with water molecules (Fig. 6, A and B). The N-terminal end of the α-helix (a9) located after Ala 189 approaches the phosphate group and partially compensates for its negative charge. The torsional angle of C3-C4-C4' in the PLP-Schiff base bond-structure is 26° indicating that the internal aldimine bond (Schiff base, C4'/H11032/N) is essentially coplanar with the PLP ring to form the conjugated system of the PLP-Schiff base, although the C4'/H11032/N bond slightly deviates from the PLP plane toward the protein side. The nitrogen atom of the Schiff base is reasonably protonated to form an intramolecular hydrogen bond with O3'/H11032 of PLP, suggesting the high pKₐ value of the Schiff base. This is consistent with the result in which tThrS showed no detectable pH-dependent spectral change with the 416-nm absorption band (protonated Schiff base) with a new absorption band at 455 nm, which was gradually shifted to 325 nm. The absorption peaks at 325 and 455 nm have been assigned to ketimine and quinonoid intermediates, respectively. In the case of tThrS, the spectra showed a marked decrease in the 414-nm absorption with new absorption bands at 316-nm (major peak), and 451 and 482-nm (minor peaks) within 1 min, and the spectra change was not detected 1 day later (Fig. 2). The major component of the cofactor-AP5 conjugate was considered to be ketimine, enamine, or a mixture of them (Fig. 9). The fitting of the cofactor-AP5 conjugate model to the corresponding residual electron density map displayed that the Cα atom of AP5 and three non-hydrogen atoms bonded to it are coplanar with the sp² hybridization of the Cα atom, and plausibly the Cy atom lies in the plane, while the C4' atom of the cofactor is out of the plane (Fig. 1). The enamine model was thus assigned to the cofactor-AP5 conjugate.

Active Site of tThrS/AP5 in the Closed Form—The 2Fo−Fc electron density map for the cofactor-AP5 conjugate is shown in Fig. 1. AP5 in which the ester oxygen of the terminal phosphate in HSerP is replaced by a methylene group forms a new covalent bond with the cofactor similarly to the substrate, HSerP. After the α-proton elimination of AP5, the catalytic reaction stops before the phosphate of AP5 is eliminated. The reaction of E. coli ThrS with AP5 has been studied by spectroscopic methods (6). At the initial stage of the reaction, the spectra showed the loss of a 416-nm absorption band (protonated Schiff base) with a new absorption band at 455 nm, which was gradually shifted to 325 nm. The absorption peaks at 325 and 455 nm have been assigned to ketimine and quinonoid intermediates, respectively. In the case of tThrS, the spectra showed a marked decrease in the 414-nm absorption with new absorption bands at 316-nm (major peak), and 451 and 482-nm (minor peaks) within 1 min, and the spectra change was not detected 1 day later (Fig. 2). The major component of the cofactor-AP5 conjugate is considered to be ketimine, enamine, or a mixture of them (Fig. 9). The fitting of the cofactor-AP5 conjugate model to the corresponding residual electron density map displayed that the Cα atom of AP5 and three non-hydrogen atoms bonded to it are coplanar with the sp² hybridization of the Cα atom, and plausibly the Cy atom lies in the plane, while the C4' atom of the cofactor is out of the plane (Fig. 1). The enamine model was thus assigned to the cofactor-AP5 conjugate.

The stereo structure and hydrogen-bonding scheme of the active site in tThrS/AP5 are shown in Figs. 5B and 6B, respectively. The binding of AP5 liberates a number of water mole-
The X-ray structure of threonine synthase reveals the molecular basis of catalysis. The enzyme consists of two domains, a large domain containing the active site and a small domain responsible for substrate binding. The active site is located in the cleft between the two domains and is formed by the covalent binding of the cofactor and a substrate analog, AP5. The dihedral angles of Nα-C4'-C4-C3, Cα-Nα-C4'-C4, and Cβ-Cα-Nα-C4' are 38°, 164°, and 46°, respectively. The released amino group of Lys61 forms a hydrogen bond with the phosphate (we use the name phosphate for convenience in place of the phosphonomethyl group) of AP5 and a water molecule W7. The amino group of Lys61 creates distances of 3.3, 3.6, 4.8, and 3.5 Å with the Cα, Cβ, and Cγ of AP5, and the C' of the cofactor, respectively, indicating that Lys61 acts as a general acid-base catalyst on Cα, Cβ, and C'. The pyridine ring of the cofactor rotates by 29° around the N1-C6 bond compared with that in the free enzyme toward the solvent side. This rotation generally observed in PLP enzymes is induced by making room for the side chain of the released Lys61 and forming a covalent bond between the cofactor and AP5, which is fixed at the substrate binding site by extensive interactions with the active site residues. The interactions of the active site residues with the cofactor observed in the open form are maintained in the closed form because most of the residues are from the large domain.

The α-helix a4 and its N-terminal loop (the residues from Ser84 to Thr88) approach the cofactor-AP5 conjugate with a large conformational change in the Ser84-Thr88-Gly86 loop. The N-terminal region, called the asparagine loop in OASS (11), forms the binding site for the carboxylate of AP5. The negative charge of the carboxylate is partially compensated for by the dipole of α-helix a4. The main chain NH groups of Thr88, Asn87, and Thr89, the OH group of Ser84, and the water molecule W5 are hydrogen-bonded to the carboxylate of AP5. The water molecule W5 is involved in extensive interactions with the carboxylate of AP5, O3' of the cofactor, the hydroxyl group of Thr85, and the NH3+ of Lys116, playing an important role in the formation of the active site. The loop (the residues from Val153 to Asn157) between the β-strand b6 and α-helix a7, which links the small and large domains, shifts toward the opposite side of the cofactor making a space to accommodate the phosphate of AP5. Lys61, Thr88, Asn154, Ser155, Arg160, and Asn188 interact with the phosphate of AP5. Arg160 forms a salt bridge with the phosphate by the end on configuration (35) to compensate for the negative charge of the phosphate together with Lys61. The large movement of the small domain residues by the open-closed conformational change greatly contributes to the formation of the recognition site for the carboxylate and phosphate groups of AP5. The side chains of Thr88 and Asn154 approach the ester oxygen of the phosphate (actually the methylene group in AP5), which is released on the γ-elimination reaction in the catalysis. Asn154, Ser155, Val156, and Asn188 located near the phosphate of AP5 form a small hole filled with water molecules leading to the molecular surface. The released phosphate of the substrate might be expelled through this hole, although the hole must become larger. Upon binding of AP5, water molecules W6 and W7 are inserted between the phosphate groups of the cofactor and AP5 and relax the repulsive force between the phosphate groups. Five water molecules (W3-W7) are coordinated to the cofactor-AP5 conjugate and are conserved in the four subunits in the asymmetric unit.

Comparison of tThrS with aThrS, yThrS and OASS—The X-ray structure of aThrS as an apoenzyme has been determined (8). tThrS and aThrS, which are classified into a class I subgroup of ThrS, are folded into homodimers, although aThrS has an additional 100 residues at the N terminus involved in the binding of the allosteric effector, S-adenosyl-methionine (7). The primary sequence of tThrS is highly identical (32%) to that of aThrS except for the N-terminal S-adenosylmethionine binding region, and 79% of the active site residues displayed in Fig. 6B are conserved in aThrS. The program DALI (36) indicated that aThrS has the highest Z-score (strength of structural
Similarity of 36.9 with an r.m.s. deviation of 2.2 Å relative to tThrS in the open form, showing that the overall structure of aThrS is most similar to that of tThrS (Fig. 7A) among the fold-type II enzymes, the structures of which have been so far determined. The primary sequence and overall-fold well conserved between tThrS in the open form and aThrS suggest that the active site of aThrS binds HSerP to close the active site using the open-closed conformational change and the substrate recognition mechanism observed in tThrS. The reaction mechanism described below (see “Mechanism”) may be reasonably applied to that of aThrS.

An x-ray crystallographic study of γThrS in the PLP form has been reported with the structure of the γThrS-HSerP complex model optimized by a molecular dynamics simulation (12). γThrS is folded into a monomer and belongs to the class II subfamily of ThrS different from that of aThrS or tThrS. The primary sequence of γThrS is less than 15.2 and 14.2% identical to those of aThrS and tThrS, respectively. The structure of tThrS was superimposed on that of γThrS with a Z-score of 22.8 and an r.m.s. deviation of 3.2 Å using the program DALI (Fig. 7B) (36). The main chain fold of the large domain in tThrS fits well with the corresponding fold in γThrS, while the small domain of tThrS shows a significant deviation in the main chain fold from the corresponding area of γThrS. The region of γThrS corresponding to the small domain of tThrS shows a thermal fluctuation twice that corresponding to the large domain of tThrS. Especially, the α-helix of γThrS corresponding to the α4 helix of tThrS, which exhibits the maximum movement on a conformational change, has temperature factors near 100.0 Å². The small domain of γThrS might thus be flexible enough to move toward the active site. However, whether the monomeric γThrS belonging to a class II subfamily shows a large domain movement to enclose the substrate could not be answered without the x-ray structure of γThrS in a complex with the substrate or analogues. The active site structure of tThrS in the open form is essentially the same as that of γThrS with 64% of the active-site residues of tThrS shown in Fig. 6B conserved in γThrS. The interactions of the cofactor PLP with the active site residues are mostly conserved between tThrS and γThrS, but several differences are observed. The interactions of the N1 and O3’ atoms of PLP with active site residues play an important role in regulating the electronic state of the delocalized system of the cofactor. The pyridine nitrogen atom of PLP in tThrS interacts with the hydroxyl group of Thr317, which is further hydrogen-bonded by Ser290. Similarly, threonine interacts with the pyridine N1 atom in γThrS suggesting that the N1 atom is not protonated, but the residue performing the same role as that of Ser290 in tThrS is not observed in γThrS. In tThrS in the open form, O3’ of the cofactor is hydrogen-bonded by the side chain of Asn252, while in γThrS, O3’ of the cofactor was suggested to be involved in a weak hydrogen bond with the neutral carboxyl group of aspartate (12).

OASS folded into a homodimer catalyzes the β-replacement of acetate in O-acetyl-l-serine by sulfide to produce 3-mercaptopropanoic acid (11, 37). In OASS, the inhibitor, L-methionine, bound to the active site induces a large conformational change in the overall molecule from the open form to the closed form. The OASS in the open and closed form showed the second highest Z scores of 27.8 and 34.1 and r.m.s. deviations of 2.4 and 2.4 Å relative to tThrS in the open and closed forms, respectively. The region corresponding to the small domain of tThrS rotates by 13° as a rigid body closing the active site as observed in tThrS, although tThrS shows a rotation (25°) of the small domain larger than that of OASS. The overall fold of OASS in the open form is located between those of tThrS in the open and closed form, resulting in the smaller rotation angle of the small domain in OASS compared with that in tThrS. The ligand-induced conformational change (2° rotation of the mobile domain as a rigid body) similar to those of OASS, and tThrS has also been observed in tryptophan synthase which is a fold-type II PLP dependent enzyme (38, 39). The open-closed conformational change might be a common strategy of fold-type II PLP-dependent enzymes in which the large active-site crevice formed between the large and small domains in the open form must be closed to recognize the substrate and promote the reaction.

Mechanism—The x-ray structures of unliganded tThrS and its complex with AP5 obtained in this study are essentially consistent with the catalytic mechanism proposed for ThrS (4–6). However, the active site structure provided new insights regarding the details of the catalytic mechanism. The N1 atom of the cofactor is not protonated and does not have a positive charge as was depicted in the x-ray structure of OASS (11). The N1–H-O neutral hydrogen bond is considered to induce a weak electron-withdrawing effect of the cofactor pyridine ring as an electron sink compared with the N1–H–O salt bridge formed in many other PLP-dependent enzymes (40). On the basis of the N1 involvement in the neutral hydrogen bond as an acceptor, we assume that the reaction proceeds through the carbamion intermediate rather than the quinonoid intermediate which requires the protonated N1 atom (Fig. 9). The side-chain amino group of the catalytic residue Lys57 is too distant from the Cγ atom of AP5 to act as a proton donor to the Cγ atom unless the bound substrate changes its orientation to a great extent. Instead of Lys57, the phosphate released from the substrate might be involved in proton transport to the Cγ atom. The mechanism proposed for tThrS is modified by considering these insights and is presented in Fig. 9.

Aspartate aminotransferase in the closed form has essentially the same overall and the active site structure irrespective of a Michaelis complex, external aldime, or ketimine intermediate (31–33, 41), implying that the whole catalytic process proceeds without changing the active site structure. Branched-chain amino acid aminotransferase from E. coli was also shown to have the same closed structure for the Michaelis complex, external aldime form and ketimine form (42, 43). Therefore, the x-ray structure of the tThrS complex with AP5 provides a base to build up the stereochemistry of the total reaction process. The orientation of the cofactor and HSerP is reliably predicted because common interactions work between the cofactor-substrate conjugate and the active site residues in various states of the complexes, although a minor rearrangement of the small domain toward the large domain cannot be ruled out after γ-elimination of the phosphate group from the substrate.

The substrate, HSerP, approaches the re-face of PLP with its α-amino group directed toward the Schiff base of PLP. Water
molecules are liberated from the active site crevice, and the protein residues interact with the substrate inducing the small domain movement to form a Michaelis complex. The complex model was constructed by replacing the cofactor and Lys61 in the closed form (enamine complex) reported herein by the cofactor-Lys61 internal Schiff base in the open (PLP) form (Figs. 8A and 9A).

The amino group of HSerP must be in the unprotonated form to carry out a nucleophilic attack on C4 of the cofactor. This structure can be generated either by binding of the substrate with a free amino group or by deprotonation of the ammonium group after binding of the zwitterionic form of the substrate. The nucleophilic attack yields an external aldimine (Fig. 9B) to release the neutral side chain of Lys 61. Through this process, the bound HSerP does not change its location, while PLP rotates its pyridine ring about 29° toward the solvent side. The /-proton on the sp3 hybridized C atom of HSerP is directed toward the Lys61. One of the /-hydrogen atoms (green) is in a favorable position to be abstracted by Lys61. C, /, -unsaturated ketimine. The inorganic phosphate released from the substrate neighbors the C atom of the substrate and Lys61.

Lys61 is within the hydrogen bond distance to the phosphate group of the substrate. The protonated amino group of Lys61 transfers its proton to the phosphate to yield a , -unsaturated ketimine and inorganic phosphate (Figs. 8C and 9F). The Ca, Na, Cβ, and Cγ atoms form a conjugated system hindering the rotation of the Cβ=Cγ double bond around the Ca–Cβ bond. This produces one of the bases to control the stereochemistry of the reaction. The next step is the proton abstraction from C4 of the cofactor by Lys61 and the transfer of the abstracted proton to Cγ of the unsaturated ketimine. The cofactor-substrate conjugate in the unsaturated ketimine form is assumed to have an orientation analogous to that in the enamine form minus the terminal phosphate because the interactions between the cofactor-substrate conjugate with the active site residues are not essential to produce the ketimine intermediate. The change from the tetrahedral arrangement around the Ca atom in the external aldimine to the coplanar one in ketimine makes the Cβ atom of the substrate approach the protein side with a distance between Cβ and the neutral amino group of Lys61 of 3.6 Å. The pro-R hydrogen on the Cβ atom directing toward Lys61 is eliminated by its amino group to produce the enamine (Figs. 5B and 9E). Importantly, the Cβ–Cγ bond of the substrate is trans to the Ca–Na bond with respect to the Ca–Cβ bond. The R-configuration on Cβ of the final product (threone) is derived from the trans configuration of the enamine induced by the orientation of the substrate bound to the active site in the Michaelis complex.
maintained. The side chain amino group of Lys$^{61}$ is at distances of 4.8 and 4.7 Å from the substrate Cγ atom in the enamine complex and the β,γ-unsaturated ketimine model. This implies that these distances are too long for Lys$^{61}$ to catalyze the proton transfer to the Cγ atom, although the conformational change, which brings Lys$^{61}$ close to the Cγ atom upon the release of the inorganic phosphate cannot be excluded. We propose the released inorganic phosphate (HPO$_4^{2−}$) as another candidate for the proton carrier to the Cγ atom. The inorganic phosphate is reasonably assumed to be present in the vicinity of the Cγ atom of the substrate and the side chain of Lys$^{61}$. Lys$^{61}$ eliminates a proton on the C4 atom in a concerted way, producing an α,β-unsaturated aldimine (Fig. 9D). HPO$_4^{2−}$ has a pK$_a$ value of 7.2 suitable for serving as a catalyst for shutting protons. Quite recently, the product-assisted catalysis has been first proposed in the reaction catalyzed by human 8-oxoguanine DNA glycosylase/lyase (44).

The stereoselective addition of a water molecule to the Ca-Cβ unsaturated bond in Fig. 9G forms the external aldimine, which is a Schiff base between PLP and threonine (Fig. 9H). In the closed form of the enzyme, the surface side (re-face side of the cofactor) of the reaction site is covered by the side chain of Thr$^{85}$, preventing water molecules from invading the re-face side of the cofactor. If the closed form changes to the open form after the elimination of the terminal phosphate of the substrate and water molecules are allowed to freely enter into the active site from the re-face side of the cofactor, the addition reaction of a water molecule cannot be stereochemically controlled. It is thus assumed that the enzyme maintains the closed form or a pseudo-closed form until the product threonine is expelled from the active site. Lys$^{61}$ abstracts a proton from a water molecule located at the si-face side of the cofactor forming the OH$^−$ anion. The OH$^−$ attacks the Cβ atom from the si-face side and Lys$^{61}$ adds its proton to the Ca atom of the substrate, resulting in the formation of the external aldimine (Fig. 9H). The most likely candidate for the water molecule added to the Ca-Cβ unsaturated bond is W7 in the tThrS-AP5 complex, which bridges the side chain amino group of Lys$^{61}$ and the phosphate of the cofactor. The reason why the OH$^−$ does not attack the Ca atom of the ketimine (Fig. 9D) or the β,γ-unsaturated ketimine (Figs. 8B and 9F) is that the side chain of Lys$^{61}$ located just under the Ca atom inhibits the access of the OH$^−$ to the Ca atom. Similarly, the fact that the Cγ of the β,γ-unsaturated ketimine escapes the attack of H$_2$O is considered to be due to the presence of the phosphate ion that blocks the access of H$_2$O to Cγ, in addition to a lack of nearby catalytic groups. This is consistent with the assumption described above in which the phosphate remains in the vicinity of Cγ after being cleaved from Cγ. Finally, the cofactor recovers the Schiff base with Lys$^{61}$ and releases the product, threonine.

The stereoselective and regioselective control of a series of reactions depicted in Fig. 9 has been clarified on the basis of the x-ray structure of the tThrS-AP5 complex. The precise control of the reaction will be made possible by the release of water molecules from the active site, the shielding of the substrate from the solvent to strengthen the electrostatic interactions, and the exact recognition of the substrate to fix the substrate and the active site residues in a favorable arrangement for the reaction to proceed. Open-closed conformational change plays an essential role in making the environment suitable for the catalytic process. The mechanism described above is proposed based on the x-ray structure of tThrS, and further studies are required to validate the mechanism. Spectroscopic and kinetic studies are now in progress to clarify the detailed mechanism of ThrS.

REFERENCES

1. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) Protein Sci. 4, 1291–1304
2. Schneider, G., Kack, H., and Lindqvist, Y. (2000) Structure 8, R1-R6
Crystal Structures of Threonine Synthase from Thermus thermophilus HB8: CONFORMATIONAL CHANGE, SUBSTRATE RECOGNITION, AND MECHANISM
Rie Omi, Masaru Goto, Ikuko Miyahara, Hiroyuki Mizuguchi, Hideyuki Hayashi, Hiroyuki Kagamiyama and Ken Hirotsu

J. Biol. Chem. 2003, 278:46035-46045.
doi: 10.1074/jbc.M308065200 originally published online September 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308065200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 5 of which can be accessed free at http://www.jbc.org/content/278/46/46035.full.html#ref-list-1