Crystal structure of glutamine amidotransferase from *Pyrococcus horikoshii* OT3

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Abstract: Glutamine amidotransferases (GATases) hydrolyze glutamine and generate ammonia. The glutamine amide nitrogen is utilized for the biosynthesis of a variety of molecules such as amino acids, coenzymes, antibiotics, purine and pyrimidine nucleotides, and glucosamine. Here, we determined the crystal structure of a GATase (PH1346) from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 at 1.89 Å resolution. Its overall structure and active site are the most similar to those of *E. coli* guanosine 5'-monophosphate (GMP) synthase and *Sulfolobus solfataricus* anthranilate synthase, respectively.

Key words: Glutamine amidotransferase; protein structure; X-ray crystallography.

Introduction. Glutamine amidotransferase (GATase) is an enzyme widespread in living organisms. It catalyzes the hydrolysis of glutamine to generate glutamic acid and ammonia. It works cooperatively with various synthases by providing ammonium nitrogen that is utilized to produce amino acids, purine and pyrimidine nucleotides, amino sugars, coenzymes and antibiotics.¹⁾ Class-I GATase contains a characteristic Cys-His-Glu triad. The carbonyl carbon of the glutamine side chain carboxamide group is attacked by the thiolate anion of Cys, and forms a tetrahedral intermediate. Then the protonation of the amino group by His collapses this intermediate, producing a thioester intermediate and ammonia.¹⁾ Three-dimensional structures of Class-I GATase have been reported in several species including an eukaryote,²⁾ ten bacteria³⁾⁻¹²⁾ and an archaeon.¹³⁾ Here, we report the crystal structure of a Class-I GATase from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 (PH1346, a putative guanosine 5'-monophosphate (GMP) synthase, E.C. 6.3.5.2)¹⁴⁾ and its comparison with the crystal structures of anthranilate synthase from *Sulfolobus solfataricus* (34% identical in sequence) and GMP synthase from *E. coli* (37% identical in sequence).

Materials and methods. The GATase from *P.*

horikoshii (PH-GATase) was overexpressed in *E. coli* BL21(DE3) cells as a fusion protein with an N-terminal His tag using the pET-28a(+) vector (Novagen). The harvested cells were sonicated in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole and then centrifuged at 40,000 *g* for 30 min. The supernatant was incubated for 30 min at 353 K and centrifuged at 40,000 *g* for 30 min. The supernatant was batch-incubated with Ni-NTA-agarose (Qiagen) for 30 min and applied to an Econopac column (Bio-Rad, Hercules, CA). The column was washed with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. Then the protein was eluted in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. The protein was further purified by size exclusion chromatography with a HiLoad 26/60 Superdex 75 pg column (Amersham) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl. The fractions containing PH-GATase were dialyzed against 5 mM Tris-HCl (pH 8.0) and concentrated to 28.6 mg/ml in Centriprep-3 concentrators (Amicon). The purity was ascertained by SDS-PAGE and the concentration was determined with the absorbance at 280 nm.

Crystallization was carried out at 278 K by the hanging-drop vapor-diffusion method using Crystal Screens 1 and 2 (Hampton Research). Each hanging-drop was

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Fig. 1. The overall structure of PH-GATase. Color coding runs from blue at the N-terminal region to red at the C-terminal region. Secondary structure assignments are labeled on the ribbon model: α , α -helix; β , β -strand; and η , 3_{10} -helix. The location of the active site triad (Cys79-His166-Glu168) is represented in sticks shown in light blue near $\alpha 5$ -helix.

Fig. 2. Stereo diagram of the GATase active sites. PH-GATase (blue), GuaA (green), and TrpG (pink) are superposed. The active-site triad, Cys79, His166 and Glu168, are conserved in these molecules. The residue number corresponds to PH-GATase.

prepared by mixing 2 μ l protein solution (20 mg/ml in 5 mM Tris-HCl pH 8.0) with 2 μ l reservoir solution and equilibrated against 500 μ l of the reservoir solution. Crystals suitable for X-ray analysis were obtained in a few days with the reservoir solution consisting of 100 mM sodium acetate pH 4.6 and 2.0 M sodium chloride. SeMet-labeled¹⁵⁾ PH-GATase was prepared with the same expression system and crystallized under the same conditions.

The crystals were soaked in the reservoir solution supplemented with 10% (v/v) glycerol as the cryoprotectant for a few seconds before flash-freezing in the cryo nitrogen gas stream. X-ray diffraction data were collected at beamline BL41XU at SPring-8 (Harima, Japan). The native dataset was collected at 0.9740 Å. The crystals diffracted X-rays beyond 1.60 Å resolution. The diffraction data to 1.89 Å were indexed and scaled with the programs HKL2000 and SCALEPACK,¹⁶⁾ respectively. The crystals belonged to space group $P3_121$ or $P3_221$ with unit cell dimensions $a = b = 65.9$ Å and $c = 116.4$ Å. The crystal contained one protein molecule per asymmetric unit according to the Matthews coefficient ($V_m = 3.0$ Å³ Da⁻¹) and solvent content of 58.7%.¹⁷⁾ The datasets for a SeMet-labeled crystal were collected at wavelengths of 0.9894 Å (remote), 0.9792 Å (peak), and 0.9794 Å (edge).

The structure of SeMet-labeled GATase was solved using the single-wavelength anomalous diffraction (SAD) method using the peak dataset. Five positions out of six Se atoms in an asymmetric unit were determined using the program SOLVE.¹⁸⁾ The program RESOLVE¹⁹⁾ was used to improve phases. The density map was obtained only when we used the space group $P3_221$. Automated model building was carried out with ARP/wARP,²⁰⁾ and the remainder was built manually with XtalView.²¹⁾ The structure was refined with Refmac5.²²⁾ The structure of the native enzyme was then determined by the molecular replacement method with the program MOLREP²³⁾ using the refined structure of SeMet-labeled PH-GATase as an initial model. Then, the structure was refined with the following programs: CNS²⁴⁾ (simulated annealing and B -factor refinements), XtalView (manual fitting), and Refmac5 (TLS²⁵⁾ and restrained refinements). The refined structure was validated with PROCHECK²⁶⁾ and visualized with PyMOL.²⁷⁾ The atomic coordinates of PH-GATase have been deposited into the Protein Data Bank with the accession number of 2D7J.

Results and discussion. The crystal structure of PH-GATase was solved at 1.89 Å resolution and refined

to an R_{factor} of 18.6% and an R_{free} of 21.0%. The structure turned out to be a monomer. The final electron density allowed modeling of 187 residues as well as 53 water molecules. N-terminal His-tag, Met1, and the side chains of Asp112 and C-terminal Leu189 were missing. Asp112 and Leu189 were modeled as Ala. The backbone geometry of the native structure was ascertained by Ramachandran plot, where 89.9% and 8.2% of the residues were in the most favored and additional allowed regions, respectively. Cys79 and Asp112 (modeled as Ala) were in the disallowed regions. The active-site Cys79 had an unusual backbone torsion angles ($\varphi = 55.9^\circ$, $\psi = 102.5^\circ$), but that is a characteristic feature of this class of enzymes.¹²⁾

The overall structure of PH-GATase is shown in Fig. 1. PH-GATase has an α/β structure consisting of 11 β -strands, five α -helices and one 3_{10} -helix. The core of the domain is a seven-stranded β -sheet ($\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 9$, $\beta 10$, $\beta 11$) surrounded by α -helices on both sides.

Two closest structural homologues to PH-GATase were found in the DALI database.²⁸⁾ One was the glutaminase subdomain (GuaA) of GMP synthase from *E. coli* (PDB code 1GPM)³⁾ and the other was the glutaminase subunit (TrpG) of anthranilate synthase from *Sulfolobus solfataricus* (PDB code 1QDL).¹³⁾ The sequence identities and root-mean-square deviations (RMSDs) were 37% and 1.8 Å (for 182 C α atoms) between PH-GATase and GuaA, and 34% and 1.6 Å (for 179 C α atoms) between PH-GATase and TrpG.

The superposition of the active sites of these molecules are shown in Fig. 2. The residues of the active-site triad consisting of Cys79, His166 and Glu168 are well conserved among the three enzymes. But there are two remarkable differences in the side-chain conformation of the active-site residues. The side-chain conformation of Glu168 is different between PH-GATase/GuaA and TrpG, but this difference does not affect the hydrogen bond between Glu168 and His 166. On the other hand, the Cys C β -S γ bond in Cys79 is rotated ca. 100° between PH-GATase/TrpG and GuaA, which suggests that the substrate recognition of PH-GATase would be closer to that of TrpG than that of GuaA. We are now trying to crystallize PH-GATase in complex with substrate (glutamine) or inhibitor to analyze the structural basis of substrate recognition and reaction mechanism of PH-GATase.

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References

- 1) Zalkin, H. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol.* **66**, 203-309.
- 2) Chaudhuri, B. N., Lange, S. C., Myers, R. S., Chittur, S. V., Davisson, V. J., and Smith, J. L. (2001) *Structure* **9**, 987-997.
- 3) Tesmer, J. J. G., Klem, T. J., Deras, M. L., Davisson, V. J., and Smith, J. L. (1996) *Nat. Struct. Biol.* **3**, 74-86.
- 4) Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M., and Rayment, I. (1997) *Biochemistry* **36**, 6305-6316.
- 5) Endrizzi, J. A., Kim, H., Anderson, P. M., and Baldwin E. P. (2004) *Biochemistry* **43**, 6447-6463.
- 6) Morollo, A. A., and Eck, M. J. (2001) *Nat. Struct. Biol.* **8**, 243-247.
- 7) Spraggon, G., Kim, C., Nguyen-Huu, X., Yee, M. C., Yanofsky, C., and Mills, S. E. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 6021-6026.
- 8) Omi, R., Mizuguchi, H., Goto, M., Miyahara, I., Hayashi, H., Kagamiyama, H., and Hirotsu, K. (2002) *J. Biochem.* **132**, 759-765.
- 9) Douangamath, A., Walker, M., Beismann-Driemeyer, S., Vega-Ferenandez, M. C., Sterner, R., and Wilmanns, M. (2002) *Structure* **10**, 185-193.
- 10) Chen, S., Tomchick, D. R., Wolle, D., Hu, P., Smith, J. L., Switzer, R. L., and Zalkin, H. (1997) *Biochemistry* **36**, 10718-10726.
- 11) Muchmore, C. R., Krahn, J. M., Kim, J. H., Zalkin, H., and Smith, J. L. (1998) *Protein Sci.* **7**, 39-51.
- 12) Korolev, S., Skarina, T., Evdokimova, E., Beasley, S., Edwards, A., Joachimiak, A., and Savchenko, A. (2002) *Proteins* **49**, 420-422.
- 13) Knochel, T., Ivens, A., Hester, G., Gonzalez, A., Bauerle, R., Wilmanns, M., Kirschner, K., and Jansonius, J. N. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9479-9484.
- 14) Kawarabayashi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S.-I., Kosugi, H., Hosoyama, A. *et al.* (1998) *DNA Res.* **5**, 55-76.
- 15) Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., and Clardy, J. (1993) *J. Mol. Biol.* **229**, 105-124.
- 16) Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307-326.
- 17) Matthews, B. W. (1968) *J. Mol. Biol.* **33**, 491-497.
- 18) Terwilliger, T. C., and Berendzen, J. (1999) *Acta Cryst.* **D55**, 849-861.
- 19) Terwilliger, T. C. (2000) *Acta Cryst.* **D56**, 965-972.
- 20) Perrakis, A., Morris, R., and Lamzin, V. S. (1999) *Nat. Struct. Biol.* **6**, 458-463.
- 21) McRee, D. E. (1999) *J. Struct. Biol.* **125**, 156-165.
- 22) Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Cryst.* **D53**, 240-255.
- 23) Vagin, A. A., and Teplyakov, A. (1997) *J. Appl. Cryst.* **30**, 1022-1025.
- 24) Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S. *et al.* (1998) *Acta Cryst.* **D54**, 905-921.
- 25) Painter, J., and Merritt, E. (2005) *Acta Cryst.* **D61**, 465-471.
- 26) Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Cryst.* **26**, 283-291.
- 27) DeLano, W. L. (2002) <http://www.pymol.org/>.
- 28) Holm, L., and Sander, C. (1993) *J. Mol. Biol.* **233**, 123-138.

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