Crystal structure of the short-chain flavin reductase HpaC from *Sulfolobus tokodaii* strain 7

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(Communicated by Masanori OTSUKA, M. J. A.)

Abstract: Flavin reductases (FRs) catalyze the reduction of free flavins using NADH or NADPH. In recent years, a new family of short-chain FRs have been identified in a variety of bacteria and archaea. Here we have determined the crystal structure of an archaeal short-chain FR, HpaC from an aerobic thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7. The HpaC molecule exists as a homodimer with one FMN for each monomer. On the other hand, PheA2, the most structurally similar protein to HpaC, contains FAD rather than FMN. Structural comparison of these proteins has revealed that the short loop at residues 82-83 and the successive η 1 helix in HpaC are closer to the bound flavin than those in PheA2. As a result, there is not sufficient space to accommodate the AMP moiety of FAD in HpaC, and thus HpaC prefers FMN to FAD.

Key words: Short-chain flavin reductase; HpaC, three-dimensional structure; NADH.

Introduction. Reduced flavins play important roles as metabolic reaction mediators in a variety of biological processes such as bioluminescence,^{1,2)} activation of ribonucleotide reductase by reducing the iron center,³⁾ degradation of aromatic compounds,⁴⁻⁷⁾ synthesis of antibiotics,⁸⁻¹⁰⁾ and fossil fuel degradation.¹¹⁾ Flavin reductases (FRs) catalyze the reduction of free flavins using NADH or NADPH. FR can be classified into various subgroups according to substrate specificity, amino acid sequence and functional properties. In recent years, a new family of short-chain FRs have been reported,^{4-7,12)} which act as the small components of the two component aromatic monooxygenase systems. The small component FRs supply reduced flavins to the large component aromatic monooxygenases. 4-hydroxyphenylacetate (4-HPA) 3-monooxygenase is an example of the two-component enzyme first characterized in *Escherichia coli* W.¹³⁾ HpaC is a NADH: flavin oxidoreductase that supplies FADH₂ to HpaB, while HpaB oxidizes 4-HPA to 3,4-dihydroxyphenylacetate (3,4-DHPA) using FADH₂, which is the first step of the

4-HPA catabolic pathway.^{4,14)} There is no apparent interaction between HpaB and HpaC.¹⁵⁾ In order to understand the molecular mechanisms of flavin reduction by HpaC and coupled monooxygenation by the partner protein HpaB, we are investigating the structure and function of HpaC and HpaB (corresponding to the open reading frames ST0723 and ST0726) two-component system from *Sulfolobus tokodaii* strain 7,¹⁶⁾ an aerobic thermoacidophilic crenarchaeon. Here we report the crystal structure of HpaC (ST0723).

Materials and methods. The gene encoding HpaC (ST0723) was amplified by PCR using the genomic DNA of *Sulfolobus tokodaii* strain 7 as the template. Protein expression was induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside). After 12 hr, the cells were harvested by centrifugation at 4500g and resuspended in Sol A (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 5 mM imidazole). The cells were disrupted by sonication and centrifuged at 40000g for 30 min. The supernatant was heated at 353 K for 30 min and centrifuged at 40000g for 30 min. Then, 0.01% polyethyleneimine was added and stirred for 30 min on ice. After centrifugation at 40000g for 30 min, the supernatant was loaded onto a Ni-NTA agarose column. The protein was

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eluted with Sol B (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 200 mM imidazole). The purified protein solution was dialyzed against 20 mM Tricine, pH 8.5 at room temperature and concentrated to 2 mg/ml. The protein concentration was measured by the Bradford method¹⁷⁾ using bovine serum albumin as the standard. For production of Se-Met labeled protein, the same host cells were grown in minimal M9 medium supplemented with amino acids and selenomethionine. At $OD_{600} = 0.2$, expression was induced with 1mM IPTG and the cells were harvested after 12 hr. Dithiothreitol (DTT; final concentration 5 mM) was added to all the buffers except the buffers for Ni-NTA purification.

Crystallization was performed using sitting-drop vapor diffusion method at 293 K. Crystals of HpaC ($0.07 \times 0.07 \times 0.2$ mm³) were obtained in a day by mixing 1 μ l of the protein solution and 1 μ l of the reservoir solution containing 7.5% (v/v) iso-propanol and 1.4–1.7 M ammonium sulfate. A drop was equilibrated against 400 μ l of the reservoir solution. Se-Met labeled crystals were obtained by the same condition as native crystals.

Se-Met labeled crystals were transferred to the reservoir solution containing 25% glycerol as a cryoprotectant and mounted for flash-cooling at 100 K. Diffraction data were collected at the beamline BL41XU of SPring-8. Native crystals were mounted in a glass capillary and X-ray diffraction data were collected at room temperature with an R-AXIS VII. The diffraction data were indexed and scaled with HKL2000.¹⁸⁾ The native and Se-Met crystals belonged to space group $P3_121$ with unit cell dimensions $a = b = 87.6$ Å, $c = 49.6$ Å and $a = b = 86.2$ Å, $c = 49.5$ Å, respectively. They contained one molecule per asymmetric unit, according to the Matthews coefficient ($V_m = 3.1$ Å³ Da⁻¹) and solvent content of 60%.¹⁹⁾

The structure of HpaC was determined using the SAD method using the peak data. The five positions of the Se atoms were determined using the program SOLVE.²⁰⁾ The program RESOLVE²⁰⁾ were used to improve phases. The obtained density map was of good quality. Automated model building was carried out with ARP/wARP²¹⁾ and approximately 70% of the residues were built. The remainder was built manually with XtalView²²⁾ and refined with Refmac 5.²³⁾ The structure of the native enzyme was then determined by the molecular replacement program MOLREP²⁴⁾ using the Se-Met structure as the initial model. Then, simulated annealing and B -factor refinement were performed with CNS,²⁵⁾ and further refinement was performed using XtalView and Refmac 5. Water molecules were added using

ARP/wARP. The final model consists of all the residues except Lys92, Asn139, Ser155 and Leu156, 35 waters and one FMN. Lys92 and Asn139 are refined as Ala and there is no density for the last two residues (Ser155 and Leu156). The B -factor is 44.5 Å² for protein atoms and 32.3 Å² for FMN atoms.

Results and discussion. The crystal structure of HpaC from *Sulfolobus tokodaii* strain 7 was solved at 2.3 Å (Fig. 1). The quality of the native structure is determined by Ramachandran plot, where 88.1% of the residues is in the most favored regions and 11.9% is in the additional allowed regions. HpaC exists as a homodimer, consisting of 2×156 amino-acid residues with a molecular weight of 2×18 K, in the crystal with approximate dimensions of $60 \times 40 \times 40$ Å³. The molecular weight of the enzyme was determined to be about 30 K by gel filtration (data not shown), indicating that it exists as a homodimer also in solution. The dimer interface is stabilized by hydrophobic interactions and several hydrogen bonds. The secondary structure of each monomer is composed of twelve β -strands, three α -helices and two 3_{10} -helices.

The color of HpaC crystals is yellow and the density map clearly shows that each HpaC subunit binds an FMN tightly. FMN is located in a pocket near the dimer interface and surrounded by $\beta 3$, $\beta 5$ and $\beta 11$ strands, $\alpha 2$, $\alpha 3$ and $\eta 1$ helices. The *si* face of the isoalloxazine ring is buried, while the *re* face is completely exposed to the substrate binding site. FMN is stabilized by twelve hydrogen bonds and hydrophobic interactions (Fig. 2). The 2,4-pyrimidinedione moiety of the isoalloxazine ring forms hydrogen bonds to the main chain atoms of Asn33 and the $\beta 5$ strand (Phe48, Asp50) and the side chain atoms of Thr34 and Asn55. The N5 atom has hydrogen bond with the amide nitrogen of Asn33. The dimethylbenzene portion of the isoalloxazine ring forms hydrophobic contacts with the enzyme. The ribityl chain of FMN forms hydrogen bonds to the main chain atoms of Thr31 and Phe79, the N⁶ of Asn55, and the N⁷¹ and N⁷² of Arg87. Thr31 and Phe79 interacting with FMN are highly conserved in the homologous proteins, but many other contacting residues are not conserved.

Structurally similar proteins were searched for in the DALI database.²⁶⁾ HpaC is most similar to PheA2 (1RZ0)²⁷⁾ from *Bacillus thermoglucosidasius* A7 with an RMS difference of 1.8 Å, but these proteins contain different kinds of flavins, FMN *vs.* FAD. Superimposition of the structures of HpaC and PheA2 revealed that the short loop at residues 82–83 and the successive $\eta 1$ helix in HpaC are closer to the bound

Fig. 1. The overall structure of HpaC. Ribbon diagram of HpaC with the two subunits colored red and blue, FMN yellow. The bound FMN is represented as sticks.

Fig. 2. Hydrogen bonds network between FMN and the enzyme. Schematic representation of FMN and the amino acid residues that interact with FMN by potential hydrogen bonds and hydrophobic contacts. Potential hydrogen bonds are represented by *dashed lines* and hydrophobic interactions by *spiked semi-circles*. The figure was generated using LIGPLOT.

flavin than those in PheA2.²⁷⁾ As a result, there is not sufficient space to accommodate the AMP moiety of FAD in HpaC, and thus HpaC prefers FMN to FAD. In conclusion, we have determined the crystal structure of HpaC from *Sulfolobus tokodaii* strain 7, and revealed the structural basis of tight FMN binding.

Acknowledgments. Synchrotron-radiation experiments were done at SPring-8 (Harima, Japan) with the approval of Japan Synchrotron Radiation Research Institute (Proposal No. 2003A0565-NL1-np). This work was supported in part by the National Project on Protein Structural and Functional Analyses of the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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(Received June 9, 2005; accepted June 14, 2005)