Supporting Information

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SI Text

Preparation of TrmFO and SHMT. The gene encoding *T. thermophilus* HB8 TrmFO (residues 1–447) was cloned into the NdeI–BamHI sites of the pET-11 vector (Novagen). The protein was expressed in *E. coli* Rosetta 2(DE3) (Novagen) and purified by heat treatment (70 °C, 20 min), followed by chromatography on DEAE-Sepharose (GE Healthcare), HiTrap Heparin (GE Healthcare), and Resource Q (GE Healthcare) columns. The selenomethionine (SeMet)-labeled protein was expressed in *E. coli* B834(DE3) (Novagen) and purified by using the same protocol as for the native protein. The mutant proteins were prepared by using the QuikChange method (Stratagene), and the sequences were verified by DNA sequencing. The mutant proteins were expressed and purified by using the same protocol as for the wild-type protein, but without the Resource Q step. The gene encoding *T. thermophilus* HB8 SHMT (residues 1–407) was cloned into the NdeI–BamHI sites of the pET-11 vector. The protein was expressed in *E. coli* Rosetta 2(DE3) and purified by heat treatment (70 °C, 20 min), followed by chromatography on CM-Toyopearl 650M (Tosoh) and Hydroxyapatite Bio-Gel HTP Gel (Bio-Rad) columns.

Crystallization. Crystallization was performed at 20 °C, using the hanging drop vapor diffusion method. Crystals of the native free-form were grown by mixing 1 μL of the protein solution [7 mg/mL in 5 mM Tris·HCl (pH 8.0)] and 1 μL of the reservoir solution [13% PEG3350, 0.2 M ammonium citrate tribasic, and 0.1 M Bicine-NaOH (pH 8.0)]. Crystals of the native TrmFO-THF complex were grown by mixing 1 μL of the protein solution [7 mg/mL in 5 mM Tris·HCl (pH 8.0) and 0.3 mM THF] and 1 μL of the reservoir solution [20% PEG8000, 0.2 M $Ca(OAc)_2$, and 0.1 M MES-NaOH (pH 6.5)]. Crystals of the SeMet-labeled TrmFO-GSH complex were grown by mixing 1 μL of the protein solution [7 mg/mL in 5 mM Tris·HCl (pH 8.0) and 10 mM GSH] and 1 μL of the reservoir solution [13% PEG3350, 0.1 M $MgCl_2$, and 0.05 M Tris·HCl (pH 8.5)].

Data Collection and Structure Determination. X-ray diffraction data were collected at 100 K on beamline BL41XU at SPring-8 and beamline NW12A at the Photon Factory Advanced Ring. Crystals of the free form were cryoprotected in the reservoir solution supplemented with 25% MPD. Crystals of the THF complex were cryoprotected in the reservoir solution supplemented with 20% ethylene glycol, 10 mM THF, and 5% dimethyl sulfoxide. Crystals of the GSH complex were cryoprotected in the reservoir solution supplemented with 20% ethylene glycol. Diffraction data were processed by using HKL2000 (HKL Research). Initially, the structure of the GSH complex was determined by the SAD method, using the 2.2-Å resolution data from a SeMet-labeled crystal. All 14 selenium atoms were located, and initial phases were calculated by using SHARP/autoSHARP (1), followed by automated model building using RESOLVE (2). The resultant model was refined by using the 1.05-Å resolution data. The model was further manually built with COOT (3) and refined by using Refmac (4) and PHENIX (5). The structure of the GSH complex was refined to R_{work}/R_{free} of 16.2%/17.8% at 1.05-Å resolution. The crystal belongs to the space group $P2_12_12_1$, with 1 molecule in the asymmetric unit. The final model contains residues 1–180, 186–212, and 220–436, 1 FAD, 1 GSH, 5 ethylene glycols, 641 water molecules, and 1 Mg^{2+} ion. The structure of the free-form was determined by molecular replacement using MOLREP (6) with the structure of the GSH complex as a search model and refined to R_{work}/R_{free} of 14.9%/20.0% at 2.1-Å resolution. The crystal belongs to the space group $C222_1$, with 1 molecule in the asymmetric unit. The final model contains residues 2–207 and 223–438, 1 FAD, 1 2-methyl-2,4-pentanediol, and 313 water molecules. The structure of the THF complex was determined by molecular replacement and refined to R_{work}/R_{free} of 16.8%/18.8% at 1.6-Å resolution. The crystal belongs to the space group $P2_1$, with 1 molecule in the asymmetric unit. The final model contains residues 1–206 and 223–436, 1 FAD, 1 THF (its pteridin moiety), 6 ethylene glycols, 378 water molecules, and 1 Ca^{2+} ion. Data collection and refinement statistics are provided in Table S1. Structural figures were prepared by using PyMol (7).

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8. Chenna R, et al. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31:3497–3500.
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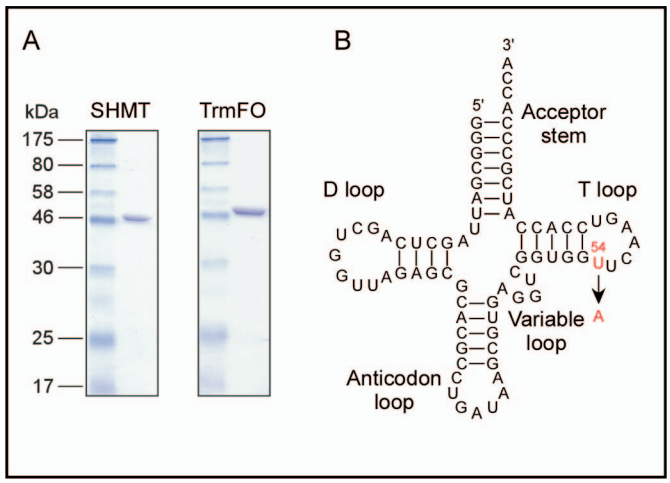

Fig. S1. Proteins and tRNA transcripts used for the in vitro methylation assay. (*A*) 15% SDS/PAGE analysis of purified *T. thermophilus* SHMT (*Left*) and TrmFO (*Right*). The gels were stained with Coomassie brilliant blue. (*B*) Sequence and secondary structure of the *T. thermophilus* $tRNA^{Ile}$ transcript.

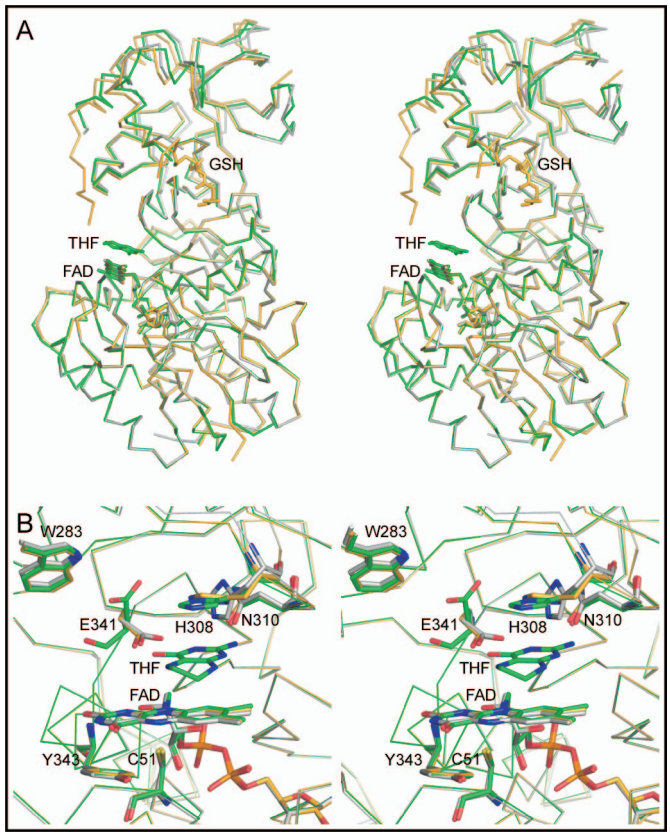

Fig. S2. Stereoview of the superposition of the TrmFO structures in 3 different forms. (*A*) Overall structure. (*B*) The active site. The structures of the free form, THF-bound form, and GSH-bound form are colored gray, green, and orange, respectively.

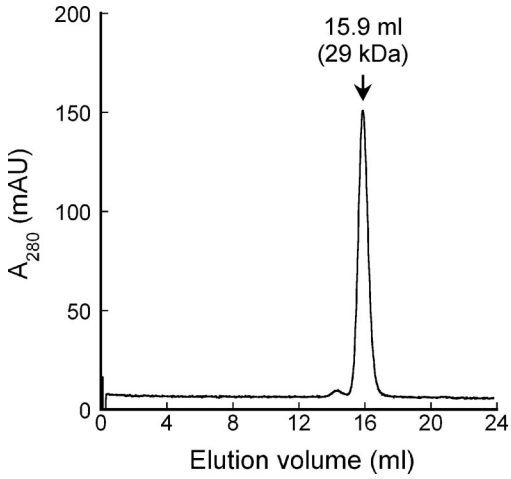

Fig. S3. Gel filtration profile of TrmFO. Gel filtration was performed on a Superdex 200 10/300 GL (GE Healthcare) column with a buffer containing 20 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 20 mM $MgCl_2$. The column was calibrated with Gel Filtration Standard (Bio-Rad).

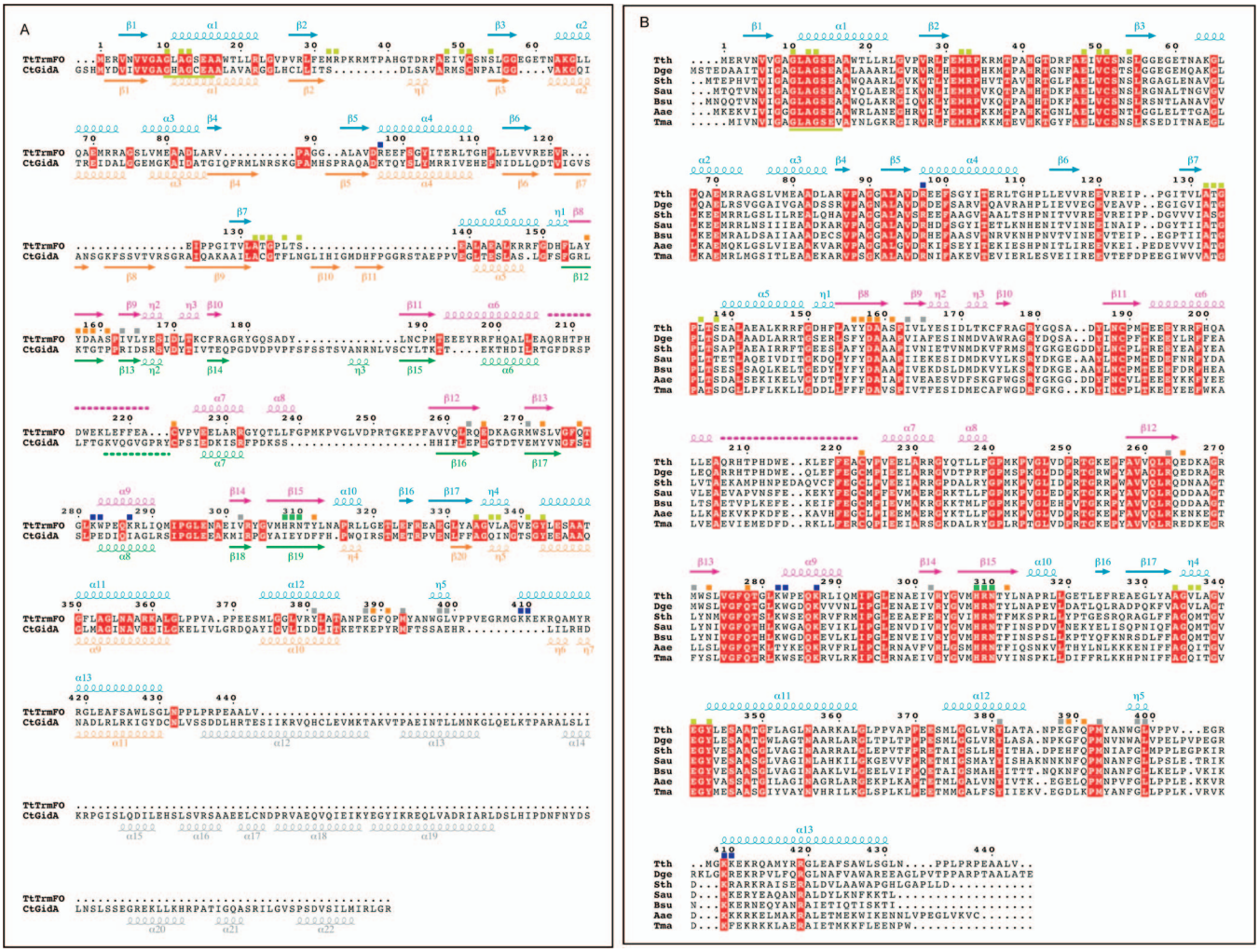

Fig. S4. Sequence alignment. (*A*) Structure-based sequence alignment of *T. thermophilus* TrmFO and *C. tepidum* GidA. (*B*) Sequence alignment of TrmFO orthologs. Conserved residues are highlighted with a red background. The secondary structures of *T. thermophilus* TrmFO and *C. tepidum* GidA are colored as in Fig. 1 and are shown above and below the alignment, respectively. Regions that are disordered in the crystal structures are indicated by dashed lines. The signature motif among the GSH reductase family members is indicated by yellow lines. Residues involved in FAD, THF, and GSH binding are indicated by yellow, green, and orange squares, respectively. Residues possibly involved in tRNA binding are indicated by blue squares. Residues contributing to the formation of the domain interface are indicated by gray squares. Tth, *T. thermophilus* TrmFO; Dge, *Deinococcus geothermalis* DGEO0484; Sth, *Symbiobacterium thermophilum* STH1482; Bsu, *Bacillus subtilis* BSU16130; Aae, *Aquifex aeolicus* AQ691; Tma, *Thermotoga maritima* TM0734. The figure was prepared by using ClustalW (8) and ESPript (9).

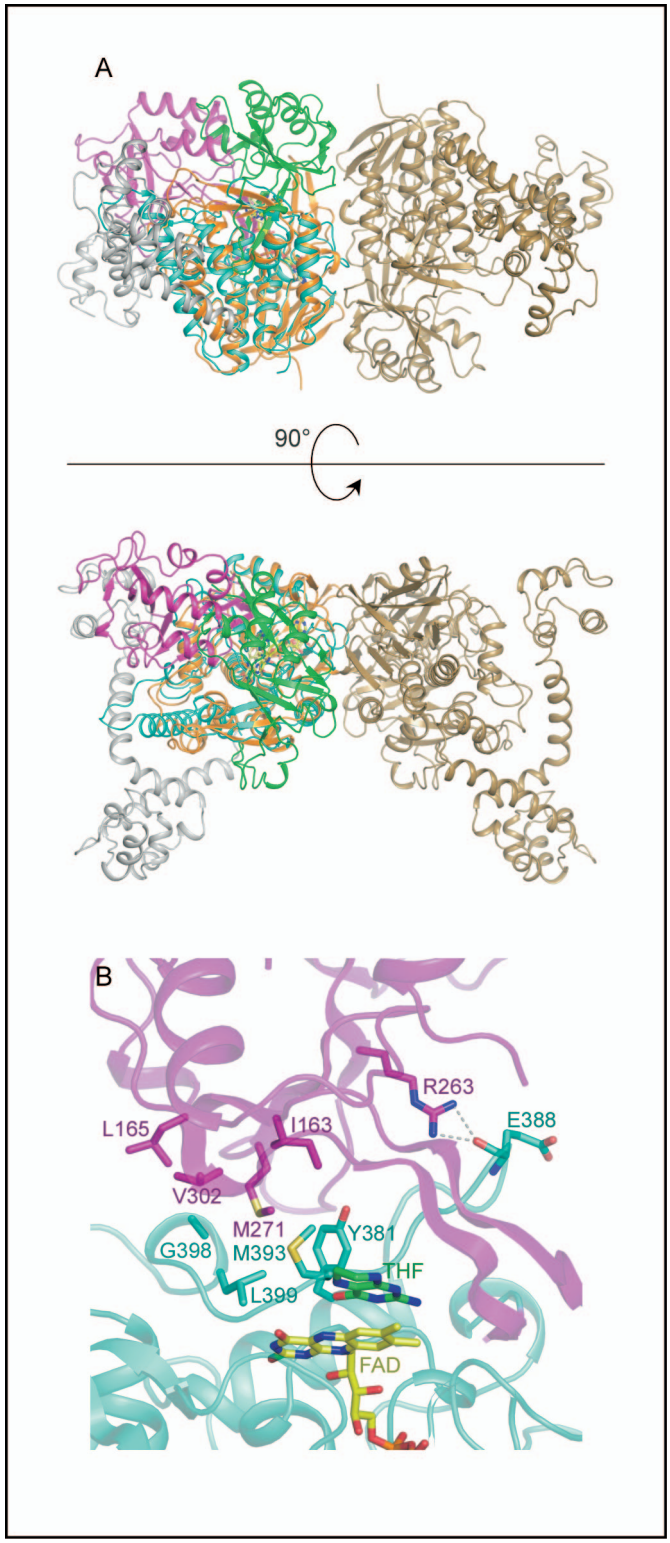

Fig. S5. (*A*) Superposition of the TrmFO monomer and the GidA homodimer, based on their FAD-binding domains. The FAD-binding and insertion domains of TrmFO are shown in cyan and magenta, respectively. The FAD-binding, insertion, and C-terminal domains of *C. tepidum* GidA are colored orange, green, and gray, respectively. The adjacent GidA monomer is colored brown. (*B*) Domain interface of TrmFO. The FAD-binding and insertion domains are shown in cyan and magenta, respectively. The residues contributing the formation of the domain interface are shown in stick representations. Hydrogen bonds are shown as dashed lines.

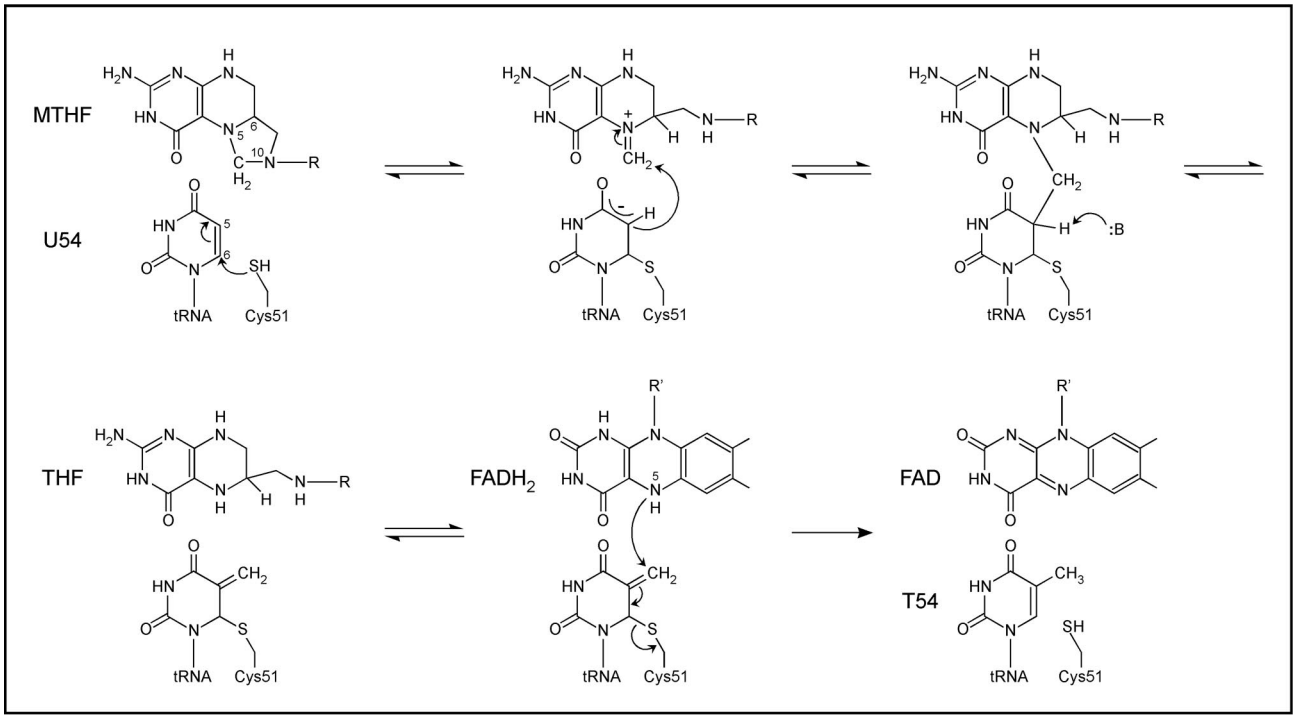

Fig. S6. Proposed catalytic mechanism. R = (*p*-aminobenzoyl)-glutamate. R′ = adenosine-5′-pyrophosphate-ribityl.

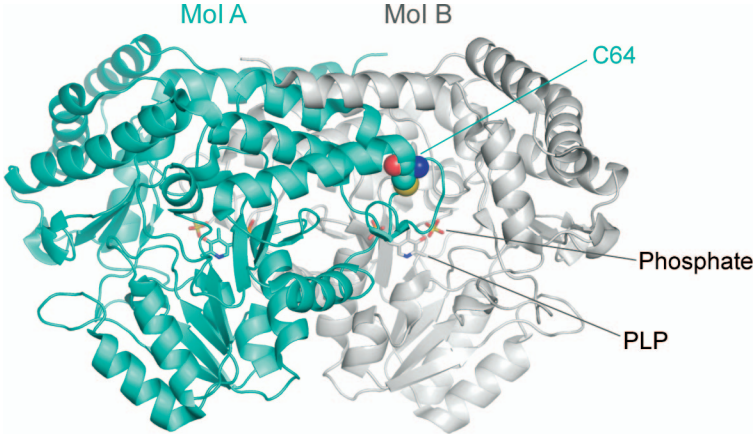

Fig. S7. Crystal structure of *T. thermophilus* SHMT (PDB ID code 2DKJ). MolA and MolB are colored cyan and gray, respectively. Pyridoxal 5′-phosphate (PLP) and phosphate are shown in stick representations. The invariant Cys-64 is shown as a space-filling model.

Table S1. Data collection and refinement statistics

Data set	Native free	Native THF-bound	SeMet GSH-bound	SeMet GSH-bound
Data collection				
Beamline	SPring-8 BL41-XU	SPring-8 BL41-XU	SPring-8 BL41-XU	PF-AR NW12
Wavelength, Å	1.0000	1.0000	0.9000	0.9793
Space group	$C222_1$	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, Å	68.4	46.8	48.3	48.1
b, Å	75.4	97	92.3	92.2
c, Å	156.1	53.8	104.5	104.4
β, °	90	101.3	90	90
Resolution, Å	50.00–2.10 (2.18–2.10)	50.00–1.60 (1.66–1.60)	50.00–1.05 (1.09–1.05)	50.00–2.20 (2.28–2.20)
No. of unique reflections	23,205	60,724	209,948	24,321
Redundancy	5.2 (3.9)	3.1 (2.5)	5.4 (3.2)	12.5 (11.2)
Completeness, %	97.1 (93.2)	97.9 (95.2)	96.8 (84.6)	99.9 (100)
$I/\sigma I$	28.5 (6.3)	24.5 (2.4)	32.8 (2.0)	42.9 (13.6)
R_{sym}, %	5.5 (16.8)	4.7 (27.7)	6.8 (29.5)	7.9 (14.6)
Refinement				
Resolution, Å	42.5–2.1	35.8–1.6	43.8–1.05	
R_{work}, %	14.9	16.8	16.2	
R_{free}, %	20.0	18.8	17.8	
No. of atoms				
Protein	3,281	3,252	3,339	
Ligand/ion	61	90	94	
Water	313	378	641	
Average B factor, Å^2				
Protein	24.6	20.6	16.2	
Ligand/ion	18.0	19.7	16.7	
Water	32.8	33.1	31.6	
rmsd from ideal values				
Bond lengths, Å	0.007	0.004	0.006	
Bond angles, °	1.1	0.9	1.2	
Ramachandran plot, %				
Most favored	93.4	92.7	93.9	
Allowed	6.3	7.0	5.8	
Generously allowed	0.3	0.3	0.3	
PDB ID code	3G5Q	3G5R	3G5S	

Values in parentheses are for the highest-resolution shell.