Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary crystallographic analysis of decameric and monomeric forms of C49S mutant thioredoxin-dependent AhpC from *Helicobacter pylori*

Supangat,[a,b] Kyung Hye Seo,[a,b,c] Ahmad Furqoni,[a,b,c] Young-Chul Kwon,[d] Myung-Je Cho,[d] Kwang-Ho Rhee,[d] Sang Yeol Lee[a,b,c] and Kon Ho Lee[a,b,c]*

[a]Division of Applied Life Science (BK21 Program), Gyeongsang National University, Jinju 660-701, Republic of Korea, [b]Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea, [c]Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea, and [d]Department of Microbiology, School of Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea

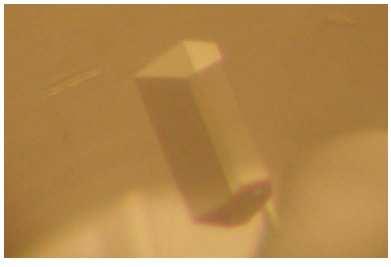
Correspondence e-mail: lkh@gsnu.ac.kr

Received 20 February 2008
Accepted 31 March 2008

Cys49Ser mutant *Helicobacter pylori* alkyl hydroperoxide reductase (C49S *Hp*AhpC) was purified under reducing conditions in monomeric and decameric forms. The monomeric form was crystallized by the hanging-drop vapour-diffusion method. The crystals diffracted to 2.25 Å resolution and belonged to space group *C*2, with unit-cell parameters a = 245.8, b = 140.7, c = 189.5 Å, $\beta = 127^{\circ}$, and contained 20 molecules in the asymmetric unit. A crystal of the decameric form was obtained by the microbatch crystallization method and diffracted to 2.8 Å resolution. It belonged to space group *C*222, with unit-cell parameters $a = 257.5$, $b = 417.5$, $c = 95.6$ Å. The structure of the monomeric form of C49S *Hp*AhpC has been solved by the molecular-replacement method.

1. Introduction

Helicobacter pylori, a curved or spiral Gram-negative microaerophilic bacterium, was first isolated from a human gastric biopsy specimen in 1983. Chronic infection with *H. pylori* is associated with various gastrointestinal diseases including chronic gastritis, gastric and duodenal ulceration and gastric cancer (Hopkins *et al.*, 1996; Sugiyama & Asaka, 2004; Stoicov *et al.*, 2004). Infection by *H. pylori* involves adhesion to and colonization of the mucosal layer of the stomach and activates the immune system and gastric inflammatory responses, including the discharge of reactive oxygen species (ROS) from macrophages and polymorphonuclear leucocytes (Olczak *et al.*, 2002; Suerbaum & Michetti, 2002; Petersen & Krogfelt, 2003). To survive this oxidative stress, *H. pylori* relies on a variety of protective enzymatic systems, including thioredoxin-dependent alkyl hydroperoxide reductase (AhpC), catalase and superoxide dismutase (Olczak *et al.*, 2002; Comtois *et al.*, 2003). *H. pylori* alkyl hydroperoxide reductase (*Hp*AhpC) is a member of the ubiquitous 2-Cys peroxiredoxin family that is involved in peroxide scavenging by reducing organic hydroperoxides and acts as a molecular chaperone that prevents protein misfolding under oxidative stress (Chuang *et al.*, 2006). *Hp*AhpC contains a conserved peroxidatic Cys (C_P) in the



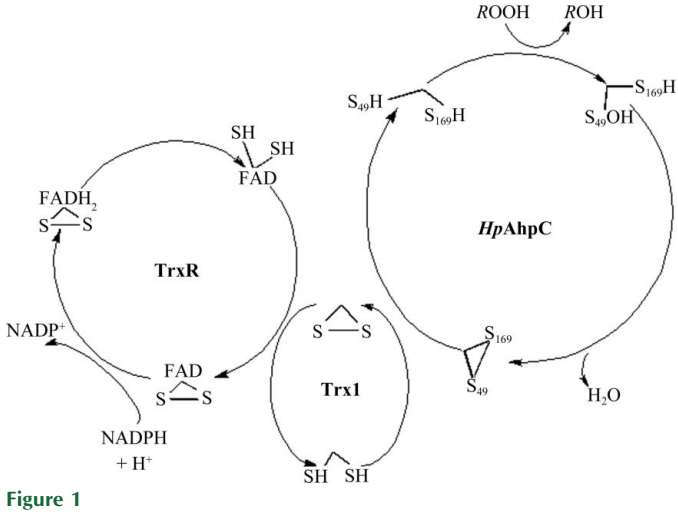


Figure 1
Pathway for the transfer of a reducing equivalent from NADPH to hydroperoxide in *Hp*AhpC.

N-terminal region (Cys49) and a resolving Cys (C_R) in the C-terminal region (Cys169) and is more closely related to the 2-Cys peroxiredoxins of higher organisms than to most other eubacterial AhpC proteins. Reduced *Hp*AhpC directly converts hydroperoxides to alcohols with the concomitant formation of sulfenic acid (C_P-SOH) and C_P and C_R condense, forming a disulfide bond (Fig. 1). The stable intersubunit disulfide bond of the oxidized *Hp*AhpC protein is then regenerated by TrxR and Trx1 (Baker *et al.*, 2001), while AhpC in *Salmonella typhimurium* is reduced by AhpF (Reynolds & Poole, 2001) and that in *Mycobacterium tuberculosis* by AhpD (Koshkin *et al.*, 2004; Parsonage *et al.*, 2005; Wood *et al.*, 2002; Fig. 1).

Here, we report the crystallization and preliminary X-ray diffraction analysis of monomeric and decameric forms of C49S mutant *Hp*AhpC protein. The structural comparison between the wild-type and C49S mutant *Hp*AhpC proteins will help in understanding the molecular mechanisms underlying their peroxidase and chaperone activities in relation to oligomerization processes.

2. Materials and methods

2.1. Expression and purification of C49S mutant *Hp*AhpC protein

The C49S mutant of the *Hp*AhpC gene was cloned into a pET-15b (Novagen, Madison, Wisconsin, USA) expression vector to produce a recombinant protein with a hexahistidine tag and a thrombin cleavage site at the N-terminus (MGSSHHHHHHSSGLVPRGSH). The plasmid was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. 50 ml aliquots of an overnight culture were subcultured into 500 ml fresh LB (Luria–Bertani) medium containing ampicillin (100 mg ml^{-1}) at 310 K with vigorous shaking until the OD_{600} reached 0.6. Protein expression was induced for 7 h with 0.4 m*M* isopropyl β-D-1-thiogalactoside (IPTG) at 310 K and cells were harvested by centrifugation (6000 rev min^{-1}, 6 min, 277 K). The harvested cells were washed with lysis buffer (50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 5 m*M* imidazole and 10 m*M* β-mercaptoethanol) and stored at 203 K until use. The frozen cells were resuspended in lysis buffer and disrupted by sonication. After centrifugation (14 000 rev min^{-1}, 1 h, 277 K), the clear supernatant was filtered (pore diameter 0.45 µm; Satorius, Goettingen, Germany) and applied onto a column of nickel–NTA beads (Qiagen, Hilden, Germany) pre-equilibrated with lysis buffer. The column was washed first with ten column volumes of the binding buffer and then with two column volumes of 50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 30 m*M* imidazole, 10 m*M* β-mercaptoethanol. The recombinant proteins were eluted with 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 300 m*M* imidazole, 10 m*M* β-mercaptoethanol. Fractions containing the proteins were pooled, concentrated and exchanged to 50 m*M* Tris–HCl pH 8.0, 10 m*M* β-mercaptoethanol by ultrafiltration (Centripep YM-10, Millipore Corporation, Bedford, Massachusetts, USA). The protein was further purified by anion-exchange chromatography with a MonoQ column (Amersham Biosciences Ltd, UK). The protein was eluted with a salt gradient and separated at ~150 m*M* NaCl pH 8.0. The fractions containing recombinant protein were concentrated by ultrafiltration (Centricon YM-10, Millipore Corporation, Bedford, Massachusetts, USA). The recombinant protein was finally purified by gel-filtration chromatography with a Superdex 200 column (Amersham Biosciences Limited, UK) in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 10 m*M* β-mercaptoethanol. The protein was eluted at 14 and 18 ml, corresponding to molecular weights of ~260 kDa (decameric form) and ~26 kDa (monomeric form). The fractions containing the decameric and monomeric forms were separately pooled and concentrated to 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 0.5 m*M* tris-(2-carboxyethyl)phosphine–HCl (TCEP–HCl) for crystallization by ultrafiltration (Microcon YM-10, Millipore Corporation, Bedford, Massachusetts, USA) (Fig. 2). Protein purity was examined by SDS–PAGE. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

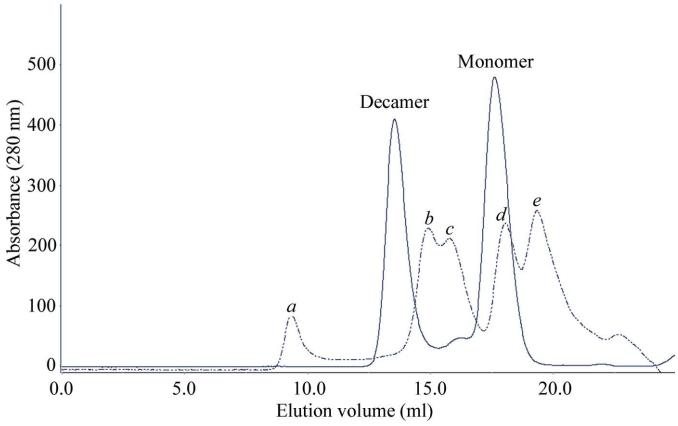


Figure 2
Gel-filtration elution analysis of the C49S mutant in the presence of 10 m*M* β-mercaptoethanol. Elution profiles are shown as a broken line for standard proteins and as an unbroken line for the C49S mutant. *a*, blue dextran (2000 kDa); *b*, catalase (232 kDa); *c*, ovalbumin (43 kDa); *d*, chymotrypsinogen A (25 kDa); *e*, ribonuclease A (13.7 kDa). C49S mutant proteins were eluted at 14 and 18 ml, corresponding to molecular weights of ~260 kDa (decamer) and ~26 kDa (monomer).

2.2. Crystallization and data collection

Crystallization of monomeric and decameric C49S mutant *Hp*AhpC protein was initially carried out with Crystal Screens I and II and Index Screen (Hampton Research, California, USA), Wizard Screens I, II, Cryo I and II (Emerald BioStructures, Bainbridge Island, Washington, USA) and laboratory solutions using a microbatch crystallization method at 291 K. Drops containing equal volumes (1 µl) of protein (10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 0.5 m*M* TCEP–HCl) and screening solution were equilibrated under Al's oil in a 72-well microbatch plate.

Screening solution No. 4 (0.2 *M* calcium oxaloacetate, 0.1 *M* HEPES pH 7.5, 40% PEG 400) from the Cryo II sparse-matrix crystallization screen produced needle-shaped and small rod-shaped crystals of the monomeric form of C49S *Hp*AhpC. Further screenings to find optimal crystallization conditions were performed by hanging-drop vapour-diffusion trials, varying the salt and precipitant concentrations and the volume of the drop. Crystals suitable for diffraction experiments were obtained at 291 K in a drop containing 3 µl 10 mg ml^{-1} protein solution and 2 µl of a mixture of 4 µl reservoir solution (0.16 *M* calcium oxaloacetate, 0.1 *M* HEPES pH 7.5, 30% PEG 400) and 2 µl 20% IPTG as an additive and had dimensions of 0.3 × 0.1 × 0.1 mm (Fig. 3*b*). Crystallization conditions were optimized further by controlling the amount of nucleation and the growth rate. The amount of nucleation was reduced by filtering the protein solution and the reservoir solution through a 0.2 µm NANOSEP MF filter (PALL Life Science, USA) and a 0.2 µm syringe filter (Satorius, Goettingen, Germany), respectively, before crystallization setup. To control the crystal-growth rate, the volume of reservoir solution was reduced from 1000 to 500 µl. The best large single crystals grew to maximum dimensions of 0.6 × 0.3 × 0.2 mm in 6–10 d (Fig. 3*c*). The crystals were flash-frozen in liquid nitrogen after soaking for 15 min in well solution.

Table 1
Data statistics for C49S *Hp*AhpC.

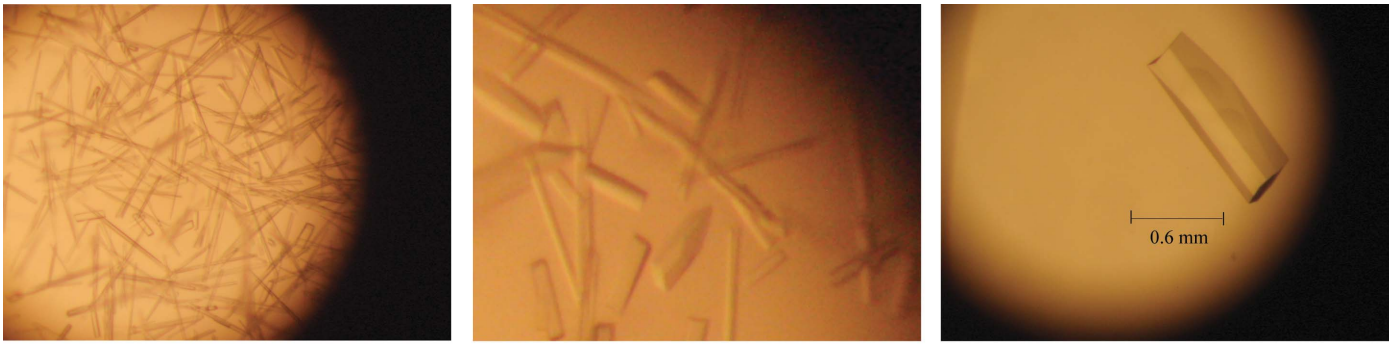
Values in parentheses are for the highest resolution shell.

	Decameric form	Monomeric form
Space group	*C*222	*C*2
Unit-cell parameters (Å, °)	a = 257.5, b = 417.5, c = 95.6	a = 245.8, b = 140.7, c = 189.5, β = 127
No. of chains in ASU	20†	20
V_M (Å^3 Da^{-1})	2.3	2.5
Solvent content (%)	46.4	50.8
Resolution (Å)	50–2.8 (2.9–2.8)	50–2.25 (2.33–2.25)
No. of reflections	124746 (12177)	242042 (23739)
Completeness (%)	98.1 (96.8)	99.3 (97.7)
R_{merge}‡ (%)	26.7 (47.7)	11.3 (15.2)
Redundancy	5.2 (4.2)	5.9 (4.6)
$\langle I/\sigma(I)\rangle$	3.2 (1.6)	13.2 (9.4)

† Number of molecules estimated according to specific volume calculation. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average

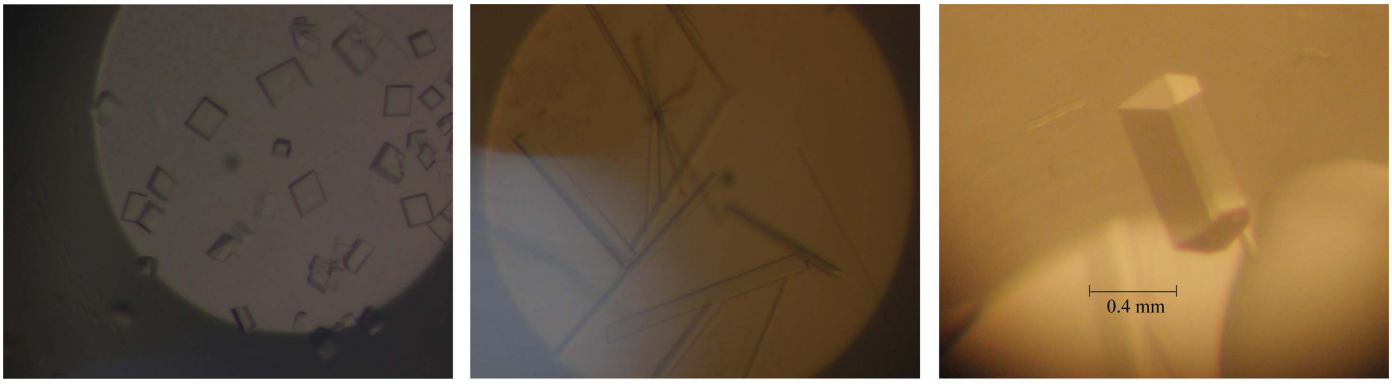
Cube-shaped crystals of the decameric form of C49S *Hp*AhpC were obtained from the initial screening in Index Screen solution No. 91 (0.15 *M* DL-malic acid pH 7.0, 20% PEG 3350; Fig. 4*a*). The condition was optimized by varying the salt and precipitant concentrations and the volume of the drop using a microbatch crystallization method at 291 K. Needle-shaped crystals suitable for diffraction experiments were obtained at 291 K in a drop containing 2 µl 10 mg ml^{-1} protein solution and 2 µl reservoir solution (0.1 *M* DL-malic acid pH 7.0, 20% PEG 3350, 15% glycerol) and had dimensions of 0.7 × 0.1 × 0.05 mm (Fig. 4*b*). However, these crystals diffracted to relatively low resolution. Further optimization was performed by screening for additives and controlling the amount of nucleation. In order to control the amount of nucleation, the protein solution was filtered using a 0.2 µm NANOSEP MF filter (PALL Life Science, USA) prior to crystallization setup. The best crystals of the decameric form of C49S *Hp*AhpC were obtained at 291 K in a drop containing 3 µl 10 mg ml^{-1} protein solution (in 20 m*M* Tris–HCl pH 8.0, 0.5 m*M* TCEP) and 3 µl of a mixture of 4 µl reservoir solution (0.1 *M* DL-malic acid pH 7.0, 20% PEG 3350, 15% glycerol) and 1 µl 0.5 *M* guanidine hydrochloride as an additive. The crystals grew to maximum dimensions of 0.4 × 0.2 × 0.2 mm (Fig. 4*c*). Crystals were soaked for 15 min in cryoprotectant solution (90 m*M* DL-malic acid pH 7.0, 18.75% PEG 3350, 20% glycerol, 0.7 m*M* Tris–HCl, 0.0175 m*M* TCEP) and flash-frozen in liquid nitrogen for data collection.

X-ray diffraction data for the monomeric form of C49S *Hp*AhpC were collected from a single flash-frozen crystal using X-rays of wavelength 1.23955 Å at station 4A of Pohang Accelerator Laboratory, Pohang, Republic of Korea. The crystal-to-detector distance was set to 220 mm and a 1° oscillation and 10 s exposure were used per image. Data set for the decameric form of C49S *Hp*AhpC were collected from a single flash-frozen crystal using synchrotron radiation of wavelength 1.23985 Å at beamline 6C of Pohang Accelerator Laboratory, Pohang, Republic of Korea. The crystal-to-detector distance was 200 mm and a 1° oscillation and 60 s exposure were used per image. All diffraction images of the monomeric and decameric forms were indexed, integrated and scaled using the *HKL*-2000 suite



(*a*) (*b*) (*c*)

Figure 3
Crystals of the reduced monomeric form of C49S *Hp*AhpC. (*a*) Needle-shaped and rod-shaped crystals obtained from initial screening. (*b*) Larger rod-shaped crystals after optimization. (*c*) The best large single crystal obtained after controlling the amount of nucleation and the growth rate.



(*a*) (*b*) (*c*)

Figure 4
Crystals of the reduced decameric form of C49S *Hp*AhpC. (*a*) Cube-shaped crystal obtained from initial screening. (*b*) Larger long needle-shaped crystal obtained after optimization. (*c*) The best large single crystal obtained after controlling the amount of nucleation and additive screening.

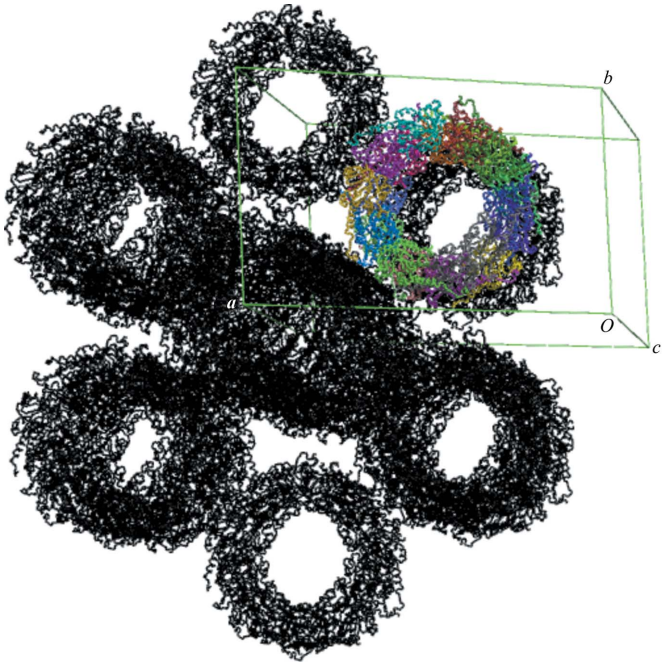


Figure 5
C49S *Hp*AhpC crystal packing. The monomeric form packed in space group *C*2. All molecules are displayed in black, except for the two doughnut-shaped decamers in the asymmetric unit which are shown with different colours for each molecule.

(Otwinowski & Minor, 1997). The phase problem was solved by the molecular-replacement method using the program *AMoRe* (Navaza, 2001) with the atomic coordinates of wild-type *H. pylori* AhpC (PDB code 1zof; Papinutto *et al.*, 2005) as a search model. The data statistics are shown in Table 1.

3. Results

The crystals of the monomeric form of C49S *Hp*AhpC diffracted to 2.25 Å resolution and belonged to the monoclinic space group *C*2, with unit-cell parameters a = 245.8, b = 140.7, c = 189.5 Å, β = 127° (Table 1). Specific volume calculations (Matthews, 1968) based on the unit-cell parameters and the molecular weight suggested that there could be 20 molecules per asymmetric unit, with a V_M value of 2.5 Å^3 Da^{-1} and a solvent content of 50.8%. Molecular replacement using wild-type *H. pylori* AhpC (PDB code 1zof; Papinutto *et al.*, 2005) as a search model found 20 molecules in the asymmetric unit (correlation coefficient of 51.4 after translation search). Two doughnut-shaped decamers are found in the asymmetric unit of the crystal of the monomeric form (Fig. 5). A 2.8 Å resolution data set was collected and processed from the decameric form crystals. The space group was found to be orthorhombic *C*222, with unit-cell parameters a = 257.5, b = 417.5, c = 95.6 Å (Table 1). According to specific volume calculations (Matthews, 1968), it appeared that there could be 20 molecules per asymmetric unit, with a solvent content of 46.4% and a V_M value of 2.3 Å^3 Da^{-1}. However, the crystal turned out to be partially twinned with a twinning fraction of 0.142 according to analysis using the *TRUNCATE* and *SFCHECK* programs from *CCP*4 (Collaborative Computational Project, Number 4, 1994). The R_{merge} for the decameric crystal was rather high and $\langle I/\sigma(I)\rangle$ was lower. This may be the consequence of partial twinning. Structure refinement and analysis of the monomeric and decameric forms of C49S *Hp*AhpC protein are in progress.

We thank the staff at beamlines 4A, 6B and 6C at the Pohang Accelerator Laboratory for help with data collection. This work was supported by the BK21 program (S and KHS), grant R15-2003-002-01001-0 to the EB-NCRC (KHL) and the Korea Research Foundation (2003-041-E00096). We thank members of PMBBRC and EB-NCRC for assistance.

References

Baker, L. M., Raudonikiene, A., Hoffman, P. S. & Poole, L. B. (2001). *J. Bacteriol.* **183**, 1961–1973.
Chuang, M. H., Wu, M. S., Lo, W. L., Lin, J. T., Wong, C. H. & Chiou, S. H. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 2552–2557.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Comtois, S. L., Gidley, M. D. & Kelly, D. J. (2003). *Microbiology*, **149**, 121–129.
Hopkins, R. J., Girardi, L. S. & Turney, E. A. (1996). *Gastroenterology*, **110**, 1244–1252.
Koshkin, A., Knudsen, G. M. & Ortiz De Montellano, P. R. (2004). *Arch. Biochem. Biophys.* **427**, 41–47.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (2001). *Acta Cryst.* D**57**, 1367–1372.
Olczak, A. A., Olson, J. W. & Maier, R. J. (2002). *J. Bacteriol.* **184**, 3186–3193.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Papinutto, E., Windle, H. J., Cendron, L., Battistutta, R., Kelleher, D. & Zanotti, G. (2005). *Biochim. Biophys. Acta*, **1753**, 240–246.
Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus, P. A. & Poole, L. B. (2005). *Biochemistry*, **44**, 10583–10592.
Petersen, A. M. & Krogfelt, K. A. (2003). *FEMS Immunol. Med. Microbiol.* **36**, 117–126.
Reynolds, C. M. & Poole, L. B. (2001). *Biochemistry*, **40**, 3912–3919.
Stoicov, C., Saffari, R., Cai, X., Hasyagar, C. & Houghton, J. (2004). *Gene*, **341**, 1–17.
Suerbaum, S. & Michetti, P. (2002). *N. Engl. J. Med.* **347**, 1175–1186.
Sugiyama, T. & Asaka, M. (2004). *Med. Electron Microsc.* **37**, 149–157.
Wood, Z. A., Poole, L. B., Hantgan, R. R. & Karplus, P. A. (2002). *Biochemistry*, **41**, 5493–5504.