Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray crystallographic analysis of enoyl-acyl carrier protein reductase from *Helicobacter pylori*

Hyung Ho Lee, Jungmin Yun, Jinho Moon, Byung Woo Han, Byung Il Lee, Jae Young Lee and Se Won Suh*

Laboratory of Structural Proteomics, School of Chemistry and Molecular Engineering, Seoul National University, Seoul 151-742, South Korea

Correspondence e-mail: sewonsuh@snu.ac.kr

Received 30 January 2002
Accepted 18 April 2002

Enoyl-acyl carrier protein reductase (ENR) catalyzes the NADH-dependent stereospecific reduction of α,β-unsaturated fatty acids bound to the acyl-carrier protein. ENR from *Helicobacter pylori* has been overexpressed in *Escherichia coli* and has been crystallized in the presence of its cofactor NADH and the inhibitor triclosan (or its analogue diclosan) at 296 K using polyethylene glycol (PEG) 400 as a precipitant. For the triclosan (or diclosan) complex, diffraction data to 2.5 (or 2.3) Å resolution have been collected using synchrotron X-rays. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 73.35$, $b = 94.91$, $c = 75.38$ Å, $\beta = 106.21^\circ$ for the triclosan complex (or $a = 73.25$, $b = 95.07$, $c = 75.02$ Å, $\beta = 106.53^\circ$ for the diclosan complex). The asymmetric unit contains one homotetramer, with a corresponding V_M of 2.10 Å^3 Da^{-1} and a solvent content of 41% by volume.

1. Introduction

Enoyl-acyl carrier protein reductase (ENR; EC 1.3.1.9) is a key enzyme of the type II fatty-acid biosynthetic system, found in plants and most prokaryotes, in which the enzymes that catalyze the individual steps are found on separate polypeptides (McCarthy & Hardie, 1984). It completes each cycle of elongation by catalyzing the stereospecific reduction of the double bond at position 2 of a growing fatty-acid chain linked to the acyl carrier protein in an NADH- or NADPH-dependent reaction (Heath & Rock, 1995 & 1996). The importance of fatty-acid biosynthesis to cell growth and function makes ENR an attractive target for the development of antibacterial or antimalarial agents. Crystal structures of EnvM (the ENR from *E. coli*) complexed with diazoborines and triclosan (Baldock *et al.*, 1996, 1998; Stewart *et al.*, 1999; Levy *et al.*, 1999; Heath *et al.*, 1999), InhA (the ENR from *Mycobacterium tuberculosis*) complexed with isoniazid (Dessen *et al.*, 1995; Rozwarski *et al.*, 1998), *Brassica napus* ENR complexed with triclosan (Rafferty *et al.*, 1995; Roujeinikova *et al.*, 1999) and *Plasmodium falciparum* ENR complexed with triclosan and its analogues (Suguna *et al.*, 2001; Perozzo *et al.*, 2002) have also been reported. Unlike diazoborines and isoniazid, which bind covalently to the NAD^+ cofactor, triclosan interacts with both ENR and NAD^+ in a non-covalent fashion.

H. pylori is a Gram-negative bacterium responsible for gastritis, peptic ulcer and gastric cancer (Covacci *et al.*, 1999). Because of its importance as one of the major human pathogens, complete genome sequences of strains 26695 and J99 have been reported (Tomb *et al.*, 1997; Alm *et al.*, 1999). ENR from *H. pylori* encoded by the *fabI* gene is a homotetramer, with each subunit comprising 275 amino-acid residues (subunit $M_r = 29\,981$). It shows amino-acid sequence identities of 44, 30 and 24%, respectively, with ENRs from *E. coli*, *M. tuberculosis* and *B. napus*. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenylether) is a broad-spectrum antibacterial agent that is widely used in consumer products such as toothpastes, soaps, cosmetics, plastics and textiles (Bhargava & Leonard, 1996). Diclosan, missing a Cl atom at position 2, is a triclosan analogue but is not apparently widely used as an antibacterial agent. Triclosan analogues have been tested as potential candidates for antimalarial drugs (Perozzo *et al.*, 2002). The structures of *H. pylori* ENR in complex with the cofactor and triclosan (or its analogue diclosan) will offer valuable information for the structure-based optimization of triclosan-like non-covalent inhibitors. As the first step toward its structure elucidation, we have overexpressed *H. pylori* ENR in the intact form and crystallized it in the presence of both NADH and the inhibitor triclosan (or its analogue diclosan). Here, we report the crystallization conditions and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The *fabI* gene encoding ENR (HP0195) was amplified from the genomic DNA of *H. pylori* strain 26695 by the polymerase chain reaction.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shells (2.64–2.50 Å for the triclosan complex and 2.34–2.30 Å for the diclosan complex).

Data	Triclosan complex	Diclosan complex
X-ray wavelength (Å)	0.9794	1.0072
Space group	$P2_1$	$P2_1$
a (Å)	73.35	73.25
b (Å)	94.91	95.07
c (Å)	75.38	75.02
β (°)	106.21	106.53
Resolution range (Å)	50–2.5	50–2.3
Total reflections	119651	130429
Unique reflections	34063	41147
Completeness (%)	99.0 (96.3)	93.7 (94.5)
$I/\sigma(I)$	9.9 (3.3)	16.1 (6.3)
$R_{\rm merge}$† (%)	5.5 (21.0)	9.2 (25.4)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

The forward and reverse oligonucleotide primers were designed using the published genome sequence (Tomb *et al.*, 1997). The amplified DNA was digested with *Nde*I and *Bam*HI and then was inserted into the *Nde*I/*Bam*HI–digested expression vector pET-22b (Novagen). The plasmid was transformed into the *E. coli* strain BL21(DE3)pLysS (Novagen) for protein expression. BL21(DE3)pLysS cells transformed with the plasmid were selected on LB-agar plates with 50 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol. A single colony was transferred into 15 ml of LB and grown overnight with vigorous shaking at 310 K. The cells were reinoculated into 1.5 l of LB–ampicillin and were grown to an A_{600} of 0.5. Protein expression was induced by 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) at 310 K. After IPTG induction, cell growth continued for 4 h at 310 K and cells were harvested by centrifugation at 4 200*g* (6000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (50 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl, 1 m*M* β-mercaptoethanol, 1 m*M* EDTA). Cells were passed through a French press twice and the crude cell extract was centrifuged at 36 000*g* (18 000 rev min^{-1}; Hanil Supra 21K rotor) for 20 min at 277 K. The supernatant was subject to ion-exchange chromatography on a Q-Sepharose column (Amersham Biosciences), which was previously equilibrated with buffer *A* (20 m*M* Tris–HCl pH 9.0, 1 m*M* β-mercaptoethanol, 1 m*M* EDTA) and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in buffer *A*. The pooled fractions were then subjected to affinity chromatography on Blue-Sepharose (Amersham Biosciences) and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in buffer *A*. Final purification was performed by gel filtration on a HiLoad XK 16 Superdex 75 prep-grade column (Amersham Biosciences), which was previously equilibrated with buffer *A* containing 200 m*M* NaCl. Homogeneity of the purified ENR was assessed by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). The protein solution was concentrated to about 8 mg ml^{-1} using a YM-10 ultrafiltration membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated molar extinction coefficient of 26 740 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

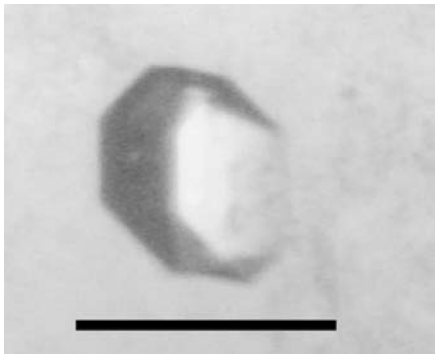

Figure 1
A monoclinic crystal of enoyl-acyl carrier protein reductase from *H. pylori*, grown in the presence of its cofactor NADH and triclosan. Approximate dimensions are 0.30 × 0.20 × 0.15 mm. The scale bar is 0.3 mm.

2.2. Crystallization and X-ray data collection

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method at 296 K using 24-well tissue-culture plates (Hampton Research). A hanging drop on a siliconized cover slip was prepared by mixing equal volumes (3 µl each) of the protein solution and the reservoir solution. It was placed over 0.5 ml of the reservoir solution. The protein solution was prepared by the addition of NADH and triclosan (or diclosan) to the apoenzyme in a 1:4:10 molar ratio, followed by 1 h incubation on ice. Initial crystallization trials were set up using Crystal Screen I (Jancarik & Kim, 1991), Crystal Screen II and MembFac screening kits (Hampton Research). Microcrystals obtained using PEG 400 as a precipitant were optimized.

X-ray diffraction data of the triclosan complex were collected at 100 K at beamline BL-18B of the Photon Factory, Tsukuba, Japan (Sakabe, 1991) with an ADSC Quantum 4R CCD detector. Crystals were flash-frozen in a liquid-nitrogen stream with 25%(*v*/*v*) ethylene glycol as a cryoprotectant. The wavelength of the synchrotron X-rays was 0.9794 Å and a 0.2 mm collimator was used. The crystal was rotated through a total of 183° with a 1.0° oscillation range per frame. X-ray data of the diclosan complex were collected at 100 K at beamline BL-6B of the Pohang Light Source, Korea, with an imaging plate as a detector (MacScience DIP 2030b). Crystals were flash-frozen in a liquid-nitrogen stream with 25%(*v*/*v*) ethylene glycol as a cryoprotectant. The wavelength of the synchrotron X-rays was 1.0072 Å and a 0.3 mm collimator was used. The crystal was rotated through a total of 200°, with a 1.0° oscillation range per frame. The raw data were processed using the program *MOSFLM* (Leslie, 1992). The resultant data were merged and scaled using the program *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results

H. pylori ENR in its intact form has been overexpressed in soluble form with a yield of approximately 35 mg of homogeneous protein from 1.5 l of culture. It has been crystallized in the presence of NADH and triclosan (or diclosan). The optimized reservoir condition is 100 m*M* sodium acetate buffer pH 4.8, 100 m*M* ammonium sulfate and 23%(*w*/*v*) PEG 400. Crystals grew to maximum dimensions of 0.3 × 0.2 × 0.15 mm within 2 d (Fig. 1).

The flash-frozen crystals of the triclosan complex initially diffracted to 2.8 Å resolution. After crystal annealing (Harp *et al.*, 1998) was performed during the 3 min incubation time, the diffraction limit slightly improved to about 2.5 Å. A total of 119 651 measured reflections were merged into 34 063 unique reflections, with an $R_{\rm merge}$ (on intensity) of 5.5%. The merged data set is 99% complete to 2.5 Å resolution. The crystal belongs to the primitive monoclinic space group $P2_1$, with unit-cell parameters $a = 73.35\ (39), b = 94.91\ (37), c = 75.38\ (48)$ Å, $\beta = 106.21\ (21)^\circ$, where estimated standard deviations are given in parentheses. The asymmetric unit contains one homotetrameric molecule of ENR, giving a crystal volume per protein mass ($V_{\rm M}$) of 2.10 Å^3 Da^{-1} and a solvent content of 41% by volume (Matthews, 1968). Data for the diclosan complex were collected without crystal annealing. Crystallographic data-collection statistics for both triclosan and

diclosan complexes are summarized in Table 1. *H. pylori* ENR structures have been solved by the molecular-replacement method using the *E. coli* ENR crystal structure as a search model. The inhibitors bound at the active site are clearly defined by the electron density and the structural details will be described in a separate paper.

We thank Professor N. Sakabe and his staff for assistance during data collection at beamline BL-18B, Photon Factory, Japan. We also thank Dr H. S. Lee for assistance during data collection at beamline BL-6B, Pohang Light Source, Korea. This work was supported by the 21C Frontier Program of the Korea Ministry of Science and Technology. SWS's laboratory is part of the Division of Chemistry and Molecular Engineering (BK21 project of the Korea Ministry of Education). HHL, BWH and BIL were supported by the BK21 Fellowship.

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