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Crystallization and preliminary X-ray analysis of the catalytic core of the alkylhydroperoxide reductase component AhpF from *Escherichia coli*

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Alkylhydroperoxide reductases (AhpR, E.C. 1.6.4.*x*) are essential for the oxygen tolerance of aerobic organisms, converting otherwise toxic hydroperoxides of lipids or nucleic acids to their corresponding alcohols. The AhpF component (521 amino-acid residues, 56.2 kDa) belongs to the family of pyridine nucleotide–disulfide oxidoreductases and channels electrons from NAD(P)H *via* a series of disulfides towards the AhpC component, which finally reduces the hydroperoxide substrates. Crystals of the proteolytically truncated AhpF component (residues Asn208–Ala521) of the alkyl hydroperoxide reductase from *Escherichia coli* were grown under oxidizing conditions. The crystals belong to space group $P3_221$, with unit-cell parameters $a = 60.4$, $c = 171.8$ Å. X-ray diffraction data were collected to 1.9 Å resolution using synchrotron radiation. A molecular-replacement solution was found using the structure of thioredoxin reductase from *Arabidopsis thaliana* as a search model.

1. Introduction

Organisms which live in aerobic environments require defence systems that prevent or minimize damage of cellular DNA or membranes by reactive oxygen species like $O_2^{\cdot -}$, H_2O_2 or $HO^{\cdot}$. These species are formed by incomplete reduction of oxygen during respiration or by other factors such as light, radiation or stimulated host phagocytes. Eubacteria such as *Salmonella typhimurium* or *E. coli* contain an alkylhydroperoxide reductase (AhpR, E.C. 1.6.4.*x*) which converts alkyl hydroperoxides, *e.g.* thymine peroxide or linoleic acid peroxide, to the corresponding alcohols using NADH or NADPH as reduction equivalents (Jacobson *et al.*, 1989). AhpR consists of two polypeptides, the AhpC component with 186 residues (M_W = 20.6 kDa) and the larger AhpF component with 521 residues and a molecular weight of 56 177 Da. The catalytic mechanism of AhpR was shown to follow a similar pattern to the thioredoxin reductase (TrxR), which is an important intracellular reduction system (Tartaglia *et al.*, 1990). The AhpF component reduces an internal redox-active disulfide bridge between Cys345 and Cys348 by electron transfer from reduced pyridine nucleotides *via* the cofactor flavin. The reduction of this disulfide bridge is followed by a cascade of disulfide-exchange reactions, first intramolecularly to a disulfide in the N-terminal region of AhpF, then intermolecularly to the disulfide Cys46–Cys165 of the AhpC component (Ellis & Poole, 1997; Li Calzi & Poole, 1997). The Ahp reductase system has a profound influence on the activity of certain antimycobacterial drugs, as the expression of the *ahpC* gene is strongly enhanced in isonicotinic acid hydrazide (INH) resistant strains of *Mycobacterium tuberculosis* (Zhang *et al.*, 1996).

The AhpF component belongs to the larger family of pyridine nucleotide–disulfide oxidoreductases, with thioredoxin reductases as the nearest relatives besides other more distantly related enzymes such as glutathione reductases, lipoamide dehydrogenases and trypanothione reductase. Sequence alignments show that both the AhpF components and thioredoxin reductases share 27–35% sequence identity among the last 300 residues. In contrast, the N-terminal extension of about 200 residues in the AhpF components from enterobacteria and Gram-positive bacteria has no counterpart in the smaller thioredoxin reductases and comprises two modules which are homologous to thioredoxins (Trx). In this work, we report the purification, crystallization and preliminary X-ray crystallographic analysis of the catalytic core of AhpF, a protein that is highly abundant in *E. coli* cells which are stressed either by the heterologous overproduction of recombinant proteins or by the high oxygen levels typically present in stationary phase cultures.

2. Experimental procedures

2.1. Bacterial expression and purification

E. coli cells which overproduce the recombinant protein sGCβ1(1–385), a fragment of the soluble guanylate cyclase from rat, were grown in TB medium containing 50 µg ml^{-1}

Table 1
Crystal data and data-collection statistics.

Values in parentheses correspond to the highest resolution shell (1.95–1.90 Å).

X-ray source and wavelength	BW6, 1.00 Å
Space group	$P3_221$
Unit-cell parameters (Å)	a = 60.44, c = 171.82
Resolution range (Å)	25–1.9
No. of observations	126257
No. of unique reflections	29657
Mosaicity	0.28°
Wilson B factor (Å^2)	13.6
$I/\sigma(I)$	20.9 (12.3)
$R_{\rm merge}$	0.045 (0.109)
Completeness (%)	99.9 (99.7)

kanamycin. After 6 h of gene expression at 297 K, the cells were harvested, resuspended in 1/100th of the culture volume with resuspension buffer (PBS, 2.0 m*M* DTT, 0.2 m*M* PMSF, 1 m*M* EDTA) and lysed by sonification three times for 20 s. The suspension was clarified by centrifugation at 25 000*g* for 30 min. The supernatant was loaded onto a 15 ml nickel–nitrilotriacetic acid (Ni^{2+}–NTA) column (Qiagen) pre-equilibrated with PBS buffer. The column was eluted with 40 volumes of PBS buffer in a linear imidazole gradient (0–250 m*M*). During this step, designed to purify His_6-tagged fusions of sGC-β1(1–385), yellow-coloured fractions containing AhpF were observed which eluted at low imidazole concentrations, ∼40 m*M*, as a major component along with other non-specifically bound contaminants from *E. coli* cytosol. The AhpF-containing fractions were further purified by anion-exchange chromatography on a Resource Q column (Pharmacia) pre-equilibrated with washing buffer (20 m*M* Tris–HCl pH 8.0). The AhpF component was eluted in a linear NaCl gradient (0–1000 m*M* NaCl in 20 m*M* Tris–HCl pH 8.0) at a salt concentration of 250 m*M* NaCl. The eluted yellow fractions were pooled and concentrated using Centriprep 10 concentrator cells (Amicon). The protein was subsequently loaded onto a Superdex 200 gel-filtration column (Pharmacia). Gel filtration was carried out at 277 K using 20 m*M* Tris–HCl pH 8.0, 0.6 *M* NaCl, 0.5 m*M* DTT as elution buffer. The estimated yield of the AhpF component was 0.5–1.0 mg purified protein per litre culture, with >95% purity. Protein concentrations were determined using the Bradford reagent (Biorad) and bovine serum albumin as standard.

2.2. Limited proteolysis

In order to optimize the solubility characteristics of AhpF for crystallization, the protein was subjected to limited proteolysis by incubating it at a concentration of 0.5 mg ml^{-1} for 30 min at 277 K with the same concentration of the serine protease proteinase K (Boehringer). The cleavage reaction was carried out in 20 m*M* Tris–HCl pH 8.0 as reaction buffer and was stopped by adding 10 m*M* PMSF (Sigma). The truncated AhpF was further purified by anion-exchange chromatography on Resource Q, where it was eluted with 60 ml of a linear NaCl gradient (0–600 m*M* NaCl in reaction buffer). The AhpF-containing fractions were concentrated to a final volume of 1.0 ml and loaded onto a Superdex 75 gel-filtration column (Pharmacia) in 20 m*M* Tris–HCl pH 8.0, 0.6 *M* NaCl. Under these conditions, the protein eluted in a volume that corresponds to a monomeric state of the AhpF fragment.

Purified proteins were N-terminally sequenced by Edman degradation and were examined by UV–VIS spectroscopy.

2.3. Crystallization and data collection

Crystals of the catalytic core of AhpF were grown by hanging-drop vapour-diffusion at 291 K by mixing 1 µl of protein solution (7 mg ml^{-1} protein in 5 m*M* Tris–HCl pH 8.0) with 1 µl of the precipitation buffer. A triclinic crystal form (space group $P1$, unit-cell parameters a = 53.0, b = 58.1, c = 61.3 Å, α = 61, β = 82, γ = 63°) and a crystal form with hexagonal/trigonal morphology were obtained from the truncated AhpF component. The triclinic form was grown as thin plates (∼0.2 × 0.2 × 0.03 mm) using 0.2 *M* lithium sulfate, 30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5 as precipitation buffer.

The second crystal form was grown using 2.0 *M* ammonium sulfate, 6% PEG 400, 0.1 *M* Tris–acetate pH 6.5 as precipitation buffer. Phase separation occurred under these conditions after 2–3 d and plate-like protein crystals grew after 1–2 weeks.

A complete 1.9 Å data set was collected from a single frozen crystal belonging to the trigonal form (space group $P3_121$ or $P3_221$, unit-cell parameters a = 60.4, c = 171.8 Å) on beamline BW6 at the DORIS III storage ring, DESY, Hamburg. Data processing was carried out using the *HKL* package (Otwinowski & Minor, 1997).

3. Results

During the heterologous overproduction of various recombinant proteins in *E. coli* BL21(DE3) cells, we regularly observed the accumulation of a yellow-coloured chromoprotein in the cytosolic fraction. Subsequent purification and protein sequencing identified the chromoprotein as the AhpF component of the *E. coli* alkylhydroperoxide reductase, which is well known to be inducible by oxidative stress, a condition that mostly prevails during overexpression at low growth temperatures or when *E. coli* cultures reach their stationary phase. Initial attempts to crystallize the intact AhpF component which was purified as a major side-product when Ni–NTA columns were used to isolate His_6-tagged fusion proteins from cytosol failed, as the protein behaved inhomogenously during further purification and showed some tendency towards aggregation.

Subsequently, the purified protein was proteolytically cleaved with a set of different serine proteases. The C-terminal catalytic core of the AhpF, which showed an apparent molecular weight of 33 kDa on 15% SDS–PAGE, was derived from limited proteolysis when the serine protease proteinase K was used. Sequencing of the truncated protein by Edman degradation showed that the first 207 residues, which contain two N-terminal Trx-like modules, were clipped off by the proteolysis reaction. The truncated protein comprised the residues Asn208–Ala521 and was further purified by anion exchange and gel filtration. An UV–VIS spectrum of AhpF-(Asn208–Ala521) was monitored under reducing conditions, which showed that the truncation of native AhpF lead to no loss of the bound cofactor FAD (data not shown). This indicates that the FAD-binding properties of the C-terminal catalytic core were unaffected by the removal of the N-terminal Trx modules which are a hallmark of eubacterial alkylhydroperoxide reductases.

Two different crystal forms were obtained from the catalytic core of the AhpF component using the hanging-drop vapour-diffusion method. Crystallographic data were collected from the trigonal crystal form using synchrotron radiation (for statistics, see Table 1). The mounted crystal was first frozen at 100 K in its growth buffer, but the diffraction pattern exhibited some ice rings, excessive mosaicity (>1°) and a mediocre diffraction limit of 2.4 Å resolution. Consequently, the crystal was rethawed by rapid transfer of the cryo-loop into a 10 µl droplet which was held on a cover slip 2 cm below the cryostream. The thawing buffer consisted of the growth buffer with 20% glycerol added. Soaking in this solution took place for about 3 s before the crystal was transferred back into the cryostream. This procedure reduced the mosaicity to 0.28° and improved the maximum diffraction limit

to 1.3 Å. However, data collection was restricted to 1.9 Å resolution in order to avoid spatial spot overlap owing to a disadvantageous orientation of the long *c* axis. Using the Matthews equation (Matthews, 1968), the solvent content of the hexagonal crystal form was estimated to be 43% with one molecule per asymmetric unit (V_m = 2.65 Å Da^{-1}), while the water content of the triclinic form, which proved inferior owing to its resolution limit of about 3.5 Å, was 67 or 33%, respectively, for one or two molecules per asymmetric unit (V_m = 4.28 or 2.14 Å Da^{-1}, respectively).

The crystal structure of AhpF-(Asn208–Ala521) was solved by molecular replacement (MR) using the program *AMoRe* as implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The structures of thioredoxin reductase from *E. coli* (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994) and from the plant *A. thaliana* (Dai *et al.*, 1996) were tested as search models, either using the complete structures or the individual FAD- and NAD(P)H-binding domains. A convincing solution was found in space group $P3_221$ only with the complete *A. thaliana* model, although there were no major differences in sequence conservation among AhpF and *E. coli* and *A. thaliana* thioredoxin reductases (34 and 33%, respectively, for 315 residues). However, the *A. thaliana* structure has an association between the FAD- and NAD(P)-binding domains that differs by 8° from the *E. coli* enzyme (Dai *et al.*, 1996). The initial correlation coefficients were 0.093 for the rotation search (next peak, 0.091; data used, 15–4 Å) and 0.158 for the translation search (*R* factor, 59.4%; data used, 15–4 Å; correlation coefficient of next peak, 0.063) while similar searches in $P3_121$ failed to yield sufficiently contrasting solutions in the translation function. Rigid-body refinement of the MR solution improved the correlation coefficient and *R* factor to 0.489 and 50.9%, respectively. After positional refinement with *CNS*, which yielded an *R* factor and $R_{\rm free}$ of 42.7 and 52.2%, respectively (25–2.5 Å data), a sequence-corrected model was automatically rebuilt using the *wARP*/*ARP* suite with all data to 1.9 Å (Perrakis *et al.*, 1999). Ten macrocycles consisting of further refinement with *CNS* and manual rebuilding reduced the *R* factor and $R_{\rm free}$ from 38.3 and 39.1%, respectively, to 20.4 and 24.6%, respectively, for data in the resolution range 25–1.9 Å. The final structure of the catalytic core of AhpF will be a useful guide in deciding whether there are any structural pecularities of alkylhydroperoxide reductases which make these enzymes efficient catalysts for intramolecular redox reactions.

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