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Crystallization and preliminary X-ray crystallographic analysis of a *ytfG* gene product from *Escherichia coli*

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The *Escherichia coli ytfG* gene product, with NAD(P)H:quinone oxidoreductase activity, was crystallized by the hanging-drop vapour-diffusion method at 296 K. A 1.78 Å data set has been collected using synchrotron radiation at Pohang Light Source, South Korea. The crystal belongs to the primitive trigonal system, with unit-cell parameters $a = b = 81.7$, $c = 76.8$ Å. Analysis of the packing density shows that the asymmetric unit probably contains one monomer, with a solvent content of 48.8%.

1. Introduction

Quinones represent a class of toxicological intermediates that can create a variety of cytotoxic effects *in vivo* (Bolton *et al.*, 2000). The cytotoxicity of quinones is known to arise from the generation of semiquinone radicals by one-electron transfer to quinones. The redox cycling of the generated semiquinone radicals can give rise to reactive oxygen species that are toxic to cells (Foster *et al.*, 2000).

Several cytoplasmic NAD(P)H-dependent oxidoreductases have been reported to participate in quinone metabolism and can be divided into two groups depending on whether they reduce quinone compounds by two-electron transfer (hydride transfer) or one-electron transfer. The enzymes of the first group contain FAD as a prosthetic group and reduce quinone compounds by two-electron transfer. In mammals, for example, the flavoenzyme NAD(P)H:quinone oxidoreductase type I (QR1, NQO1 and DT-diaphorase) catalyzes the two-electron reduction of quinones to hydroquinones with NADPH or NADH as an electron donor (Cui *et al.*, 1995; Ernster *et al.*, 1962; Segura-Aguilar *et al.*, 1992). The crystal structure of rat QR1 (Li *et al.*, 1995) gave insights into the sequential mechanism of two-electron reductions by hydride transfers: firstly from NAD(P)H to FAD and then from $FADH_2$ to a quinone. The first half reaction consists of a hydride transfer from C4 of the reduced nicotinamide of NADPH to N5 of the flavin of FAD. This reaction generates a negative charge at O2 of the flavin, which is stabilized by sequential proton relay from His161 to Tyr155 and then finally to O2 of flavin, which is hydrogen bonded to Tyr155. Since the NADPH and quinone share the same site in the enzyme, the process mentioned above is reversed in the second half reaction.

Numerous studies have proposed that QR1 serves a protective role against quinone toxicity without the production of semiquinone radicals which are the major toxic species. For instance, QR1-null mice show higher sensitivity to menadione toxicity and increased lipid peroxidation and DNA adducts, which are known to be cytotoxic effects of quinones, compared with wild-type mice (Joseph *et al.*, 2000; Radjendirane *et al.*, 1998). Similarly, the flavoenzyme NAD(P)H:quinone reductase (NQR), a functional homologue of mammalian QR1 in higher plants, is also believed to protect against oxidative action of semiquinones (Sparla *et al.*, 1996, 1999; Trost *et al.*, 1995).

The enzymes of the second group, such as ζ-crystallin, a major lens protein found in hystricomorph rodents and camelids (Piatigorsky & Wistow, 1989), have no prosthetic group and reduce quinone compounds by one-electron transfer, producing semiquinone radicals (Rao *et al.*, 1992). A family of proteins homologous to ζ-crystallin is widely distributed from bacteria to mammals (Babiychuk *et al.*, 1995; DeRisi *et al.*, 1997; Huang *et al.*, 1990). In bacteria, to date, NADPH-dependent quinone oxidoreductases from *Escherichia coli* (EcQOR) and from *Thermus thermophilus* HB8 (TtQOR) have been shown to be homologous to the ζ-crystallin-type quinone oxidoreductases (Edwards *et al.*, 1994; Shimomura *et al.*, 2002). Despite the universal distribution of ζ-crystallin-type quinone oxidoreductases, the physiological role of their univalent quinone-reduction activity is yet unclear.

Recently, we cloned and purified a soluble NAD(P)H:quinone oxidoreductase from *Streptomyces seoulensis* that shows no sequence homology to the previously known cytoplasmic quinone oxidoreductases (unpublished results). A BLAST (Altschul *et al.*, 1997)

database search revealed that an *E. coli ytfG* gene codes a protein homologous to the *S. seoulensis* NAD(P)H:quinone oxidoreductase. Because of its ease of manipulation, we studied the *E. coli* YtfG as a model system for the characterization of the new type of oxidoreductase. YtfG consists of 286 amino-acid residues with a calculated molecular weight of 29.73 kDa. As expected, YtfG is able to reduce benzoquinone with NAD(P)H as an electron donor. In the reaction of YtfG with benzoquinone as a substrate, a semiquinone radical was detected by EPR spectroscopy (data not shown), a definite indication of a one-electron transfer reaction. In addition, no prosthetic group was found in YtfG. Thus, YtfG can be classified as a member of ζ-crystallin-type oxidoreductases. The oligomeric conformation of YtfG as well as the primary sequence, however, is distinct from those of other ζ-crystallin-type oxidoreductases. The purified YtfG exists as a monomer in solution while QR1, QOR and *Arabidopsis thaliana* P1 ζ-crystallin are dimers and ζ-crystallins from mammalian lenses have a tetrameric quaternary structure (Duhaiman *et al.*, 1995; Huang *et al.*, 1987; Li *et al.*, 1995; Mano *et al.*, 2000; Thorn *et al.*, 1995).

According to the database of clusters of orthologous groups of proteins (COGs; Tatusov *et al.*, 2001), YtfG contains a nucleoside-diphosphate-sugar epimerase domain. Sugar-nucleotide epimerases such as UDP-galactose-4-epimerase bind NAD^+ and possess conserved Y*xxx*K and G*xx*G*xx*G sequences that are an active-site motif and nucleotide-binding motif, respectively (Mulichak *et al.*, 1999; Thoden *et al.*, 2000). In YtfG, the nucleotide-binding motif is found at its N-terminus but the active-site motif is absent, which is consistent with the results that YtfG can use NAD(P)H as a cofactor and shows no sugar-nucleotide epimerase activity.

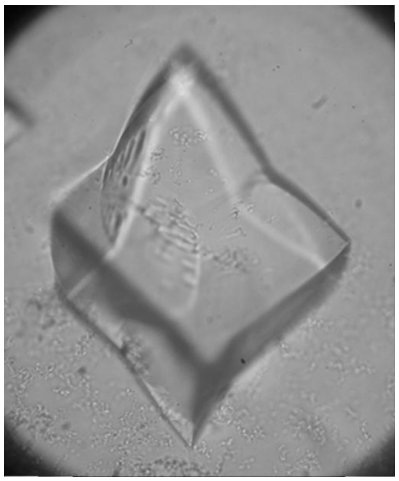

Figure 1
Hexagonal crystal of YtfG from *E. coli* MG1655.

Thus far, the molecular mechanism of one-electron transfer from NAD(P)H to quinone in ζ-crystallin-type oxidoreductases has remained elusive, although the crystal structure of EcQOR has been reported (Thorn *et al.*, 1995). To investigate the one-electron transfer reaction without any prosthetic group at the atomic level, we plan to determine the structure of YtfG. In spite of their functional similarity, the disparity in primary sequence and the oligomeric conformation compared with other ζ-crystallin-type oxidoreductases also arouses interest in the three-dimensional structure of YtfG. Here, we report the crystallization and preliminary X-ray crystallographic analysis of YtfG as a first step towards the structure determination.

2. Protein purification and characterization

2.1. Preparation of recombinant YtfG

The *ytfG* gene was amplified by polymerase chain reaction using *E. coli* MG1655 genomic DNA as a template. The gene was inserted downstream of the T7 promoter of pET-3a (Novagen) and the plasmid was transformed into *E. coli* strain BL21 (DE3). Cells were grown to an OD_{600} of approximately 0.6 in Luria–Bertani media containing 0.1 mg ml^{-1} ampicillin (Duchefa) at 310 K and expression was induced with 0.4 m*M* isopropyl-β-D-thiogalactoside (Duchefa). After 3 h induction, cells were harvested and resuspended in 50 m*M* potassium phosphate (Fluka) pH 7.5 containing 0.1 m*M* ethylenediamine tetraacetic acid (EDTA, Fluka). The cells were disrupted by sonication and the cell debris was discarded by centrifugation at 20 000*g* for 30 min. The ammonium sulfate (Fluka) was added to the supernatant to 45% saturation. After stirring the solution for 2–3 h, the precipitate was collected by centrifugation at 20 000*g* for 30 min and additional ammonium sulfate was added to the supernatant fraction to 65% saturation. The precipitate was collected after 2–3 h by centrifugation at 20 000*g* for 30 min and dissolved in 50 m*M* potassium phosphate buffer pH 7.5 containing 0.1 m*M* EDTA and 150 m*M* NaCl (Fluka). The enzyme solution was loaded onto a Superdex 75 HR 16/60 column pre-equilibrated with the same buffer. The fractions with quinone oxidoreductase activity were pooled and dialyzed against 50 m*M* potassium phosphate buffer pH 7.5 containing 0.1 m*M* EDTA. The partially purified protein was loaded again onto Protein-Pak DEAE 5PW and YtfG was eluted with washing buffer (50 m*M* potassium phosphate buffer pH 7.5 containing 0.1 m*M* EDTA). The purified protein was dialyzed against 20 m*M* Tris–HCl buffer pH 7.5 and then concentrated to approximately 20 mg ml^{-1} for crystallization.

Table 1
Crystal information and data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.78–1.87 Å).

Source	BL-6B, PLS
Wavelength (Å)	1.12714
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	a = 81.7, b = 81.7, c = 76.8
Resolution range (Å)	20.0–1.78
Completeness (>1σ) (%)	97.9
R_{sym}† (%)	6.2 (29.4)
$I/\sigma(I)$	9.9 (3.1)

† $R_{sym} = \sum |I_{obs} - I_{avg}|/I_{obs}$.

2.2. Enzyme-activity assay

The oxidoreductase activity of YtfG was assayed by the absorption decrease of NADPH (Sigma) monitored at 340 nm. The reaction mixture contained 0.2 m*M* *p*-benzoquinone (Sigma) and 0.2 m*M* NADPH in 20 m*M* potassium phosphate buffer pH 7.5.

3. Crystallization and X-ray analysis

YtfG was crystallized by the hanging-drop vapour-diffusion method using 24-well Costa plates at 296 K. The first crystallization screening was performed with Crystal Screen, a sparse-matrix screening kit (Hampton Research, USA). Several bundles of rod-shaped crystals were produced under conditions containing 2.0 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5 and 2% polyethyleneglycol 400 (PEG 400) in 2 d.

The initial crystallization condition was optimized by using PEG 4000 instead of PEG 400; subsequent addition of $CuCl_2$ greatly improved the quality of the crystals. Finally, a large single crystal was found in droplets containing 1.5 µl of protein sample (15 mg ml^{-1}) and an equal volume of precipitant solution containing 10 m*M* $CuCl_2$, 1.4 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5 and 0.17% PEG 4000. The droplets were equilibrated against 1 ml of the same precipitant solution at 296 K and the crystals grew to maximum size in 1 d (0.3 × 0.3 × 0.3 mm; Fig. 1). For data collection (Table 1), crystals were frozen at 100 K using an Oxford Cryosystem after

brief immersion in a cryoprotectant solution containing 20% glycerol in the same precipitant solution. A 1.78 Å data set was obtained using a Bruker Proteum 300 CCD at beamline 6B at Pohang Light Source (PLS), South Korea. Using the autoindexing program *SMART* and examining the diffraction data set with *SAINT* and *PROSCALE*, we found that the crystals belong to the primitive trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 81.7$, $c = 76.8$ Å. Analysis of the packing density shows that the asymmetric unit probably contains one monomer, with a solvent content of 48.8%. Efforts toward structure determination using MAD with selenomethionine-substituted YtfG crystals are in progress.

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