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Crystallization and preliminary crystallographic analysis of major nitroreductase from *Escherichia coli*

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NADPH:nitrocompound oxidoreductase from *Escherichia coli*, NfsA, has been crystallized in the presence of FMN by the vapor-diffusion method using polyethylene glycol 6000 as a precipitant. The crystals belonged to the triclinic space group *P*1 with cell dimensions, $a = 52.2$, $b = 52.7$, $c = 53.3$ Å, $\alpha = 75.1$, $\beta = 60.1$, $\gamma = 60.5°$. The crystals are expected to contain two NfsA molecules per asymmetric unit. The crystals diffracted X-rays to at least 2.3 Å resolution and are appropriate for structural analysis at high resolution.

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1. Introduction

Nitroheterocyclic and nitroaromatic compounds are diverse natural products in the environment, in preservative and additive agents for food, and in coloring matters. These xenobiotics are transformed to carcinogens, mutagens and clastogens by nitroreductases derived from enterobacteria (Howard *et al.*, 1983; McCalla, 1983).

Enterobacterial nitroreductases are classified into two types (Asnis, 1957; Peterson *et al.*, 1979; Djuric *et al.*, 1986; McCalla *et al.*, 1975): oxygen-sensitive and oxygen-insensitive enzymes. The oxygen-sensitive enzymes catalyze the reduction of nitrocompounds by a one-electron transfer mechanism and form radical intermediates in the reduction process, which are oxidized by oxygen and return to the initial substrates. The other type of nitroreductases are oxygen-insensitive enzymes that catalyze reduction by a two-electron transfer mechanism and are not inhibited by oxygen.

In *E. coli*, three proteins, NfsA, NfsB and NfsC, are identified as the oxygen-insensitive nitroreductases (Bryant *et al.*, 1981). Biochemical studies have suggested that NfsA is an FMN-containing flavoprotein with a molecular mass of approximately 26 kDa and can use only NADPH as an electron donor (Zenno, Koike, Kumar *et al.*, 1996), in contrast to NfsB, which can use either NADH or NAPDH as an electron donor (Anlezark *et al.*, 1992; Zenno, Koike, Tanokura *et al.*, 1996).

The three-dimensional structure has been determined for none of nitroreductases yet. In order to elucidate the reaction mechanism and the substrate specificity, a high-resolution three-dimensional structure has to be determined for the enzymes. Here we report the crystallization and the preliminary X-ray crystallographic analysis of NADPH: nitrocompound oxidoreductase, NfsA, from *E. coli*.

2. Experimental

E. coli JM83 cells, carrying a plasmid pAJ102 overexpressing NfsA (Kumar & Jayaraman, 1991), were cultured in LB media containing 100 μg ml^{-1} ampicillin at 310 K for 7 h. Then cells were harvested by centrifugation at 10 000*g* for 5 min, suspended in 50 ml of 50 m*M* Tris–HCl buffer (pH 7.0) per liter culture and sonicated for 10 min at 277 K using a Sonifier 250D (Branson). Sonicated suspensions were centrifuged at 40 000*g* at 277 K for 30 min, and ammonium sulfate was added to the resultant supernatant, which was fractionated between 40 and 70% saturation (1.57 and 2.75 *M*) of ammonium sulfate. The pellet containing NfsA enzyme was dissolved into 50 m*M* Tris–HCl buffer (pH 7.0). The crude enzyme was dialyzed against 50 m*M* Tris–HCl (pH 7.0) for 12 h, was loaded onto a Q-Sepharose column equilibrated with 50 m*M* Tris–HCl (pH 7.0), and were eluted with a linear gradient of 0 to 1 *M* NaCl. The active fractions were collected and dialyzed against 50 m*M* Tris–HCl (pH 7.0) containing 200 m*M* NaCl and then applied to a gel-filtration column (Sephacryl H-100, Pharmacia) equilibrated with the same buffer. Ammonium sulfate was added to the active fractions to 1.0 *M* concentration. The resultant solution was then loaded onto a phenyl Sepharose 6FF column (Pharmacia) equilibrated with 50 m*M* Tris–HCl (pH 7.0) containing 1.0 *M* ammonium sulfate and eluted with a linear gradient of 1.0–0 *M* ammonium sulfate. The purity was analyzed by SDS–PAGE (Laemmli, 1970). The yield of the purified enzyme was 150–200 mg from 3 l culture medium.

NfsA catalytic activity was measured by the procedure described (Zenno, Koike, Kumar *et al.*, 1996). The standard assay solutions contained, in a final volume of 3.0 ml, 50 m*M* Tris–HCl (pH 7.0), appropriate amounts of enzyme, 100 μ*M* nitrofurazone. Reactions were

carried out at 296 K using a Shimadzu UV2200A recording spectrophotometer. The activity was monitored by recording the nitrofurazone reduction at 400 nm (ε = 12.96 mM^{-1} cm^{-1}).

Crystallization was accomplished at 278 K as follows. Crystals were grown using the sitting-drop vapor-diffusion method by mixing equal volumes of a 9 mg ml^{-1} protein solution with a reservoir solution that contained 22.5% polyethylene glycol 6000 as a precipitant and 2–15% ethylene glycol, polyethylene glycol 400 or isopropanol as an additive in 0.1 *M* MES buffer (pH 6.5). Crystals appeared over about 10 d, which had a plate-like shape (Fig. 1).

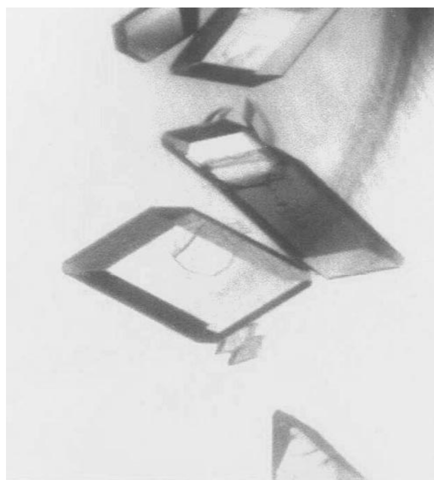

Figure 1
Plate-like yellow crystals of NfsA with the size of 0.3 × 0.3 × 0.2 mm in a sitting drop.

3. Results and discussion

The X-ray diffraction patterns were recorded using a Rigaku imaging-plate detector system, R-AXIS IIc, with double mirror-focused Cu $K\alpha$ radiation from a Rigaku RU-200R X-ray generator. A cooling device was used to keep the crystal at 100 K (Rigaku CN2364B2). Ethylene glycol or methanol was used as an anti-freeze reagent for cryo-measurement, which was introduced into the crystal by dialysis. According to the program, *hklplot* in the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994), there was no systematic absence in the *hk*0, *h*0*l*, and 0*kl* plane. The crystals belonged to space group *P*1 with cell dimensions of a = 52.2, b = 52.7, c = 53.3 Å, α = 75.1, β = 60.6, γ = 60.5°. Assuming that there are two molecules per unit cell, the crystal volume per unit molecular mass (V_m) and the solvent content are 2.08 Å^3 Da^{-1} and 41.0%, respectively (Matthews, 1968). The self-rotation function had only one peak at ω = 90.7, φ = 180.0, κ = 180.0° (Fig. 2). Since biochemical experiments have suggested that this enzyme acts as a dimer in solution (Zenno, Koike, Kumar *et al.*, 1996), it seems proper that the crystals contain one dimer in a unit cell.

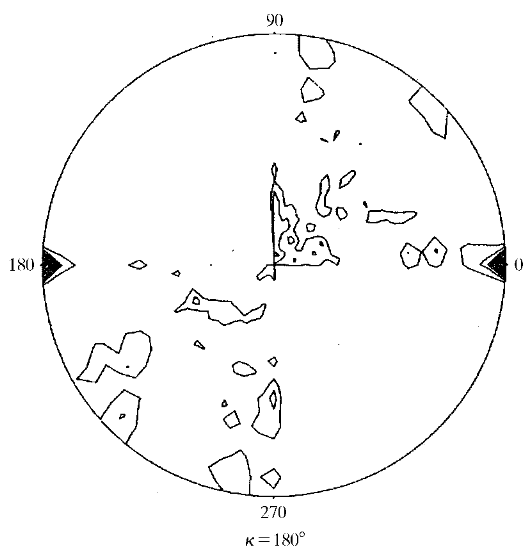

Figure 2
The section at κ = 180° of self-rotation function calculated for the diffraction data of NfsA crystals.

Intensity data were collected at BL6A and BL6B stations in the Photon Factory at High Energy Accelerator Research Organization (Tsukuba, Japan), using the Weissenberg camera for macromolecular crystallography with synchrotron radiation (Sakabe, 1983, 1991). The wavelength was set to 1.0 Å and a beam collimator of 0.1 mm was used. Three-dimensional data sets to 2.3 Å resolution were collected. Data evaluation was performed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The merging $R(I)$ factor $(\sum_i |I_i - \langle I_i \rangle| / \sum_i I_i$, where $\langle I_i \rangle$ is the average of I_i over all symmetrical equivalents) was 0.079 and the data was 96.1% complete to 2.3 Å resolution. The completeness in the resolution shell between 2.38 and 2.3 Å was 94.4% and the $\langle I \rangle / \langle \sigma(I) \rangle$ value in this range was 8.2. Since NfsA has 51% identity in amino-acid sequence with NADPH:FMN oxidoreductase from *Vibrio harveyi* (Tanner *et al.*, 1996), work is now under way to determine the structure of NfsA using the molecular-replacement method.

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References

Anlezark, G. M., Melton, R. G., Sherwood, R. F., Coles, B., Friedlos, F. & Knox, R. J. (1992). *Biochem. Pharmacol.* **44**, 2289–2295.
Asnis, R. E. (1957). *Arch. Biochem. Biophys.* **66**, 208–216.
Bryant, D. W., McCalla, D. R., Leeksma, M. & Laneuville. P. (1981). *Can. J. Microbiol.* **27**, 81–86.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Djuric, Z. D., Potter, W., Heflich, R. H. & Beland. F. A. (1986). *Chem. Biol. Interact.* **59**, 309–324.
Howard, P. C., McCoy, E. C. & Rosenkranz, H. S. (1983). *Mutagenesis*, **2**, 431–432.
Kumar, A. N. & Jayaraman, R. (1991). *J. Biosci.* **16**, 145–159.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
McCalla, D. R. (1983). *Environ. Mutagenesis*, **5**, 745–765.
McCalla, D. R., Olive, P., Tu, Y. & Fan, M. L. (1975). *Can. J. Microbiol.* **21**, 1485–1491.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Peterson, F. J., Mason, R. P., Hovsepian, J. & Holtzman, J. L. (1979). *J. Biol. Chem.* **254**, 4009–4014.
Sakabe, N. (1983). *J. Appl. Cryst.* **16**, pp, 542–547.
Sakabe, N. (1991). *Nucl. Instrum. Methods*, **303**, 448–463.
Tanner, J. J., Lei, B., Tu, S.-C. & Krause, K. L. (1996). *Biochemistry*, **35**, 13531–13539.
Zenno, S., Koike, H., Kumar, A. N., Jayaraman, R., Tanokura, M. & Saigo. K. (1996). *J. Bacteriol.* **178**, 4508–4514.
Zenno, S., Koike, H., Tanokura, M. & Saigo, K. (1996). *J. Biochem.* **120**, 736–744.