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# Crystallization and preliminary X-ray studies of azurin-I and azurin-II from denitrifying bacterium *Alcaligenes xylosoxidans* GIFU 1051

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## Abstract

Two distinct azurins named azurin-I and azurin-II were isolated from *Alcaligenes xylosoxidans* GIFU 1051 (AzG-I and AzG-II). They have been purified and crystallized. The crystals of AzG-I belong to the monoclinic crystal system, space group $C2$, unit-cell parameters $a = 130.6$, $b = 54.4$, $c = 74.7$ Å, $\beta = 96.1°$. The crystals of AzG-II belong to the tetragonal crystal system, space group $P4_122$ or $P4_322$, unit-cell parameters $a = b = 52.6$, $c = 100.7$ Å. Both crystals diffract up to 2.0 Å resolution and are suitable for X-ray crystallographic studies.

## 1. Introduction

Azurins are blue-copper proteins that function in electron transfer in biological systems (Ryden, 1984; Adman, 1985). These monomeric proteins are of molecular mass about 14 kDa. They exhibit a very intense absorption band in the visible spectra ($\lambda_{max} = 595$–$630$ nm, $\varepsilon \simeq 5000\ M^{-1}\ cm^{-1}$), unusually high redox potentials (240–400 mV), and a characteristically narrow hyperfine splitting in the electron paramagnetic resonance spectra ($A_{\parallel} \simeq 0.006\ cm^{-1}$) (Gray & Solomon, 1981). It has been known for a long time that only one azurin is obtained from one species of bacteria, except for the case of *Methylomonas J.* (Ambler & Tobari, 1989). Recently, two azurins were found instead of the single previously identified one in both *Alcaligenes xylosoxidans* NCIMB 11015 and GIFU 1051 (Yamaguchi, Nakamura, Shidara, Iwasaki & Suzuki, 1995; Dodd, Hasnain, Abraham, Eady & Smith, 1995). The amino-acid sequence of azurin-II from *A. xylosoxidans* NCIMB 11015 (AzN-II) has an homology of 65.1% to that of azurin-I from the same bacterium (AzN-I). The results of partial amino-acid sequence experiment revealed that AzG-I and AzG-II were quite similar to their counterparts from *A. xylosoxidans* NCIMB 11015. However, residue 31 in AzG-II is valine instead of methionine in AzN-II (Ambler, 1996; Yamaguchi, Nakamura, Shidara, Iwasaki & Suzuki, unpublished). Both the absorption spectra of AzG-I and AzG-II display two peaks at 279 and 620 nm. However, the molar absorption coefficient ($\varepsilon_{279} = 10\,700\ M^{-1}\ cm^{-1}$) of AzG-I at 279 nm is considerably smaller than that ($\varepsilon_{279} = 14\,500\ M^{-1}\ cm^{-1}$) of AzG-II. Moreover, the circular dichroism (CD) spectrum of AzG-I is clearly distinguishable from that of AzG-II. The positive sharp CD band at 291 nm of AzG-I is not observed in AzG-II, and the CD spectrum of AzG-I in the 250–300 nm region is different from that of AzG-II (Yamaguchi *et al.*, 1995). More details of the functions of these two azurins are under investigation.

Crystal structures of several bacterial azurins have been reported. They are azurins from *P. aeruginosa* (Adman & Jensen, 1981; Nar, Messerschmidt, Huber, Van de Kamp & Canters, 1991), *A. denitrificans* (Baker, 1988) and *P. denitrificans* (Korszun, 1987). The crystal structure of AzN-II was first reported at 2.5 Å in 1994 (Inoue *et al.*, 1994) and then at 1.9 Å in 1995 (Dodd *et al.*, 1995). However, no crystal structure information is available for azurin-I. Although structures of the active centers of azurins have been discussed intensively to explain their distinct properties, finding of two azurins from one strain gives a new interest to investigate their classification and structure–function relationships. Here, we present our recent work on the crystallization and preliminary X-ray studies of AzG-I and AzG-II.

## 2. Methods and results

All reagents in this experiment were of chemical grade and used without further purification. The cultivation of *A. xylosoxidans* GIFU 1051 and the isolation and purification of AzG-I and AzG-II were carried out by the means previously reported (Yamaguchi *et al.*, 1995). Purified AzG-I and AzG-II were stored at approximately 20 mg ml$^{-1}$ in 0.1 *M* potassium phosphate buffer (pH 6.0) at 277 K. Crystallization experiments for both proteins were performed using the hanging-drop vapor-diffusion method at 293 K.

Crystals of azurins are usually obtained by the hanging-drop vapor-diffusion method using ammonium sulfate as precipitant. However, we failed to get the crystals of AzG-I by this method. So other precipitants such as polyethylene glycol (PEG), 2-methyl-2,4-pentanediol (MDP) and phosphate salts were screened, and the pH was tested from 4.0 to 9.0. PEG 4000 yielded the most promising crystals over a pH range 7.5–8.5 in Tris–HCl buffer. A 6 µl droplet of 10 mg ml$^{-1}$ azurin-I solution containing 0.1 *M* Tris–HCl buffer (pH 8.5) and 15%(*w*/*v*) PEG and a 500 µl reservoir solution containing 0.1 *M* Tris–HCl buffer (pH 8.5) and 28%(*w*/*v*) PEG were used. Needle-shaped crystals with maximum dimensions of 0.08 × 0.08 × 0.2 mm appeared in the droplet after 10 d. They were suitable for X-ray crystallographic studies.

For AzG-II, a 6 µl droplet of 10 mg ml$^{-1}$ protein solution containing 0.1 *M* potassium phosphate (pH 7.0) and 1.0 *M* ammonium sulfate was used and equilibrated with a 500 µl reservoir solution containing 0.1 *M* potassium phosphate (pH 7.0) and 2.0 *M* ammonium sulfate. After 1 d, many needle crystals with maximum dimensions of 0.01 × 0.01 × 0.2 mm appeared in the droplet. Because the crystals were too small to be used for X-ray analysis, macroseeding was employed to obtain larger crystals. High-quality crystals were chosen as seeds. After being washed by the solution containing 0.1 *M* potassium phosphate (pH 7.0) and 1.0 *M* ammonium sulfate, they were put into a 6 µl droplet of 10 mg ml$^{-1}$ protein solution containing 0.1 *M* potassium phosphate (pH 7.0) and 1.2 *M* ammonium sulfate against the same reservoir solution as we

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mentioned above to obtain the small crystals. One day later the seed crystal grew to about twice the size. The process was repeated four or five times and finally AzG-II crystals with maximum dimensions of 0.10 × 0.10 × 1.5 mm were obtained.

The X-ray diffraction data for both azurins were collected on an R-AXIS IIc image-plate system (Rigaku) using Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å), with crystal-to-detector distances of 80 and 100 mm, respectively. Crystals of AzG-I diffracted to at least 2.0 Å resolution. The crystals did not show any sign of decay during the data collection, so they were stable under the X-ray irradiation. The space group of AzG-I was determined to be monoclinic $C2$, unit-cell parameters $a = 130.6$, $b = 54.4$, $c = 74.7$ Å, $\beta = 96.1°$. Four molecules of AzG-I are included in the asymmetric unit, the crystal density $V_m$ is 2.36 Å$^3$ Da$^{-1}$, which is close to the average for the protein crystals reported (Matthews, 1968).

Crystals of AzG-II diffracted to at least 2.0 Å resolution. Like the case of AzG-I, the crystals also did not show any sign of decay during the data collection, so they were stable in the X-ray beam. The space group was determined to be tetragonal $P4_122$ or $P4_322$, unit-cell parameters $a = b = 52.6$, $c = 100.7$ Å. The asymmetric unit includes one AzG-II molecule. The crystal density $V_m$ is 2.4 Å$^3$ Da$^{-1}$. The structure analyses for both azurins are in progress.

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