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Crystallization and preliminary X-ray study of iso-2 azurin from the methylotrophic bacterium, *Methylomonas* J

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The obligate methylotroph *Methylomonas* J possesses two distinct azurins. The iso-2 azurin, which functions as an electron acceptor for methylamine dehydrogenase, has been crystallized using two kinds of precipitants: PEG 4000 and ammonium sulfate. The crystals precipitated with PEG belong to the monoclinic system, space group $P2_1$, with unit-cell parameters $a = 32.96$, $b = 33.67$, $c = 47.34$ Å and $\beta = 101.35°$. The crystals precipitated with ammonium sulfate belong to the orthorhombic system, space group $C222_1$, with unit-cell parameters $a = 31.52$, $b = 62.49$ and $c = 135.41$ Å. The crystals diffract to 1.6 and 1.9 Å resolution, respectively, and were suitable for X-ray crystallographic studies. A Patterson search is being conducted using the recently reported structure of *Alcaligenes xylosoxidans* NCIMB 11015 as a starting model.

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1. Introduction

Methylomonas J is an obligate methylotroph, and can grow on either methanol or methylamine, but unlike several other methylotrophs such as Organism 4025 (Lawton & Anthony, 1985), *Methylobacillus flagellatum* KT (Gak *et al.*, 1995), *Paracoccus denitrificans* (Husain & Davidson, 1985) and *Thiobacillus versutus* (Houwelingen *et al.*, 1985), it cannot use methane for its carbon and energy sources (Ambler & Tobari, 1989). Methylamine dehydrogenase (MADH) is the enzyme that oxidizes methylamine to formaldehyde in those bacteria that grow on methylamine (Eady & Large, 1968; Haywood *et al.*, 1982; Husain & Davidson, 1987; Janvier *et al.*, 1985; Kenny & McIntire, 1983; Kirukhin *et al.*, 1990; Lawton & Anthony, 1985). Two types of electron acceptors, *c*-type cytochrome and blue copper proteins, are thought to be used to accept electrons from MADH. Three classes of blue copper proteins are found in methylotrophs: amicyanins, azurins and pseudoazurins (Auton & Anthony, 1989; Dinarieva & Netrusov, 1989; Husain & Davidson, 1985; Lawton & Anthony, 1985; Tobari & Harada, 1981; Tobari, 1984; Houwelingen *et al.*, 1985).

Blue copper proteins from the obligate methylotrophs were first isolated and studied by Tobari & Harada (1981). In the methylotrophic bacterium *M. extorquens* AM1, it has been shown that the electron acceptor from MADH is a blue copper protein called amicyanin. At high copper concentrations, the bacterium produces a large amount of an azurin-type protein called pseudoazurin (Tobari, 1984; Ambler & Tobari, 1985; Inoue, Kai *et al.*, 1994; Inoue, Shibata *et al.*, 1994). *Methylomonas* J produces two blue copper proteins, as does *M. extorquens* AM1 (Ambler & Tobari, 1989). These azurins are functionally equivalent to the two blue copper proteins, amicyanin and pseudoazurin, found in *M. extorquens* AM1. When cells were grown on both methanol and methylamine, the bacteria produced iso-1 azurin which could not function as the primary acceptor to MADH. On the other hand, when cells were grown on only methylamine, the bacteria produced iso-2 azurin which is a good acceptor of electrons from methylamine dehydrogenase (Tobari, 1984). More details of the functions of iso-1 and iso-2 azurins are under investigation.

Azurins are small copper-containing proteins (Adman, 1985) that function as electron-transfer agents in the redox systems of some bacteria (Ryden, 1984). They exhibit a very intense absorption band in the visible region of the electromagnetic spectrum (λ_{max} = 595–630 nm, $\varepsilon_M = 5000\ M^{-1}\ cm^{-1}$), unusually high redox potentials (240–400 mV) and a characteristically narrow hyperfine splitting in the electron paramagnetic resonance spectra ($A_{\parallel} = 0.006\ cm^{-1}$; Gray & Solomon, 1981). It was assumed for a long time that only one azurin could be obtained from each species of bacteria. However, two azurins were found in the methylotrophic bacterium *Methylomonas* J and two in the denitrifying bacteria *Alcaligenes xylosoxidans* NCIB 11015 and GIFU 1051 (Yamaguchi *et al.*, 1995; Dodd *et al.*, 1995). The iso-1 azurin of *Methylomonas* J shows 59–65% identity with other azurins from fluorescent pseudomonas and various species of *Alcaligenes*, whereas the iso-2 azurin shows only 47–53% identity. The azurins show 52% identity with each other.

A comparison of the amino-acid sequences of 15 azurins from different sources reveals

that the iso-2 azurin and azurin from *Methylobacillus flagellatum* KT (Gak *et al.*, 1995) have unique amino-acid sequences which differ from those conserved in other azurins. For example, both azurins have Gly10 instead of the Asn10 or Thr10 conserved in other azurins. Gly43 is also unique only in the methylotroph azurins, while the corresponding residue in other azurins is valine or alanine. Both azurins have Pro23 instead of the conserved Asp23 or Ser23, and Pro85 instead of the conserved Lys85 or Ser85. Arg18, Arg122 and Leu33 (corresponding to Lys18, Lys122 and Phe33 in other azurins, respectively) are also unique in the two methylotroph azurins. Moreover, the (+) or (−) charged amino-acid residues at positions 74, 83, 93, 98 and 101 are replaced by non-charged or oppositely charged residues in the methylotroph azurins. Residue 61 is threonine or serine in the other azurins; however, lysine is found in two methylotroph azurins. Striking differences in charge distribution on the molecular surface and the recognition mechanism with MADH are expected in the azurins of methylotroph bacteria.

Several crystal structures of bacterial azurins have previously been reported. These are azurins from *P. aeruginosa* (Nar *et al.*, 1991), *A. denitrificans* (Baker, 1988) and *P. denitrificans* (Korszun, 1987). Very recently, the crystal structure of azurin II from *A. xylosoxidans* (NCIMB 11015; Dodd *et al.*, 1995) was determined at 1.9 Å resolution. Although the active-site structures of these azurins, including their mutants, have been investigated in order to explain their distinct properties, the finding of two azurins in one bacterial strain provides a new interest in their structure–function relationship. Here, we present our recent work on the crystallization and preliminary X-ray studies of iso-2 azurin from the obligate methylotroph *Methylomonas* J.

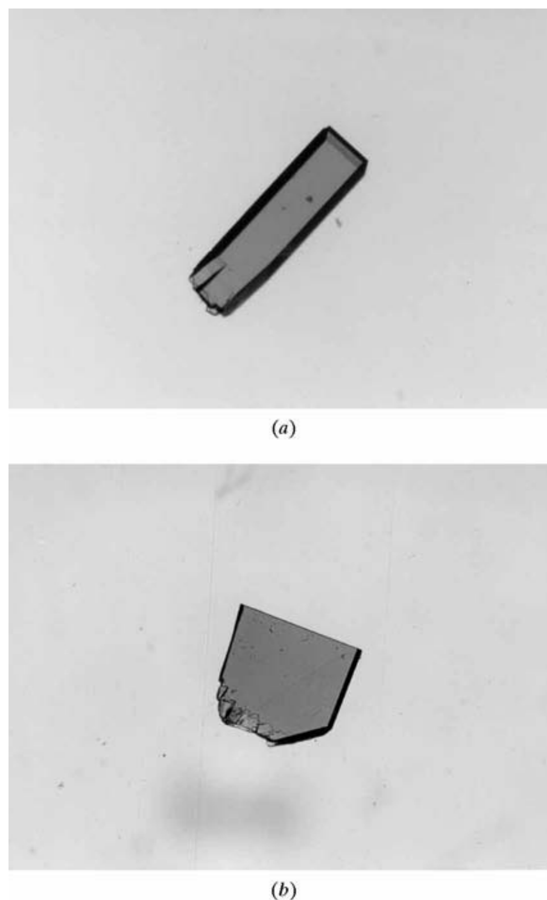

Figure 1
(*a*) A crystal of iso-2 azurin with maximum dimensions 0.3 × 0.3 × 1.2 mm appeared in the droplet of PEG solution (inner solution contains 6% protein, 12.5% PEG 4000 and 50 m*M* cacodylate buffer pH 5.0; outer solution contains 25% PEG 4000 and 100 m*M* cacodylate buffer pH 5.0) after 3 d. (*b*) A plate crystal of iso-2 azurin with maximum dimensions 0.6 × 0.7 × 0.1 mm appeared in the droplet of ammonium sulfate solution (inner solution contains 6% protein, 34% saturated ammonium sulfate and 50 m*M* cacodylate buffer pH 5.0; outer solution contains 68% saturated ammonium sulfate and 100 m*M* cacodylate buffer pH 5.0) after 1 d.

2. Materials and methods

All chemicals used were of chemical grade and were used without further purification. The cultivation of *Methylomonas* J and the isolation and purification of iso-1 and iso-2 azurins were carried out as previously reported (Ambler & Tobari, 1989). Purified iso-1 and iso-2 azurins were stored at approximately 12 mg ml^{-1} in 100 m*M* sodium cacodylate buffer (pH 5.0) at 277 K. Crystallization experiments for both proteins were performed using the hanging-drop vapour-diffusion method at 293 K.

Azurins are usually crystallized using ammonium sulfate (AS) as a precipitant. AS or PEGs of various molecular weights were used as precipitants in the crystallization of iso-1 azurin, together with $MgCl_2$ or $CaCl_2$, in the pH range 3.6–9.0. Needle-like crystals of iso-1 azurin were obtained; however, they were too thin for crystallographic data analysis. On the other hand, crystals of iso-2 azurin obtained by using AS and PEG 4000 as precipitants were of higher quality. PEG 4000 yielded the most promising crystals at pH 5.0 in cacodylate buffer. A 6 µl hanging drop of 6 mg ml^{-1} iso-2 azurin solution containing 100 m*M* cacodylate buffer (pH 5.0) and 12.5%(*w*/*v*) PEG 4000 was eqilibrated against 500 µl reservoir solution containing 100 m*M* cacodylate buffer (pH 5.0) and 25%(*w*/*v*) PEG 4000. Single crystals with maximum dimensions 0.3 × 0.3 × 1.2 mm appeared in the drop after 3 d (PEG-form crystal). The crystals are suitable for X-ray crystallographic studies (Fig. 1*a*). Iso-2 azurin was also crystallized by AS in a conventional way (AS-form crystal). A 6 µl hanging drop of 6 mg ml^{-1} protein solution containing 100 m*M* cacodylate (pH 6.0) and 34% saturated AS was equilibrated against 500 µl reservoir solution containing 100 m*M* sodium cacodylate (pH 5.0) and 68% saturated AS. After 1 d, single crystals suitable for X-ray crystallographic studies appeared with maximum dimensions 0.7 × 0.6 × 0.1 mm (Fig. 1*b*).

3. Data collection and results

The X-ray diffraction data for both crystals were collected at room temperature on an R-AXIS IIc imaging-plate system (Rigaku) using Cu $K\alpha$ radiation, λ = 1.5418 Å. Data was collected from a PEG-form crystal with a crystal-to-detector distance of 54 mm, while two AS-form crystals were used to collect two data sets with crystal-to-detector distances of 100 and 140 mm. The PEG-form crystal diffracted up to at least 1.6 Å resolution and was stable in the X-ray beam. The space group was determined to be monoclinic, $P2_1$, with unit-cell parameters a = 32.96, b = 33.67, c = 47.34 Å and β = 101.3°. If we assume there to be one molecule of iso-2 azurin in the asymmetric unit, the crystal density (V_m) is calculated to be 1.84 Å^3 Da^{-1}, which is close to the average for proteins (Matthews, 1968). A data set to 1.6 Å resolution has been collected with 88.2% completeness. A total of 34240 reflections collected were reduced to 12059 independent reflections with an $R_{\rm merge}$ of 5.3%. Image data were processed using the *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996) software.

AS-form crystals diffract to at least 1.9 Å resolution and are stable to X-ray exposure. The space group was determined to be orthorhombic, $C222_1$, with unit-cell parameters a = 31.52, b = 62.49 and c = 135.41 Å. The asymmetric unit contains one iso-2 azurin molecule. The crystal density V_m is 2.42 Å^3 Da^{-1}, which is also close to the average for proteins (Matthews, 1968). A total of 59724 reflections and 10191 independent reflections were recorded to a

resolution of 1.9 Å with 92.5% completeness. This gave an R_{merge} of 7.8%.

The molecular-replacement method for the structural analysis with *AMoRe* (Navaza, 1994) in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) is in progress using the structure of azurin from *A. denitrificans* as a starting model.

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