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Crystallization and preliminary X-ray crystallographic studies of mavicyanin from *Cucurbita pepo medullosa*

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Mavicyanin isolated from *Cucurbita pepo medullosa* is a glycosylated protein containing a single polypeptide chain of 109 amino-acid residues and is a member of the phytocyanin subclass of cupredoxins. Non-glycosylated recombinant mavicyanin, which was expressed in *Escherichia coli*, was crystallized by the hanging-drop vapour-diffusion method with ammonium sulfate as the precipitant at pH 5.5. The crystals belonged to the hexagonal space group $P6_1$ (or $P6_5$), with unit-cell parameters a = 64.0, c = 245.0 Å, four molecules per asymmetric unit and a solvent content of 59%. X-ray diffraction data were collected to 1.6 Å resolution. To solve the structure of mavicyanin, the MAD method as well as a Patterson search method using the structure of stellacyanin as a starting model are presently being utilized.

1. Introduction

Mavicyanin isolated from *Cucurbita pepo medullosa* (zucchini) peel is a member of the family of proteins known as cupredoxins (Marchesini *et al.*, 1979; Maritano *et al.*, 1997). Proteins in this family are characterized by an intense electronic absorption band near 600 nm, which gives them their characteristic blue to green-blue colour, and unusual small hyperfine coupling constants in their EPR spectra. The Cu sites in cupredoxins have common structural features: a loop containing three of the ligands of the Cu ion relatively close together in the sequence Cys-X_n-His-X_m-Met (where n and m vary from one protein to the next) and a fourth ligand which is a histidine located considerably toward the amino-terminus (Adman, 1991). All Cu^{II} sites in cupredoxins have similar distorted trigonal pyramidal or tetrahedral geometries. On the basis of sequence similarity, cupredoxins may be categorized into four main structural groups: (i) plastocyanin-related proteins, including amicyanin and pseudoazurin, (ii) azurins, (iii) soluble Cu_A domains derived from cytochrome oxidases and (iv) phytocyanins, small blue copper proteins from non-photosynthetic tissue of plants including stellacyanin, umecyanin, cucumber basic protein (CBP), putative blue copper protein in pea pods (PBP), cucumber-peeling cupredoxin (CPC) and blue copper protein from *Arabidopsis thalami* (BCB; Ryden & Hunt, 1993). As electron-transfer proteins, cupredoxins accept and donate a single electron to their redox partners, during which process the protein-bound copper oscillates between Cu^{II} and Cu^{I}. However, all the roles of phytocyanins have yet to be elucidated.

Phytocyanins share a remarkably high degree of sequence identity and are distinctly different from other groups of cupredoxins. Based on their spectroscopic properties, precursors, mature protein domain organization and identity or availability of resides involved in blue copper binding, phytocyanins are classified into four subfamilies: stellacyanins, plantacyanins, uclacyanins and early nodulins (Nersissian *et al.*, 2001; Nersissian & Shipp, 2002). The crystal structures of two phytocyanins, CBP (plantacyanin; Guss *et al.*, 1996) and cucumber stellacyanin (Hart *et al.*, 1996), support their classification as members of a distinct family of phytocyanins.

Several years ago, the sequence of zucchini mavicyanin was first published; it consisted of 108 amino-acid residues, lacking the first amino-acid residue (methionine). The sequence has 50.5% identity with stellacyanin and 45.8% with CBP (Schinina *et al.*, 1996). Comparisons of the primary structure of mavicyanin with those of other cupredoxins show that three copper ligands (two His residues and one Cys residue) are conserved, while the fourth ligand is probably the amido O atom of a Gln residue, as in stellacyanin. The domain architecture of mavicyanin indeed reveals that it is a member of the stellacyanin subfamily (Nersissian *et al.*, 1998).

A gene coding for the 109 amino-acid residue non-glycosylated form of mavicyanin has been synthesized in previous studies. Recombinant mavicyanin was expressed in

Escherichia coli, with a molecular weight of 11 745.6 Da as measured by MALDI-TOF mass spectroscopy. Its spectroscopic properties are identical to those of glycosylated wild mavicyanin isolated from zucchini peel. A mutant form of mavicyanin in which the putative ligand (Gln95) has been replaced with Met has been prepared by site-directed mutagenesis. Comparison of the spectroscopic and electrochemical properties of the recombinant and mutant proteins with those of wild-type mavicyanin show that the characteristic properties of mavicyanin (a rhombic EPR signal, a similar CD spectra to plastocyanins and a positive shift of 187 mV for the midpoint potential) arise from the replacement of the fourth ligand (Met95 instead of Gln95; Kataoka *et al.*, 1998). A mutant of *Achromobacter cycloclastes* pseudoazurin in which the Met86 copper ligand has been replaced by Gln (Met86Gln) shows electronic absorption and CD spectra that are very similar to those of mavicyanin and stellacyanin. The EPR signal of the mutant pseudoazurin has an axial character, whilst mavicyanin and stellacyanin show rhombic signals (Kataoka *et al.*, 2000). The $E^{\circ\prime}$ of mavicyanin is found to be sensitive to two acid–base equilibria at the extremes of pH. One occurs below pH 4 and is related to protonation and detachment from the Cu^{I} centre of a histidine ligand. The other, which is observed above pH 8, causes a remarkable change in the electrostatic potential and/or the field strength around copper (Battistuzzi *et al.*, 2001). These findings reveal that the Cu site containing Gln might have a distorted trigonal geometry, showing distinctive redox properties in phytocyanins.

In this study, we have carried out a crystallographic study to establish the relationship between the spectroscopic and electrochemical properties and the three-dimensional structure of mavicyanin. In this paper, we report the crystallization and preliminary X-ray diffraction analyses of recombinant mavicyanin expressed in *E. coli*.

2. Crystallization of mavicyanin

Overexpression and purification of mavicyanin was carried out under previously reported conditions (Kataoka *et al.*, 1998). Purified mavicyanin was solubilized in 50 m*M* sodium cacodylate pH 7.0 and concentrated to 20 mg ml^{-1}. Crystallization of mavicyanin was performed using the hanging-drop vapour-diffusion method at 293 K. The crystallization conditions were initially screened by a sparse-matrix sampling method (Jancarik & Kim, 1991) using Crystal Screen I (Hampton Research). Each hanging droplet on a siliconized cover slip consisted of 2 µl protein solution (20 mg ml^{-1}) plus 2 µl of one of the precipitating reagents. The reservoir contained 0.5 ml of the same reagent. Of the 50 crystallization conditions instituted, crystallites appeared in tube #47 (2.0 *M* ammonium sulfate, 0.1 *M* sodium acetate trihydrate pH 4.6) within two weeks at 293 K. We then optimized the pH and the concentration of ammonium sulfate. Crystals of mavicyanin were obtained using a reservoir solution containing 2.25 *M* ammonium sulfate and 100 m*M* sodium cacodylate pH 5.5. Single crystals of mavicyanin with maximum dimensions of 0.4 × 0.4 × 0.7 mm appeared in the droplet after one week and were suitable for X-ray crystallographic studies (Fig. 1).

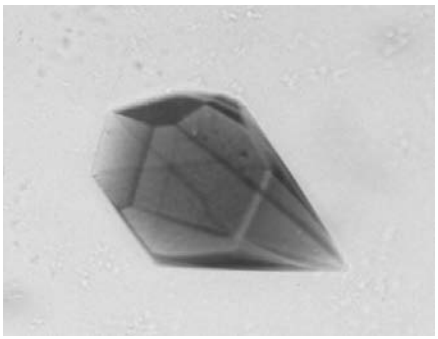

Figure 1
Typical crystal of mavicyanin, with maximum dimensions of 0.4 × 0.4 × 0.7 mm.

3. Data collection and processing

X-ray diffraction data from a single crystal of mavicyanin were collected at beamline 18B for structural biology using synchrotron radiation at the High Energy Accelerator Research Organization (KEK). A crystal of mavicyanin was mounted in a rayon loop in a stream of liquid nitrogen at 100 K. Prior to data collection, the crystal was soaked in a cryoprotectant solution consisting of 2.97 *M* ammonium sulfate, 100 m*M* sodium cacodylate pH 5.5 and 20%(*v*/*v*) glycerol. The diffraction patterns were recorded on a Fuji imaging plate using Sakabe's Weissenberg camera for macromolecules (Sakabe, 1991). The wavelength, camera distance, oscillation range and exposure time were 1.000 Å, 200 mm, 1.0° and 60 s, respectively. A complete data set was collected from 60 images, covering 60° in total (Fig. 2).

Diffraction intensity data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The mavicyanin crystals belonged to the hexagonal space group $P6_1$ (or $P6_5$), with unit-cell parameters a = 64.0, c = 245.0 Å. The resolution cutoff was defined so that 50% of reflections in the highest shell had $I > 1\sigma(I)$. From 314 972 accepted observations to 1.6 Å resolution, 37 768 independent reflections were obtained. The completeness of the data was 93.2%, with an overall $R_{\rm merge}$ of 3.8% (in the highest resolution shell, 1.66–1.60 Å resolution, the completeness was 81.3% and $R_{\rm merge}$ was 17.5%). The statistics

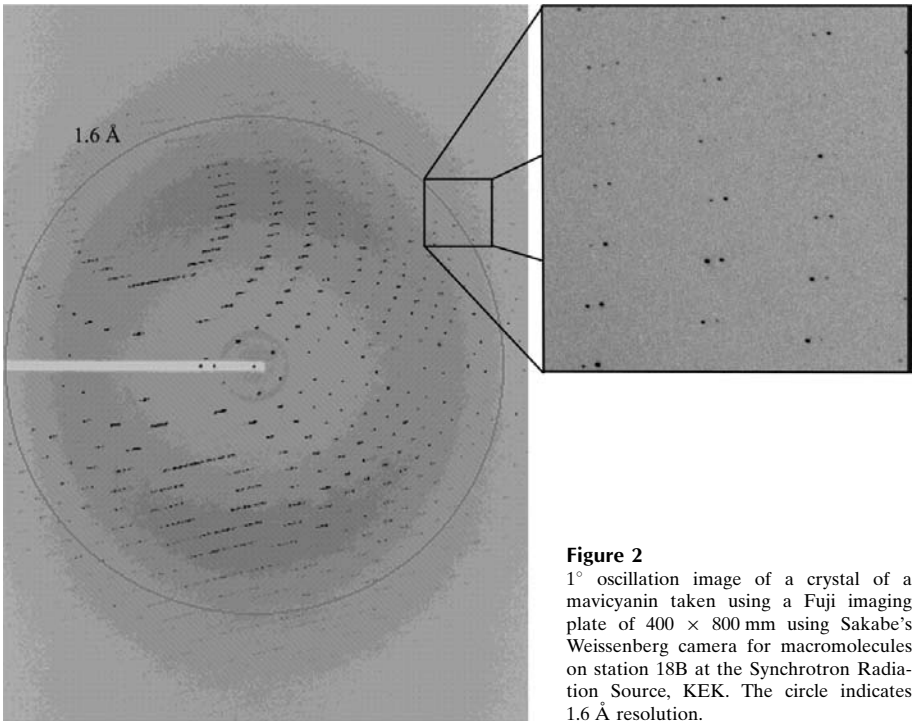

Figure 2
1° oscillation image of a crystal of a mavicyanin taken using a Fuji imaging plate of 400 × 800 mm using Sakabe's Weissenberg camera for macromolecules on station 18B at the Synchrotron Radiation Source, KEK. The circle indicates 1.6 Å resolution.

Table 1
Crystal parameters and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell (1.66–1.60 Å).

Crystal system	Hexagonal
Space group	$P6_1$ (or $P6_5$)
Unit-cell parameters (Å)	
a	64.0
c	245.0
Resolution range (Å)	40.0–1.60
No. of molecules per AU	4
V_M (Å^3 Da^{-1})	3.02
V_{solv} (%)	59
No. of measured reflections	314972
No. of unique reflections	37768
R_{merge}† (%)	3.8 (17.5)
Completeness (%)	93.2 (81.3)
Average $I/\sigma(I)$	13.9 (2.3)

† $R_{merge} = \sum |I(k) - I| / \sum I(k)$, where $I(k)$ is value of the kth measurement of the intensity of a reflection, I is the mean value of the intensity of that reflection and the summation is over all measurements.

of the diffraction data are shown in Table 1.

The self-rotation function calculated with the *POLARRFN* program from the *CCP4* program package (Collaborative Computational Project, Number 4, 1994) suggested that four mavicyanin molecules were correlated by two non-crystallographic twofold axes in the asymmetric unit, giving a Matthews coefficient of 3.02 Å^3 Da^{-1} and a solvent content of 59% (Fig. 3).

Preliminary molecular-replacement calculations were performed with the *AMoRe* program (Navaza, 1994) from the *CCP*4 program package using the structure of stellacyanin (PDB code 1jer) as a search model (Hart *et al.*, 1996). The highest correlation coefficients in the cross-rotation solutions were further examined using a translation search. However, no consistent solutions were obtained and a search for heavy-atom derivatives has been initiated to solve the structure. MAD data collection using Cu atoms is also in progress.

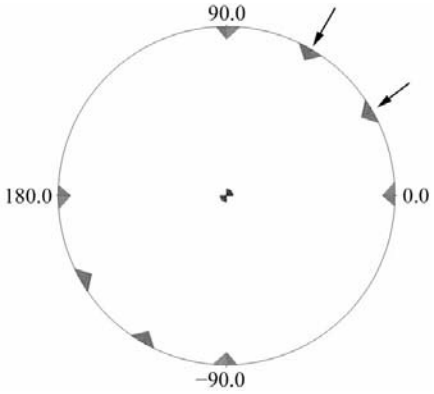

Figure 3
κ = 180.0° section of the self-rotation function calculated on a native data set from a mavicyanin crystal. The resolution of the normalized structure factors of the data used was 10.0–2.0 Å, with a radius of integration of 25 Å. The peaks shown by the two arrows correspond to the directions of the two non-crystallographic twofold axes of the dimer.

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