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A preliminary time-of-flight neutron diffraction study on amicyanin from *Paracoccus denitrificans*

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Crystals of the blue copper protein amicyanin suitable for neutron diffraction were grown by the sitting-drop method, followed by repeated macroseeding using solutions prepared with D_2O. Although the crystal sizes were the same, crystals grown using solutions made up in H_2O in the initial stages of macroseeding and solutions with D_2O in later stages did not diffract neutrons well. However, when the protein was initially exchanged with buffered D_2O and then crystallized and also macroseeded using solutions made up in D_2O throughout, the crystals diffracted neutrons to high resolution. One of those crystals was used to collect a data set to a resolution of 1.9 Å.

1. Introduction

Abundantly found in nature, blue copper proteins function as rapid long-range electron-transfer centers in biological systems (Guckert *et al.*, 1995). Amicyanin belongs to the Type I blue copper proteins, which contain a single copper site with two histidines, one cysteine and one methionine providing the copper ligands (Adman, 1991). The molecular weight of amicyanin is 11.5 kDa. In *Paracoccus denitrificans*, amicyanin plays a very important role as an obligatory intermediate in the transfer of electrons from methylamine dehydrogenase, a quinoprotein containing the prosthetic group tryptophan tryptophylquinone (TTQ), to *c*-type cytochromes (Husain & Davidson, 1985, 1986). Amicyanin is induced in this bacterium during growth on methylamine as a carbon source and is located in the periplasmic space (Husain & Davidson, 1985). Extensive X-ray crystallographic studies have been carried out on amicyanin, its binary complex with methylamine dehydrogenase and its ternary complex with methylamine dehydrogenase and cytochrome c_{551i} (Cunane *et al.*, 1996; Chen *et al.*, 1992, 1994). Single crystal polarized absorption spectroscopy studies have established that all of these complexes are functionally active in the crystalline state (Merli *et al.*, 1996; Ferrari *et al.*, 2004).

The mid-point potential (E_m) of free amicyanin in solution varies with pH (Zhu *et al.*, 1998). In the reduced state of amicyanin at pH 4.6, one of the ligands of Cu^{2+}, His95, rotates by 180° about the $C^{\beta}-C^{\gamma}$ bond relative to its position in the oxidized state (pH 4.8) and thereby moves away from the copper coordination sphere. It has been postulated that His95 is doubly protonated in the reduced state and singly protonated in the oxidized state (Zhu *et al.*, 1998). At pH 7.7, the reduced state of amicyanin exhibits dual conformations for the side chain of His95, one close to the oxidized state and the other close to the reduced state at the lower pH. There is a lengthening of the hydrogen bond between Cys92 S^{γ} and Asn54 N from 3.6 Å in the oxidized to 3.8 Å in the reduced structure. Furthermore, it has been possible to alter the E_m of amicyanin by site-directed mutagenesis, which appears to alter the hydrogen-bonding interactions between Cys92 and other residues in the metal-binding site (Carrell *et al.*, 2004). Thus, small differences in the hydrogen-bonding pattern and different protonation states of the residues around the copper site appear to significantly influence the electronic properties of amicyanin.

Although crystals of amicyanin diffracted X-rays to high resolution (1.3 Å) and extensive biophysical and structural studies have been made, the precise mechanisms by which the protein environment

tunes the redox potential and electron-transfer properties of the Type I copper site is not known and their study would greatly benefit from detailed information on the position of H atoms. This situation has led us to apply the neutron diffraction technique, the best available technique to locate the positions of H atoms accurately, to amicyanin. As a first step and as a feasibility study, neutron diffraction data were collected to 1.9 Å resolution from the oxidized state of amicyanin.

2. Crystallization and data collection

Amicyanin was purified from *P. denitrificans* cells and purified as described elsewhere (Husain & Davidson, 1985). In the original crystallization protocol (Lim *et al.*, 1986), amicyanin crystallized spontaneously by hanging-drop vapor diffusion using ammonium sulfate (pH 5–6) as a precipitant. Although these crystals were unsuitable for data collection, they could be used to prepare diffraction-quality crystals by repeated macroseeding from protein solution equilibrated against 3 *M* phosphate solution (and stored under mineral oil), even though spontaneous crystallization using phosphate as a precipitant could not be achieved. In the present case, blue-colored crystals of amicyanin were obtained by the sitting-drop method at room temperature. Initially, the protein was dialyzed against 5 m*M* sodium monobasic/potassium dibasic phosphate buffer pH ∼6.6. Scanning around the original crystallization condition was performed in the pH range 5–6 and the ammonium sulfate concentration range 1.6–3.2 *M* using an ammonium sulfate grid screen (Hampton Research, Aliso Vieja, CA, USA). The initial crystals were obtained from 2.4 *M* ammonium sulfate, 100 m*M* citric acid pH 5 by the vapor-diffusion method. The droplet (8 µl) was formed using a protein concentration of ∼16 mg ml^{-1} mixed with reservoir solution in a 1:1 proportion. The crystals formed within 3 d. The crystals grown in ammonium sulfate were used as seeds for subsequent macroseeding in phosphate solutions. Instead of crushing the crystals as originally suggested, macroseeding was adopted, as the initial crystals grown in ammonium sulfate were fairly large in size. The seeds were washed and transferred to a freshly prepared droplet (24 µl) formed of a 1:2 ratio of protein of concentration ∼16 mg ml^{-1} with 3 *M* sodium monobasic/potassium dibasic phosphate pH $\simeq$ 4.8 and in D_2O, pD $\simeq$ 4.8.[1]

Subsequently, the crystals were transferred repeatedly to newly prepared drops at least once per week. The current procedure did not use mineral oil as suggested in the original protocol. Crystals of up to ∼2 mm in size were obtained by repeated macroseeding.

Initially, the macroseeding was carried out by using buffer and salts prepared in H_2O. However, when the crystals size reached ∼0.45 mm, all subsequent macroseeding was carried out using solutions prepared in D_2O. Using D_2O solutions rather than H_2O in the crystallization allows accessible and labile H atoms in the protein to be replaced by D atoms. This increases the scattering power of the crystal for neutrons at medium resolution, as the negative scattering density of H atoms tends to cancel out the positive density of other atoms. It also reduces the amount of incoherent scattering from H atoms in the crystal, which tends to contribute to background scattering and reduces the signal-to-noise ratio in the diffraction signals. Three crystals of dimensions ∼2 × 1 × 1 mm grown by this method were very carefully mounted in a thin-walled quartz capillary tube (Hampton Research, Aliso Viejo, CA, USA) with deuterated mother liquor and their neutron diffraction quality was checked at the Protein Crystallography Station (PCS) at the neutron facility at Los Alamos Neutron Science Center (Langan *et al.*, 2004). The mounting was performed in a conventional manner, but care was taken to complete the mounting process in the shortest possible time in order to prevent back-exchange of H atoms. As D_2O is hygroscopic in nature, it can readily exchange with H_2O from the surrounding atmosphere through a diffusion process. All three crystals gave diffraction patterns and the best one diffracted to ∼6 Å resolution. The low-angle reflections were radially streaked. Thus, the poor diffraction quality primarily arises from a large mosaic spread, possibly a consequence of the stress produced by buffer mismatch. It may be concluded that both these factors became predominant when the crystal dimensions are large, as at various stages of crystal growth, both before and after the use of D_2O solutions, in order to ascertain the quality of the crystals, a good small crystal (∼0.1–0.2 mm in size) from the setup where crystals were growing was mounted and tested in the X-rays at the NE-CAT 8BM beamline at Advanced Photon Source, Argonne National Laboratory, Illinois, USA and every time it was found to diffract beyond 1.3 Å resolution.

At this point, the crystallization strategy was changed. Prior to crystallization, the amicyanin was first extensively exchanged with buffered D_2O to replace any solvent-exchangeable H atoms, ions and water molecules from the protein. Though the D_2O is the only source of deuterium, the repeated dilution in buffered D_2O followed by concentration by ultrafiltration will ensure the H to D exchange. The crystals of amicyanin were then grown using solutions prepared only in D_2O. The initial crystals were obtained by replicating the successful condition obtained from the earlier ammonium sulfate fine grid screen, but in D_2O. The pD of the 5 m*M* phosphate buffer used for dialysis was ∼7.2, while that of the 3 *M* phosphate solution used for reservoir was ∼4.8. Repeated macroseeding, similar to the procedure described above, yielded crystals of dimensions 2 × 1.3 × 1 mm in approximately five months (Fig. 1).

Again, six crystals were mounted in quartz capillaries by an identical procedure to that used in the previous attempt and their neutron diffraction quality was checked at the PCS. This time, two crystals were found to diffract to 1.9 Å. The crystal which produced the best reflection profiles was used for full data collection. Data were collected for a quadrant of reciprocal space by scanning the crystal around its spindle axis at various κ and ω settings of the goniometer as described previously (Hanson *et al.*, 2004; Li *et al.*, 2004; Langan & Greene, 2004). Wavelength-resolved Laue patterns were collected at 32 different settings at room temperature over a period of three weeks of beam time. The distance from the sample to the center of cylindrical detector was 70 cm.

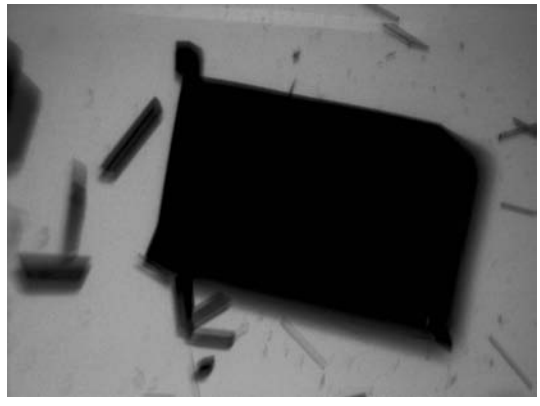

Figure 1
Crystals of amicyanin; the largest has dimensions of 2 × 1.3 × 1 mm.

[1] The pD of deuterated buffers was measured using a standard pH meter and the value of pD is obtained by adding 0.4 to the observed pH value (Glasoe & Long, 1960).

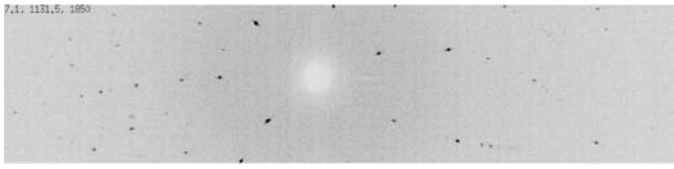

Figure 2
Neutron Laue diffraction data from a crystal setting for amicyanin.

Table 1
Preliminary data and data-collection parameters for amicyanin.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 28.5, b = 55.9, c = 27.2, β = 95.6
Wavelength range (Å)	1.1–5
Crystal settings	32
$R_{\rm merge}$ (%)	11.6
No. of measured reflections	11808
No. of unique reflections	5075

Table 2
$R_{\rm merge}$, completeness (%Comp), $I/\sigma(I)$ and $\langle I/S_{\rm A}\rangle$ *versus* resolution for amicyanin.

The cumulative $R_{\rm merge}$ and completeness are also given (Cum$R_{\rm merge}$ and Cum%Comp, respectively). Only reflections with $I/\sigma(I) > 1.5$ are included in the above terms. $S_{\rm A}$ corresponds to the standard deviation in merging reflections as opposed to $\sigma(I)$, the error involved in measuring the individual intensities.

Resolution	$R_{\rm merge}$	Cum$R_{\rm merge}$	%Comp	Cum%Comp	$I/\sigma(I)$	$\langle I/S_{\rm A}\rangle$
∞–5.69	0.046	0.046	91.8	91.8	10.7	24.4
5.69–4.02	0.065	0.058	87.7	89.1	9.4	19.9
4.02–3.29	0.079	0.066	84.2	86.9	8.9	13.7
3.29–2.85	0.238	0.078	80.8	84.7	5.1	7.0
2.85–2.55	0.190	0.089	75.7	82.2	3.8	4.2
2.55–2.32	0.204	0.098	69.0	79.0	3.4	3.0
2.32–2.15	0.208	0.104	62.0	75.5	3.4	2.4
2.15–2.01	0.257	0.111	56.0	71.9	2.7	1.8
2.01–1.90	0.267	0.116	48.8	68.2	2.6	1.5
Overall	0.116	0.116	68.2	68.2	5.7	6.5

3. Results and discussion

Fig. 2 illustrates a typical neutron diffraction picture for amicyanin. The image shows an overlay of all time intervals for a particular setting and corresponding to a conventional Laue pattern. The preliminary data and the data-collection parameters are summarized in Table 1.

Data in the wavelength range 1.1–5 Å were processed using a version of *d*TREK* modified for wavelength-resolved neutron Laue protein crystallography (Langan & Greene, 2004). The processed intensities were then scaled and wavelength-normalized using *LAUENORM* (Helliwell *et al.*, 1989). Although the diffraction spots near the beam center were slightly smeared by mosaicity, this did not affect the data processing. In order to obtain a reasonable value of $R_{\rm merge}$, the wavelength range was restricted to 1.1–5 Å and only reflections with $I > 3\sigma(I)$ were used in determining the wavelength-scaling normalization curve. Reflections in this wavelength range were binned into 16 wavelength intervals and the normalization curve was determined from a Chebyshev polynomial of order 5 (Arzt *et al.*, 1999). The *LAUENORM* data were then output in unmerged form so that *SCALA* could be used for statistical analysis (Collaborative Computational Project, Number 4, 1994; Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997; Weiss, 2001). The X-ray data statistics for each individual shell are listed in Table 2.

The high-resolution 1.3 Å X-ray structure of amicyanin (Cunane *et al.*, 1996) was used directly for positional refinement procedures against neutron data using *CNS* (Brünger *et al.*, 1998). Currently, the refinement of the structure is in progress.

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