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Crystallization and preliminary X-ray diffraction analysis of a complex between the electron-transfer partners hexameric Cu-containing nitrite reductase and pseudoazurin

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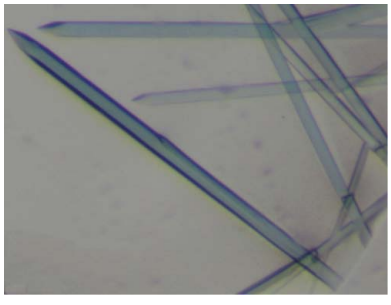
The complex between Cu-containing nitrite reductase (HdNIR) and its electron-donor protein pseudoazurin (HdPAz) from *Hyphomicrobium denitrificans* has been crystallized. The crystals were obtained from a mixture of the two proteins using the hanging-drop vapour-diffusion method in the presence of polyethylene glycol (PEG) and 2-methyl-2,4-pentanediol (MPD) as precipitants. SDS–PAGE analysis demonstrated that the crystals contained both proteins. The X-ray diffraction experiment was carried out at SPring-8 and diffraction data were collected to 3.3 Å resolution. The crystals were tetragonal (space group $P4_12_12$), with unit-cell parameters $a = b = 130.39$, $c = 505.55$ Å. Preliminary analysis indicated that there was one HdNIR and at least two HdPAz molecules in the asymmetric unit of the crystal.

1. Introduction

Long-range electron-transfer (ET) reactions between ET proteins and redox enzymes are involved in energy-producing biological processes such as photosynthesis and respiration. Specific interactions between these proteins are critical for the adequate orientation of their redox centres and to prevent incorrect electron flows, although most soluble redox proteins are considered to make weak associations with each other. However, relatively few examples of ET partner complexes have been crystallized and structurally characterized. Four classes of ET proteins, cytochromes *c*, ET flavoproteins, ferredoxins and cupredoxins, have been structurally characterized as complexes with partner enzymes. In the case of cupredoxins, there have only been three structural reports: binary complexes of azurin with the quinoprotein aromatic amine dehydrogenase (Sukumar *et al.*, 2006) and of amicyanin with methylamine dehydrogenase (Chen *et al.*, 1992) and a ternary complex between amicyanin, methylamine dehydrogenase and cytochrome c_{551i} (Chen *et al.*, 1994). Structural information is important for understanding ET mechanisms and the features of the specific protein–protein interactions.

Denitrification (anaerobic nitrate respiration) is the dissimilatory reduction of nitrate or nitrite usually to dinitrogen in prokaryotic organisms; namely, $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. In these processes, one-electron reduction of NO_2^- to NO, which is catalyzed by nitrite reductase (NIR), is a key step in the denitrification process; this is the first step leading to the gaseous products NO, N_2O or N_2. Cu-containing NIRs (CuNIRs) have a trimeric structure in which a monomer (~35 kDa) contains one type 1 Cu and one type 2 Cu (Adman *et al.*, 1995; Dodd *et al.*, 1997; Inoue *et al.*, 1998; Murphy *et al.*, 1997). The type 1 Cu atom binds to one of the two β-barrel domains in the monomer by coordinating to four amino-acid residues (two His, one Cys and one Met). The type 2 Cu atom, which is coordinated by three His residues, is located at the interface between two adjacent monomers. The distance between the two Cu atoms connected through the sequence segment Cys-His is about 12.5 Å. The type 1 Cu site relays an electron from the electron-donor protein to the type 2 Cu site, where NO_2^- is reduced to NO.

Pseudoazurin (PAz) is a cupredoxin that is found in denitrifying bacteria and methylotrophs. PAz consists of eight β-strands forming a



β-barrel and two helices at the C-terminus. The four ligands, like those in CuNIR, coordinate the type 1 Cu with distorted tetrahedral geometry (Petratos *et al.*, 1987; Williams *et al.*, 1995; Inoue *et al.*, 1999). PAz is an electron donor for CuNIRs in denitrifying bacteria. The ET reaction and the interaction between CuNIR and PAz have been studied extensively. An electrostatic interaction between the basic amino-acid residues of PAz from *Alcaligenes faecalis* and the acidic amino-acid residues of its cognate CuNIR has been proposed from X-ray crystal structure analyses, kinetics (Kukimoto *et al.*, 1995) and NMR measurements (Impagliazzo *et al.*, 2005, 2007). Although the structure of the complex between CuNIR and PAz from *A. faecalis* has been studied using paramagnetic NMR spectroscopy (Vlasie *et al.*, 2008), no crystal structure of the CuNIR–PAz complex is yet available.

Recently, we have reported the structural and functional characterization of CuNIR (HdNIR) and its electron-donor protein PAz (HdPAz) from *Hyphomicrobium denitrificans* A3151 (Yamaguchi *et al.*, 2004; Nojiri *et al.*, 2007; Hira *et al.*, 2007). HdNIR is composed of a 15 kDa N-terminal cupredoxin domain and a 35 kDa C-terminal CuNIR domain with one type 1 Cu and one type 2 Cu as in common CuNIRs. The HdNIR molecule shows a trigonal prismatic shape, in which a monomer containing three Cu atoms is organized into a unique hexamer (*i.e.* a tightly associated dimer of trimers). Based on the proposed complex between CuNIR and PAz from *A. faecalis* (Vlasie *et al.*, 2008), the possible 'PAz-binding site' in the C-terminal domain of HdNIR is covered by the N-terminal domain. Therefore, we have undertaken crystal structural analysis in order to elucidate the ET reaction mechanism and the interaction between HdNIR and HdPAz. In this paper, we report the crystallization and preliminary X-ray diffraction analysis of the complex of HdNIR with HdPAz.

2. Materials and methods

2.1. Protein purification

The proteins HdNIR and HdPAz were purified according to previously described procedures (Deligeer *et al.*, 2002; Hira *et al.*, 2007). The concentrations of the two proteins were determined from the absorbances of oxidized HdNIR at 605 nm (ε = 6300 M^{-1} cm^{-1}) and of oxidized HdPAz at 585 nm (ε = 2200 M^{-1} cm^{-1}) at pH 6.0. Purified HdNIR and HdPAz were concentrated to 20 and 45 mg ml^{-1}, respectively, in 10 m*M* potassium phosphate buffer pH 6.0. The samples were plunged into liquid nitrogen and then stored at 193 K.

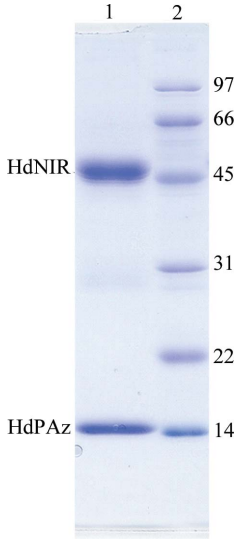


Figure 1
SDS–PAGE profile of the crystals obtained from Crystal Screen II condition No. 30 [10%(*v*/*v*) MPD, 10%(*w*/*v*) PEG 6000, 0.1 *M* HEPES pH 7.5]. Lane 1, dissolved crystals from condition No. 30. Lane 2, molecular-weight markers; the size of each protein (in kDa) is shown on the right.

2.2. Crystallization of the complex of HdNIR with HdPAz

Crystallization trials were performed using the hanging-drop vapour-diffusion method at 277 K and the crystallization conditions were initially screened using Hampton Research Crystal Screens I and II and PEG/Ion Screens. Each drop was composed of 1.0 µl of a mixture of HdNIR and HdPAz and 1.0 µl reservoir solution and was equilibrated against 300 µl reservoir solution. To screen a wide range of conditions, the composition of the protein mixture was varied using molar ratios (HdNIR:HdPAz) of 1:3 (18:3.0 mg ml^{-1}), 1:20 (10:11 mg ml^{-1}), 1:20 (5.0:5.5 mg ml^{-1}) and 1:40 (10:22 mg ml^{-1}). Rod-shaped crystallites appeared in several conditions, *e.g.* Crystal Screen I conditions No. 14 [0.2 *M* calcium chloride, 28%(*w*/*v*) PEG 400, 0.1 *M* HEPES pH 7.5] and No. 28 [0.2 *M* sodium acetate, 30%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5] and Crystal Screen II conditions No. 30 [10%(*v*/*v*) MPD, 10%(*w*/*v*) PEG 6000, 0.1 *M* HEPES pH 7.5] and No. 46 [0.1 *M* sodium chloride, 20%(*v*/*v*) PEG MME 550, 0.1 *M* Bicine pH 9.0]. The solutions obtained from dissolution of the crystals were analyzed by SDS–PAGE; the crystals were first rinsed with reservoir solution.

2.3. Preliminary X-ray diffraction analysis of the complex crystal

Single crystals were picked up using nylon loops (Hampton Research) and then cryoprotected in crystallization solution with 20% cryoprotectant. They were frozen and stored in liquid nitrogen. The crystals were unstable when transferred to high concentrations of cryoprotectant solution, resulting in an unbearably high mosaicity. Therefore, after testing several cryoprotectants such as glycerol, ethylene glycol, PEG 400, sucrose and DMSO, MPD was finally chosen as the best cryoprotectant solution [5%(*w*/*v*) PEG 4000, 30%(*v*/*v*) MPD, 50 m*M* HEPES pH 7.5] and was added by overnight dialysis using a dialysis button (Hampton Research). Diffraction data were collected using a Bruker DIP-6040 imaging-plate detector on beamline 44XU at SPring-8 (Japan Synchrotron Radiation Research Institute). The crystals were maintained at 100 K during data collection. The crystal-to-detector distance was set to 650 mm. A total

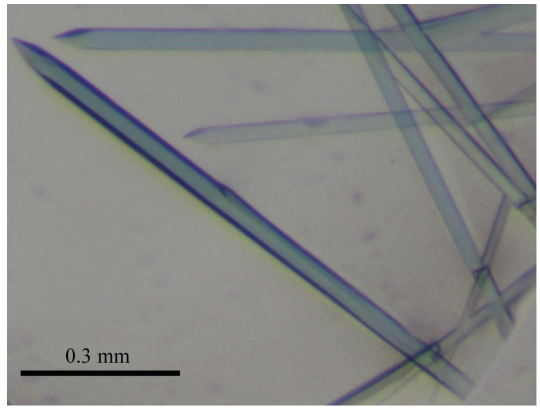


Figure 2
Crystals of the complex between HdNIR and HdPAz. The crystals were estimated to be approximately 1.0 mm in their longest dimension. Most of the crystals have a similar oblong shape.

of 180 images were collected with 0.5° oscillations and an exposure time of 1 s per image. The diffraction data were processed with the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results

SDS–PAGE analyses of the solution obtained from dissolution of the crystals demonstrated that both HdNIR and HdPAz molecules were contained in the crystals from Crystal Screen II condition No. 30 (Fig. 1). The crystals obtained from the other conditions were composed of HdNIR or HdPAz only. Several rounds of optimization of the initial crystallization condition were set up to produce optimally sized crystals. The best crystallization condition used a solution of HdNIR and HdPAz in a 1:20 molar ratio (HdNIR:HdPAz, 10:11 mg ml^{-1}) and a reservoir solution containing 8%(*w*/*v*) PEG 4000, 9%(*v*/*v*) MPD and 50 m*M* HEPES pH 7.5. Crystals suitable for X-ray data collection appeared within a week (Fig. 2).

Crystals of the complex diffracted to 3.30 Å resolution (Fig. 3). The reflection conditions 00*l*, *l* = 4*n* and *h*00, *h* = 2*n* identified the crystals as belonging to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$, with unit-cell parameters *a* = *b* = 130.39, *c* = 505.55 Å. Crystal and data-processing statistics are summarized in Table 1. Considering the molecular weights of hexameric HdNIR (289.0 kDa) and HdPAz (13.6 kDa), the calculated V_M values (Matthews, 1968) were 3.72, 3.55 and 3.40 Å^3 Da^{-1}, corresponding to solvent contents of 66.9, 65.4 and 63.8%, respectively, assuming the presence of one hexameric HdNIR molecule only, one HdNIR and one HdPAz molecule, and one HdNIR and two HdPAz molecules in the asymmetric unit, respectively.

Molecular replacement with the structure of HdNIR (PDB code 2dv6) as a search model was performed using *Phaser* (Read, 2001), which gave a log-likelihood gain (LLG) of 16 373 for $P4_12_12$ (after rigid-body refinement). This value was significantly higher than that of the solution for $P4_32_12$, which had an LLG of 3545. We have presently positioned one hexameric HdNIR molecule in the asymmetric unit in space group $P4_12_12$ (using data in the resolution range 50–3.3 Å; R_{work} = 30.8%). The interpretable electron densities of at least two HdPAz molecules were observed in the difference Fourier $2F_o - F_c$ and $F_o - F_c$ maps calculated using phases from the partial refinement of HdNIR. Refinement using *CNS* (Brünger *et al.*, 1998) and manual rebuilding of the HdNIR–HdPAz complex model using *XtalView* (McRee, 1992) are in progress.

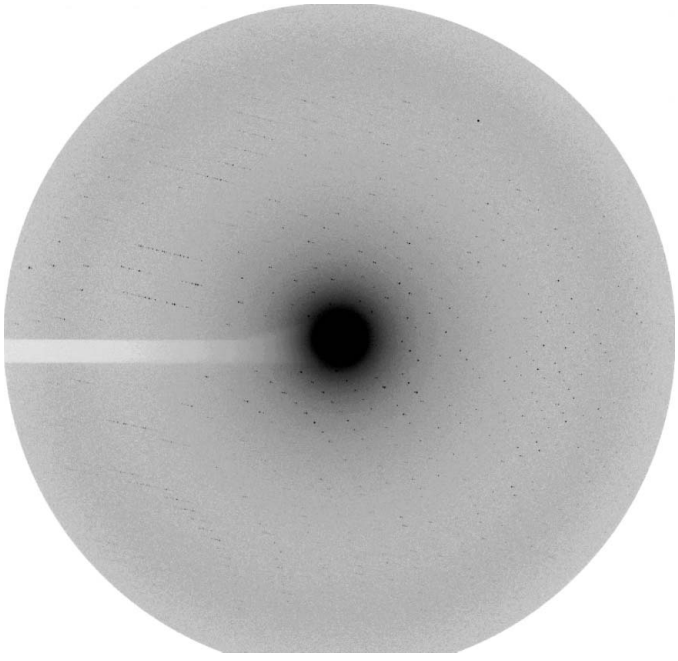


Figure 3
A typical diffraction pattern of the complex crystals using a 0.5° rotation oscillation. The edge of the detector corresponds to a resolution of 3.0 Å. The direct beam was stopped in every image by using a beam-stop during the experiment. The shadow of the beam-stop is missing at the centre as a consequence of the increased background intensity used to clearly visualize the diffraction spots.

Table 1
Crystal parameters and data-processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9
Space group	$P4_12_12$
Unit-cell parameters (Å)	*a* = *b* = 130.39, *c* = 505.55
Resolution (Å)	50.00–3.30 (3.42–3.30)
No. of observed reflections	969689
No. of unique reflections	66848 (6553)
Completeness (%)	99.8 (99.9)
$\langle I/\sigma(I)\rangle$	11.4 (3.9)
R_{merge}†	17.4 (49.8)
Redundancy	6.0 (5.7)
Mosaicity (°)	0.18

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations *i* of reflection *hkl*.

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