Crystallization and preliminary X-ray crystallographic studies of dissimilatory nitrite reductase isolated from *Hyphomicrobium denitrificans* A3151

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Dissimilatory nitrite reductase isolated from *Hyphomicrobium denitrificans* A3151 (*Hd*NIR) is a novel copper-containing nitrite reductase (CuNIR) composed of six identical subunits. One plastocyanin-like domain and one green CuNIR-like domain are connected to each other, suggesting that the *Hd*NIR subunit structure resembles a complex of green CuNIR and pseudoazurin (or azurin). Recombinant *Hd*NIR protein was crystallized using the hanging-drop vapour-diffusion method with PEG 4000 as the precipitant at pH 8.9. X-ray diffraction data were collected to 2.35 Å resolution. The *Hd*NIR crystal belonged to the tetragonal space group $P4_1$ (or $P4_3$), with unit-cell parameters $a = b = 221.9$, $c = 165.2$ Å, giving 12 molecules (two hexamers) per asymmetric unit and a solvent content of 64%. A mutant form of *Hd*NIR, C260A, which lacks the type I copper ion in the CuNIR-like domain, was prepared and crystallized under wild-type *Hd*NIR conditions. The C260A mutant crystal belonged to the cubic space group $P4_332$ (or $P4_132$), with unit-cell parameters $a = b = c = 153.7$ Å, giving one molecule per asymmetric unit and a solvent content of 59%. X-ray diffraction data were collected to 3.5 Å resolution. To solve the crystal structure of *Hd*NIR, the multiwavelength anomalous dispersion (MAD) method and the molecular-replacement method are currently being used.

1. Introduction

Dissimilatory nitrite reductase, which utilizes a Cu ion redox-activity center (CuNIR), is a key enzyme catalyzing the one-electron reduction of nitrite (NO_2^-) to nitric oxide (NO) in the denitrification process, leading to a significant loss of fixed nitrogen from the terrestrial environment (Payne, 1985). CuNIRs have a strong band near 600 nm arising from (Cys) S→Cu^{II} charge transfer, which is characteristic of a type I Cu site. The ratio of the intensity of this band to a second charge-transfer absorption band near 460 nm determines whether a CuNIR is blue or green in colour. Therefore, CuNIRs are usually categorized into two subgroups: blue CuNIRs and green CuNIRs (Han *et al.*, 1993). At present, the molecular structures of the green CuNIR from *Achromobacter cycloclastes* (*Ac*NIR; Godden *et al.*, 1991; Adman *et al.*, 1995), the green CuNIR from *Alcaligenes faecalis* (*Af*NIR; Kukimoto *et al.*, 1994; Murphy *et al.*, 1997) and the blue CuNIR from *Alcaligenes xylosoxidans* (*Ax*NIR; Dodd *et al.*, 1997; Inoue *et al.*, 1998) have been determined. These CuNIRs are homotrimers, in which a monomer (∼35 kDa) contains one type I Cu and one type II Cu site. These CuNIRs show more than 60% sequence similarity and their crystal structures are very similar to each other. Intramolecular electron transfer from the type I Cu site to the type II Cu site that occurs at pH 6.0 in the absence or presence of nitrite was observed by pulse radiolysis in both *Ac*NIR and *Ax*NIR (Suzuki *et al.*, 1994; Kobayashi *et al.*, 1999). The type I Cu site accepts one electron from an external electron-transfer protein and transfers it to the type II Cu site, which is bound by three His residues and one water molecule in a tetrahedral geometry that constitutes the nitrite-reduction centre. The high-resolution crystal structure of a type II copper–nitrosyl complex of *Af*NIR reveals an unprecedented side-on binding mode in which the N and O atoms are nearly equidistant from the copper cofactor. Comparison of this structure with that of a nitrite-bound form of CuNIR revealed the coordination change of the type II Cu ion between copper–oxygen and copper–nitrogen during catalysis (Tocheva *et al.*, 2004). CuNIR accepts an electron donated by its redox partner, an electron-transfer protein such as cytochrome c_{550} or cytochrome c_{552} and azurin or pseudoazurin, to accomplish one-electron reduction of NO_2^- (Miyata & Mori, 1969; Kakutani *et al.*, 1981; Liu *et al.*, 1986; Zumft *et al.*, 1987, Dodd *et al.*, 1995). There is a striking difference in the overall surface-charge distribution between blue and green CuNIRs, providing a neat structural explanation for their different reactivities to pseudoazurin or

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azurin and supporting the view that electron transfer proceeds *via* complex formation (Dodd *et al.*, 1998). The first comprehensive electron-donation experiments from the redox partners pseudoazurin or azurin to two types of CuNIRs proved that pseudoazurin can readily donate electrons to both blue and green CuNIRs. In contrast, azurins are very sluggish electron donors with respect to the green CuNIR. On the basis of the results, which were discussed in terms of the surface compatibility of the component proteins, complex formation, overall charge distribution, hydrophobic patches and redox potentials, one docking model of the *Af*NIR–pseudoazurin complex and one docking model of the *Ax*NIR–azurin complex were proposed to show the interactions between the CuNIR and its redox partner (Murphy *et al.*, 2002). However, the structure of the complex has still not been determined. How CuNIR accepts a donated electron from pseudoazurin (or azurin) to accomplish conversion of NO_2^- to NO is not known.

In contrast to these CuNIRs, a novel CuNIR from *Hyphomicrobium denitrificans* A3151 (*Hd*NIR) reveals some distinctive properties, as this CuNIR is composed of six identical subunits, which contain a polypeptide chain of 447 amino-acid residues ($\sim$50 kDa), two type I copper ions and one type II copper ion (Deligeer *et al.*, 2002). The intramolecular electron-transfer reaction from the type I Cu to the type II Cu is not observed at pH 6.0 in the absence of nitrite. Studies of the *Hd*NIR gene found two type I Cu ligand motifs and one type II Cu ligand motif, suggesting the existence of an extra type I Cu site in addition to the coupled type I Cu and type II Cu sites present in common CuNIRs (K. Kataoka, A. Fukui, M. Kabayashi, K. Yamaguchi & S.Suzuki, unpublished work; DDBJ accession No. AB076606). Moreover, the N-terminal 92 amino-acid sequence (40–131) is 32% identical to plastocyanin from green alga (Merchant *et al.*, 1990) and the C-terminal 284 amino-acid sequence (164–447) is 35–38% identical to common CuNIRs (Suzuki *et al.*, 1999). To elucidate the domain structure and the coordination centres of two type I Cu motifs, *Hd*NIR was proteolyzed to two protein fragments: an N-terminal fragment ($\sim$14 kDa) and a C-terminal fragment ($\sim$35 kDa). The spectroscopic properties of the solutions of the two fragments revealed that the N-terminal fragment has only one type I Cu site with an axially elongated bipyramidal geometry and the C-terminal fragment has one type II Cu site and one type I Cu site with a flattened

tetrahedral geometry. The C-terminal fragment can accept one electron from cytochrome c_{550} and exhibits a larger nitrite-reduction activity than the common CuNIRs, suggesting that the C-terminal domain is the essential region for the intermolecular electron-transfer and catalytic reactions of *Hd*NIR. The absorption spectrum of the C-terminal 35 kDa fragment of *Hd*NIR has two peaks at 454 and 597 nm and a shoulder near 700 nm and has a green colour resembling that of *Ac*NIR (Suzuki *et al.*, 1997). These results imply that the structure of *Hd*NIR may resemble the proposed complex of green CuNIR and pseudoazurin or azurin (Yamaguchi *et al.*, 2003).

Preparation and characterization of the C114A mutant lacking the type I copper ion in the N-terminal domain and the C260A mutant lacking the type I copper ion in the C-terminal domain also proved that the colours of the type I Cu ions in the N- and C-terminal domains are blue and green, respectively. The C114A mutant shows catalytic activity, but the C260A mutant shows hardly any nitrite-reduction activity. The cognate cytochrome c_{550} functions as an electron donor to the C114A mutant, not the C260A mutant, proving that the electron-acceptor site of *Hd*NIR is type I Cu in the C-terminal domain is coupled with type II Cu and not type I Cu in the N-terminal domain (Suzuki *et al.*, 2003). On the basis of the results of previous studies, the N-terminal domain should be proposed as the electron-donating partner of the C-terminal domain. However, whether the type I copper ion in the N-terminal domain directly donates an electron to the type I copper or the type II copper ion in the C-terminal domain has not yet been elucidated. In this

work, crystallographic studies were performed to show the relationship between the function and structure of *Hd*NIR. The interactions between the N- and C-terminal domains of *Hd*NIR may give some information to help understand the interactions between CuNIR and its electron-transfer partner, pseudoazurin or azurin. In this paper, we report the results from crystallographic studies of *Hd*NIR and the C260A mutant.

2. Experimental

2.1. Crystallization of wild-type and the C260A mutant of *Hd*NIR

Overexpression and purification of *Hd*NIR was carried out using previously reported conditions (Deligeer *et al.*, 2002). Purified protein was solubilized in 20 m*M* Tris–HCl pH 7.5 and was concentrated to 20 mg ml^{-1}. The crystallization conditions were initially screened using a sparse-matrix sampling method (Jancarik & Kim, 1991) with Crystal Screen (Hampton Research). Each hanging droplet on a siliconized cover slip consisted of 2 µl protein solution plus 2 µl of one of the precipitating reagents. The reservoir contained 0.5 ml of the same reagent. Of the 50 crystallization conditions tested, crystallites appeared in tubes 6 [0.2 *M* magnesium chloride hexahydrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w/w*) PEG 4000], 17 [0.2 *M* lithium sulfate monohydrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w/w*) PEG 4000], 22 [0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w/w*) PEG 4000] and 41 [0.1 *M* Na HEPES pH 7.5, 10%(*v/v*) 2-propanol, 20% (*w/w*) PEG 4000] within 24 h at 293 K. The crystallization conditions were then optimized. Crystals of
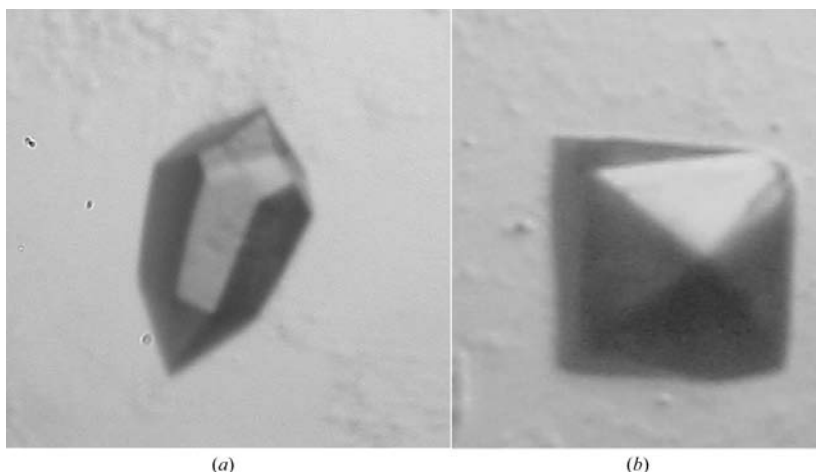

Figure 1
(*a*) A typical crystal of wild-type *Hd*NIR, with approximate dimensions 0.2 × 0.2 × 0.3 mm; (*b*) a typical crystal of the C260A mutant, showing the tetragonal-like pyramid, with approximate dimensions 0.15 × 0.15 × 0.15 mm.

HdNIR were obtained using a reservoir solution containing 18%(*w/w*) PEG 4000 and 100 m*M* Tris–HCl pH 8.9 after 3 d at 293 K. Preparation of the C260A mutant was performed under the conditions reported previously (Suzuki *et al.*, 2003) and it was crystallized using the same crystallization conditions as those used for wild-type HdNIR. Crystals of C260A mutant appeared in the droplet after 3 d. These crystals were suitable for X-ray crystallographic studies (Fig. 1).

2.2. Diffraction data collection and processing

X-ray diffraction data from a crystal of wild-type HdNIR were collected using beamline BL41XU, SPring-8. The diffraction images were recorded using a MAR CCD 165 area detector (MAR Research, Gemany). A crystal of HdNIR was mounted on a rayon loop in a stream of gaseous nitrogen at 100 K. Prior to data collection, the crystal was soaked for up to 20 s in cryoprotectant solution consisting of 20%(*w/w*) PEG 4000, 0.1 *M* Tris–HCl pH 8.9 and 10%(*v/v*) 2-methyl-2,4-pentanediol. The wavelength, camera distance, oscillation range and exposure time were 0.9793 Å, 175 mm, 0.8° and 8 s, respectively. One data set was collected from 200 images covering 160° in total.

X-ray diffraction data for a crystal of the C260A mutant were collected using beamline BL40B2, SPring-8 at 100 K. The diffraction images were recorded using a Quantum 4 CCD detector (ADSC, USA). Prior to data collection, the crystal was soaked in the cryoprotectant solution described above. The wavelength, camera distance, oscillation range and exposure

time were 1.000 Å, 270 mm, 1.0° and 10 s, respectively. One data set was collected from 90 images covering 90° in total.

X-ray diffraction intensity data were indexed, scaled and merged using the programs *MOSFLM* (v.6.2.3; Leslie, 1992), *SCALA* and *TRUNCATE* from the *CCP*4 (Collaborative Computational Project, Number 4, 1994) program package.
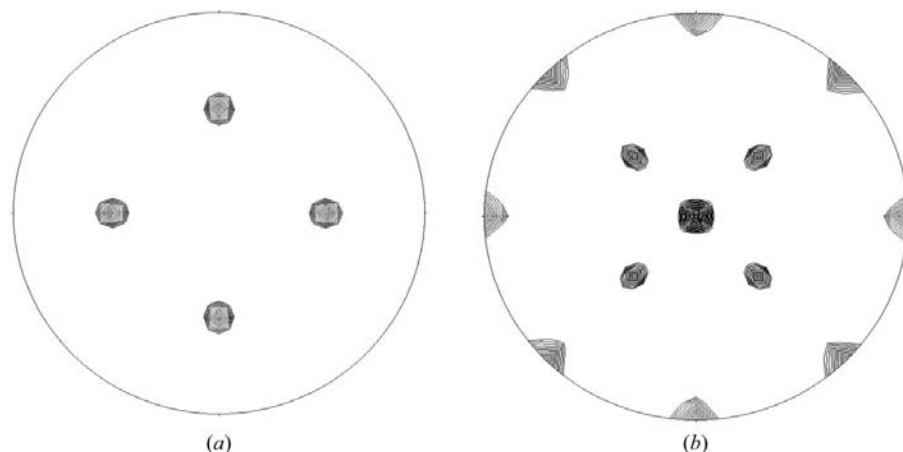
3. Results and discussion

The crystallographic data and statistics of the diffraction data for wild-type HdNIR and its C260A mutant are summarized in Table 1. The self-rotation function (Fig. 2) calculated using the *POLARRFN* program from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) suggested that two hexamers (12 molecules) were correlated by two non-

crystallographic twofold axes and one threefold axis in the asymmetrical unit. The two twofold axes are found at $\varphi = 0°$ and $\varphi = 90°$ in the $\kappa = 180°$ section, suggesting that the Laue symmetry of the wild-type crystal is P4/*mmm*. However, processing the X-ray diffraction data in P4/*mmm* gives an overall R_{merge} of 48.6%. On the other hand, processing the X-ray diffraction data in P4/*m* gives an overall R_{merge} of 4.0%. Moreover, results calculated by the *HKLPLOT* program (Collaborative Computational Project, Number 4, 1994) also suggest that the crystal belongs to space group $P4_1$ (or $P4_3$). There are 12 molecules (two hexamers, one hexamer being constructed from two trimers) in the asymmetric unit, giving a Matthews coefficient of 3.3 Å^3 Da^{-1} and a solvent content of 64%. To solve the crystal structure of HdNIR, analysis using the multiwavelength anomalous dispersion (MAD) method and the molecular-replacement method is currently in progress.

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

Values in parentheses are for the highest resolution shell.

	Wild type	C260A mutant
X-ray source	BL41XU, SPring-8	BL40B2, SPring-8
Wavelength (Å)	0.9793	1.0000
Crystal system	Tetragonal	Cubic
Space group	$P4_1$ (or $P4_3$)	$P4_332$ (or $P4_132$)
Unit-cell parameters (Å)		
a	221.9	153.7
c	165.2	
No. molecules per AU	12 (two hexamers)	1
V_M (Å^3 Da^{-1})	3.3	3.1
V_{solv} (%)	64	59
Resolution range (Å)	34.3–2.35 (2.48–2.35)	54.2–3.50 (3.69–3.50)
No. measured reflections	647798	165097
No. unique reflections	314385	15557
R_{merge} (%)†	4.0 (21.7)	8.2 (35.2)
Completeness (%)	95.0 (85.1)	100.0 (100.0)
Average $I/\sigma(I)$	6.4 (2.2)	7.9 (2.2)

† $R_{merge} = \sum_h \sum_i |I(h, i) - \langle I(h) \rangle| / \sum_h \sum_i I(h, i)$, where $I(h, i)$ is the intensity of the *i*th measurement of reflection h and $\langle I(h) \rangle$ is the mean value of $I(h, i)$ for all i measurements.

(a)   *(b)*

Figure 2
A plot of the self-rotation function calculated using wild-type HdNIR data in the resolution range 15–4 Å with a 20 Å radius of integration in (*a*) the $\kappa = 120°$ section and (*b*) the $\kappa = 180°$ section. These results suggest two non-crystallographic twofold axes and one threefold axis in the asymmetrical unit of the wild-type HdNIR crystal.

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