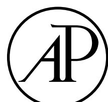

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Characterization of two Cu-containing protein fragments obtained by limited proteolysis of *Hyphomicrobium denitrificans* A3151 nitrite reductase☆

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Abstract

The unusual *Hyphomicrobium denitrificans* nitrite reductase containing two type 1 Cu sites and one type 2 Cu site (MW, 50 kDa) has been proteolyzed to two protein fragments (14 and 35 kDa) with subtilisin. The visible absorption, CD, and EPR spectra of these proteins imply that the blue 14-kDa protein fragment has one type 1 Cu site, which is axially elongated trigonal bipyramidal, and the green 35-kDa protein fragment has one type 1 Cu site having a flattened tetrahedral geometry with one type 2 Cu site. The 35-kDa fragment shows the nitrite reduction activity a little higher than to that of native HdNIR. The redox potentials of the 14- and 35-kDa fragments are +345 and +353 mV vs. NHE at pH 7.0, respectively. Moreover, the intermolecular electron transfer rate constant of the 35-kDa fragment from an electron donor, cognate cytochrome c_{550}, is nearly the same as that of the native enzyme.
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Keywords: Nitrite reductase; Copper protein; Nitrite reduction; Electron transfer

Denitrification is dissimilatory reduction of nitrate or nitrite usually to produce dinitrogen by prokaryotic organisms, being comprised of anaerobic reduction processes ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), which are caused either in the cytoplasmic membrane or in the periplasm by the corresponding oxidoreductases containing transition metal ions [1]. Dissimilatory nitrite reductase (NIR) located in the periplasm catalyzes one-electron reduction of nitrite to nitric oxide. Copper-containing nitrite reductase (CuNIR) occurs in about one-third of the denitrifying bacteria, and other denitrifiers have hemes *c* and d_1-containing NIR [1–3]. Generally, CuNIRs are a trimmer, in which a monomer (ca. 37 kDa) contains two types of Cu, a type1 Cu ion and a type 2 Cu ion [4]. The type 1 Cu is bound by four ligands (2His, Cys, and Met), while the type 2 Cu site has a solvent ligand and a total of three His ligands from each of the two adjacent monomers. The distance between the type 1 and type 2 Cu sites bound by the sequence segment (–Cys–His–) is ca. 12.5 Å [5]. The enzyme receives one electron at the type 1 Cu site from an electron donor protein and catalyzes one-electron reduction of NO_2^- to NO at the type 2 Cu site.

Recently, we reported the spectroscopic and functional characterization of a unique CuNIR from *Hyphomicrobium denitrificans* A3151 (HdNIR) [6–8]. HdNIR is composed of six identical 50-kDa subunits. The electronic absorption, CD, and EPR spectra of HdNIR suggested that the enzyme has two type 1 Cu ions and one type 2 Cu ion per one subunit. The apparent first-order rate constant of the intramolecular electron transfer reaction from the type 1 Cu to the type 2 Cu in HdNIR was obtained to be $2.4 \times 10^{-2}\,s^{-1}$ at pH 6.0 in the presence of nitrite ion. This value is

☆ *Abbreviations:* SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CuNIR, copper-containing nitrite reductase; HdNIR, copper-containing nitrite reductase from *Hyphomicrobium denitrificans* A3151; AcNIR, copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM1013; AxNIR, copper-containing nitrite reductase from *Alcaligenes xylosoxidans* GIFU1051; Az-iso2, azurin from *Methylomonas* sp. strain J; CD, circular dichroism; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode.

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substantially smaller than those of AcNIR and AxNIR ($1.9–2.0 \times 10^3\,s^{-1}$ at pH 6.0) [3]. *H. denitrificans* cytochrome c_{550} donates one electron to HdNIR with an intermolecular electron transfer rate constant (k_{inter}) of $3.7 \times 10^5\,M^{-1}\,s^{-1}$ at pH 5.5 and 25 °C. The electron donors of AcNIR and AxNIR are cognate pseudoazurin ($k_{inter} = 7.3 \times 10^5\,M^{-1}\,s^{-1}$ at pH 7.0) [9] and cognate cytochrome c_{551} ($k_{inter} = 4.0 \times 10^5\,M^{-1}\,s^{-1}$ at pH 6.0) [7], respectively.

The gene of HdNIR has quite recently been cloned and sequenced [10]. Two type 1 Cu ligand motifs (His77, Cys104, His109, and Met114; His219, Cys260, His268, and Met273) and one type 2 Cu ligand motif (His224, His259, and His416) are found, suggesting the existence of an extra type 1 Cu center with a couple of type 1 and type 2 Cu centers of common CuNIRs. Interestingly, N-terminal 92 amino-acid sequence (40–131) is 32% identical to *green alga* plastocyanin [11] and C-terminal 284 amino-acid sequence (164–447) is 35–38% identical to common CuNIRs [3]. It is likely that HdNIR is composed of a plastocyanin-like (blue copper) domain and a NIR domain.

In order to elucidate the domain structure and the coordination centers of two type 1 Cu ions in HdNIR, we have proteolyzed HdNIR with a protease, subtilisin, and obtained two major protein fragments (14 and 35 kDa). The spectroscopic and electrochemical properties and enzymatic activities of these fragments were compared to those of undigested HdNIR.

Materials and methods

Materials. H. denitrificans A3151 cells were grown in a mineral salt medium containing 1% methanol and 0.5% potassium nitrate at 30 °C for 3 days [12]. The isolation and preparation of the enzyme were carried out by the previous method [9]. The purified enzyme was reconstituted with Cu^{2+} ion [13]. The concentration of the protein was determined by the use of Bio-Rad Protein Assay Reagent. Standard proteins for SDS–PAGE and subtilisin were purchased from Bio-Rad and Sigma, respectively. All other chemicals were of analytical grade.

Physical measurements. The electronic absorption and CD spectra were measured at room temperature with a Shimadzu UV-2200 spectrophotometer and a JASCO J-500A spectropolarimeter, respectively. The EPR spectra were recorded with a JEOL JES-FE1X X-band spectrometer at 77 K. Copper content was examined by using a Nippon Jarrel Ash AA-880 Mark-II atomic absorption spetrophotometer. Cyclic voltammetric analyses were carried out by using a Bioanalytical Systems Model CV-50W voltammetric analyzer with a three-electrode system consisting of an Ag/AgCl reference electrode, a gold-wire counter electrode, and a bis(4-pyridyl)disulfide-modified gold working electrode under Ar atmosphere at 25.0 °C. Amino-acid sequence analysis was performed by Edman degradation with an Applied Biosystems 476A gas–liquid phase protein sequencer.

Determination of NIR activity. The nitrite reduction activities of HdNIR and proteolyzed protein fragments were typically assayed in 100 mM phosphate buffer (pH 5.5) containing 0.5 mM benzyl viologen and 1.5 mM sodium nitrite at 25.0 °C [14]. Benzyl viologen reduced with sodium dithionite was used as an electron donor, and the oxidation of reduced benzyl viologen in the presence of the enzyme was spectrophotometrically monitored at 550 nm. In order to exclude oxygen gas, the preparation of the reaction mixture was carried out in a glove box. One unit of activity is defined as the amount of enzyme that is required to reduce 1 μmol of nitrite/min.

Results and discussion

The proteolysis of HdNIR with subtilisin was carried out at 30 °C in 100 mM potassium phosphate buffer (pH 7.0) at a protease-to-substrate ratio of 1:250 (w/w). A 10-μl aliquot of the reaction mixture was taken and mixed with *p*-aminophenylmethylsulfonyl fluoride to stop the digestion, and then analyzed by SDS–PAGE. As shown in Fig. 1, HdNIR was proteolyzed for 1 h to give rise to several polypeptide fragments. Two main fragments (14 and 35 kDa) were further purified by DEAE TOYOPEARL (TOSOH) and Resource Q (Amersham Biosciences) columns equilibrated with 20 mM phosphate buffer (pH 7.0) (0–0.5 M KCl continuous gradient). The purity of the blue 14-kDa and the green 35-kDa protein fragments was confirmed by SDS–PAGE (Fig. 1).

According to the sequence analysis of the fragments eluted from the gel, the 14-kDa fragment had N-terminal sequence identical with that of HdNIR, and the 35-kDa fragment had the amino-acid sequence alignment of S–S–G–A–D–I–T–R–D–P at its N-terminus. The results clearly show that subtilisin hydrolyzed the peptide bond between Lys139 and Ser140 of HdNIR, which is located at the position between two type 1 Cu ligand motifs (Fig. 2). Moreover, the 14- and 35-kDa fragments contain 0.84 ± 0.05 and 1.53 ± 0.05 mol of Cu per subunit, respectively. Copper ions would be slightly released by the proteolysis of HdNIR with subtilisin. These results indicate that HdNIR consists of a N-terminal 14-kDa domain containing one type 1 Cu and a C-ter-

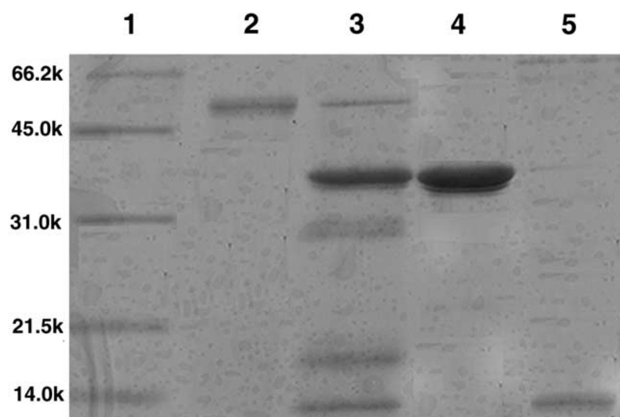

Fig. 1. SDS–PAGE (12.5%) of samples before and after proteolysis of HdNIR with subtilisin. Lane 1, standard proteins; lane 2, native HdNIR (undigested); lane 3, HdNIR digested for 1 h; lane 4, purified 35-kDa fragment; and lane 5, purified 14-kDa fragment.

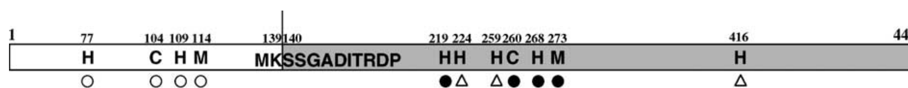

Fig. 2. Schematic diagram of the amino-acid sequence alignment of HdNIR. The type 1 copper ligands are indicated with open circles and closed circles, and type 2 copper ligands are indicated with triangles.

minal 35-kDa domain containing one type 1 Cu and one type 2 Cu.

The electronic absorption, CD, and EPR spectra of the fragments are compared to undigested HdNIR in Figs. 3–5, respectively. The sum of the absorption, CD, or EPR spectra of the 14- and 35-kDa fragments is similar to the corresponding spectrum of HdNIR, respectively. The UV–visible spectrum of the 14-kDa fragment is clearly distinguishable from that of the 35-kDa fragment (Fig. 3). The absorption spectrum of the 14-kDa fragment possessing two peaks at 458 and 602 nm in the visible region is similar to that of azurin (Az-iso2) from *Methylomonas* sp. strain J ($\lambda_{max} = 460$ and 616 nm) [15], while the absorption spectrum of the 35-kDa fragment having two peaks at 454 and 597 nm and a shoulder near 700 nm resembles that of AcNIR ($\lambda_{max} = 460$, 584, and 690 nm) [13]. The CD spectrum of the 14-kDa fragment exhibits two positive peaks at 390 and 570 nm, and two negative peaks at 460 and 710 nm, being analogous to those of Az-iso2 (390(+), 460(−), 570(+), and 740(−) nm) [15]. The X-ray crystal analysis

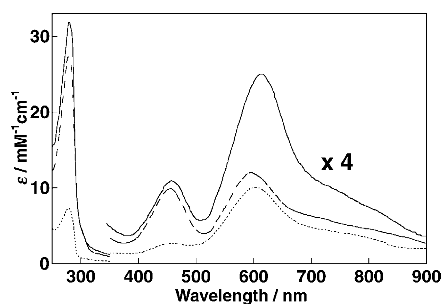

Fig. 3. UV–visible electronic absorption spectra of HdNIR (solid line), 14-kDa fragment (dotted line), and 35-kDa fragment (broken line) in 100 mM phosphate buffer (pH 7.0) at 25 °C.

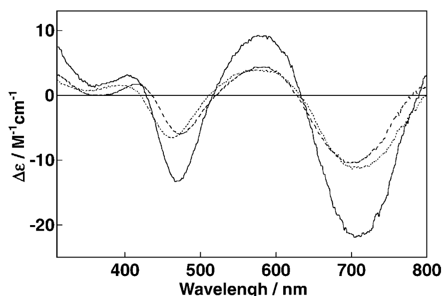

Fig. 4. CD spectra of HdNIR (solid line), 14-kDa fragment (dotted line), and 35-kDa fragment (broken line) in 100 mM phosphate buffer (pH 7.0) at 25 °C.

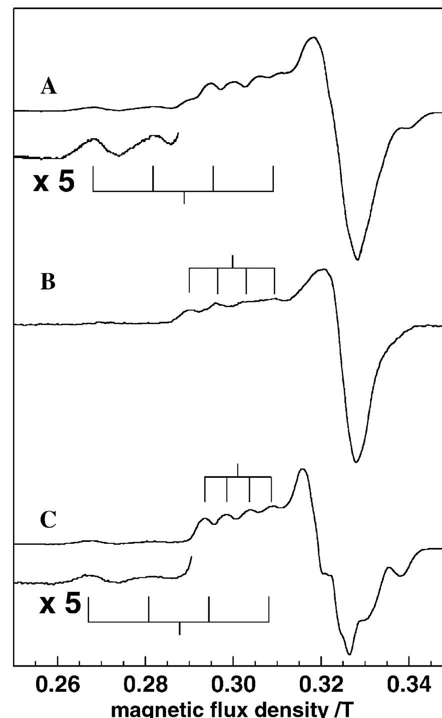

Fig. 5. X-band EPR spectra of HdNIR (A), 14-kDa fragment (B), and 35-kDa fragment (C) in 100 mM phosphate buffer (pH 7.0) at 77 K.

of Az-iso2 demonstrated that the Cu site has an axially elongated bipyramidal geometry (2His and Cys ligands in trigonal plane and two axial ligands of Met and main-chain carbonyl oxygen), in which the bond length between Cu atom and carbonyl oxygen is especially longer than those of general azurins [15]. Accordingly, the type 1 Cu site in the 14-kDa fragment would be axially elongated bipyramidal. The CD spectrum of the 35-kDa fragment exhibits two positive peaks at 414 and 580 nm, and two negative peaks at 470 and 700 nm, being considerably similar to those of AcNIR (394(+), 462(−), 560(+), and 674(−) nm) [13]. Therefore, it is considered that the type 1 Cu site in the 35-kDa fragment has a flattened tetrahedral geometry like that of AcNIR [5,16]. In Fig. 5B, the 77-K EPR spectrum of the 14-kDa fragment shows the axial signal ($g_{\parallel} = 2.23$, $A_{\parallel} = 6.0$ mT, and $g_{\perp} = 2.06$) having somewhat rhombic character [15]. The EPR spectrum of the 35-kDa fragment reveals both the type 1 Cu ($g_z = 2.21$ and $A_z = 5.0$ mT) and type 2 Cu ($g_{\parallel} = 2.31$ and $A_{\parallel} = 14.0$ mT) signals (Fig. 5C) and the former exhibits a rhombic character [13].

Table 1
Effect of 14-kDa fragment on catalytic activity of 35-kDa fragment at pH 5.5 and 25.0 °C

14-kDa fragment/35-kDa fragment ratios	0	0.2	0.5	0.7	1.0
Specific activities (10^4 U/mol)	1.96 ± 0.10	1.79 ± 0.05	1.70 ± 0.03	1.47 ± 0.20	1.41 ± 0.04

The nitrite reduction activities of the fragments were determined by the steady-state method using dithionite/benzyl viologen as an electron donor. Although the 14-kDa fragment had no detectable activity, the 35-kDa fragment displayed a specific activity of $(1.96 \pm 0.10) \times 10^4$ U/mol of fragment at pH 5.5 and 25.0 °C. The value is slightly larger than that of native HdNIR $((1.44 \pm 0.05) \times 10^4$ U/mol of subunit). The nitrite reduction activity of the 35-kDa fragment is a little affected in the presence of the 14-kDa fragment. Moreover, the addition of the 14-kDa fragment led to a slight decrease in the nitrite reduction activity (Table 1). These findings suggest that the 14-kDa domain might not be essential to the enzyme activity of HdNIR.

The cyclic voltammograms of the 14- and 35-kDa fragments show well-defined responses with midpoint potentials of $E_{1/2} = +345$ and +353 mV vs. NHE and peak to peak separations of $\Delta E = 78$ and 66 mV at pH 7.0, respectively. Both the $E_{1/2}$ values of the fragments are close to the potential (+357 mV at pH 7.0) of HdNIR. Moreover, we have examined the intermolecular electron transfer reactions from the 14-kDa fragment or cognate cytochrome c_{550} to the 35-kDa fragment in the presence of nitrite ion by cyclic voltammetry (Fig. 6). The catalytic currents were hardly detected when the 14-kDa fragment was employed, and hence, no electron transfer reaction occurs from the 14-kDa fragment to the 35-kDa fragment. The appearance of catalytic current, however, was observed, when cytochrome c_{550} was used instead of the 14-kDa fragment. The second-order rate constant of the electron transfer process from cytochrome c_{550} to the 35-kDa fragment was calculated to be $4.1 \times 10^5\,M^{-1}\,s^{-1}$ at pH 5.5 and 25.0°C, being almost the same as that from cytochrome c_{550} to HdNIR $(3.7 \times 10^5\,M^{-1}\,s^{-1})$ [8].

In conclusion, 50-kDa HdNIR is proteolyzed to two protein fragments with subtilisin; the 14-kDa protein fragment containing a type 1 Cu site only and the 35-kDa protein fragment containing a couple of the type 1 Cu and type 2 Cu sites. The type 1 Cu sites in the 14- and 35-kDa fragments probably have an axially elongated bipyramidal geometry and a flattened tetrahedral geometry, respectively. The 35-kDa fragment shows the nitrite reduction activity somewhat higher than that of the native enzyme. Moreover, the 35-kDa fragment can accept one electron from cytochrome c_{550} like the native enzyme. The C-terminal 35-kDa domain will be the essential region for the intermolecular electron transfer and catalytic reactions of HdNIR. The role of the N-terminal 14-kDa domain is under investigation.

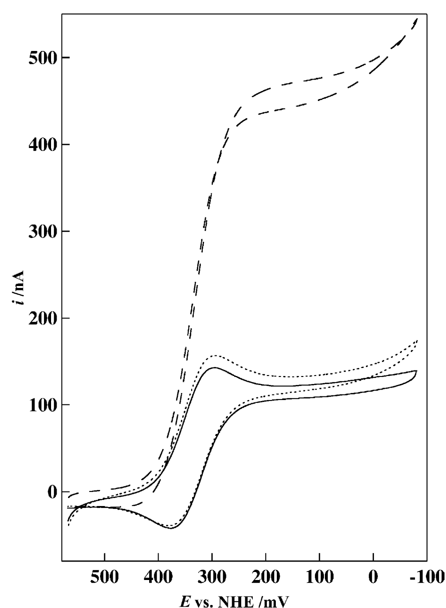

Fig. 6. Voltammetric behavior of cyt c_{550} (solid line), after addition of nitrite (dotted line), and after addition of nitrite and 35-kDa fragment (broken line) in 100 mM phosphate buffer (pH 5.5) at 25 °C. cyt c_{550}, 300 μM; 35-kDa fragment, 1 μM; sodium nitrite, 50 mM; scan rate, 2 mV/s.

Acknowledgments

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