Structure and function of a hexameric copper-containing nitrite reductase

Masaki Nojiri*, Yong Xie†, Tsuyoshi Inoue†, Takahiko Yamamoto‡, Hiroyoshi Matsumura¶, Kunishige Kataoka¶, Deligeer#, Kazuya Yamaguchi#, Yasushi Kai##, and Shinnichiro Suzuki###

*Department of Chemistry, Graduate School of Science, Osaka University, Osaka 560-0043, Japan; †Department of Materials Chemistry, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan; ¶Division of Material Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan; and ‡Department of Chemistry and Environmental Science, Inner Mongolia Normal University, Hohhot 010022, Inner Mongolia, People’s Republic of China

Edited by Norman Sutin, Brookhaven National Laboratory, Upton, NY, and approved January 5, 2007 (received for review October 18, 2006)

Dissimilatory nitrite reductase (NIR) is a key enzyme in denitrification, catalyzing the first step that leads to gaseous products (NO, N₂O, and N₂). We have determined the crystal structure of a Cu-containing NIR from a methylotrophic denitrifying bacterium, Hyphomicrobium denitriticans, at 2.2-Å resolution. The overall structure of this H. denitrificans NIR reveals a trinodal fold in which a monomer consisting of 447 residues and three Cu atoms is organized into a unique hexamer (i.e., a tightly associated dimer of trimers). Each monomer is composed of an N-terminal region containing a Greek key β-barrel folding domain, cupredoxin domain I, and a C-terminal region containing cupredoxin domains II and III. Both cupredoxin domains I and II bind one type 1 Cu and are combined with a long loop comprising 31 amino acid residues. The type 2 Cu is ligated at the interface between domain II of one monomer and domain III of an adjacent monomer. Between the two trimeric C-terminal regions are three interfaces formed by an interaction between the domains I, and the type 1 Cu in the domain is required for dimerization of the trimer. The type 1 Cu in domain II functions as an electron acceptor from an electron donor protein and then transfers an electron to the type 2 Cu, binding the substrate to reduce nitrite to NO. The discussion of the intermolecular electron transfer process from cytochrome c₁₅₃₀ to the H. denitrificans NIR is based on x-ray crystallographic and kinetic results.

The terrestrial nitrogen cycle sustained by some bacteria plays an important role in all organism kingdoms (1–3). Inorganic nitrogen is introduced into the biosphere by the biological fixation of atmospheric dinitrogen to produce NH₃ and is removed through the process of denitrification. During the mutual conversion of NH₃, NO, and NO₂ by bacteria, inorganic nitrogen is changed into the organic nitrogen required by all organisms. Finally NO3⁻ or NO₂⁻ is reduced to dinitrogen via the gaseous nitrogen oxides, NO and N₂O, by dissimilatory denitrification.

In denitrifying bacteria, reduction of NO₂⁻ to NO, which is catalyzed by nitrite reductase (NIR), is a key step in the denitrification process, because this step leads to a gaseous intermediate. There are two main categories of NIR: the heme-containing and Cu-containing enzymes (1). Generally, Cu-containing NIRs (CuNIRs) from Achromobacter cycloclastes (green AcNIR) (4, 5), Alcaligenes faecalis (green AfNIR) (5, 6), and Alcaligenes xylosoxidans (blue AxNIR) (5, 7, 8) fold a trimeric structure in which a monomer (≈37 kDa) contains one type 1 Cu and one type 2 Cu. The monomer is composed of two consecutive Greek key β-barrel folding domains. The type 1 Cu ligated by four amino acid residues (two His, one Cys, and one Met) is bound to one of the two β-barrel domains inside the monomer. The type 2 Cu site with three His ligands and a solvent ligand is bound to the interface between two adjacent monomers. The distance between the two Cu sites connected through the sequence segment (Cys–His) is ≈12.5 Å. The enzyme receives one electron at the type 1 Cu site from an electron donor protein and catalyzes one electron reduction of NO₂⁻ to NO at the type 2 Cu. Moreover, a hydrogen bond network including Asp and His around the type 2 Cu functions as the proton donation to the substrate (2, 6, 7, 9). These active site features have been also observed in the major anaerobically induced outer membrane Cu protein from pathogenic Neisseria gonorrhoeae (10) (AniA). A phylogenetic analysis indicated that AniA is a member of a previously uncharacterized class of CuNIRs, class II CuNIRs, whereas AcNIR, AfNIR, and AxNIR are class I CuNIRs.

Compared with these well characterized CuNIRs, the enzyme from a methylotrophic denitrifying bacterium, Hyphomicrobium denitriticans A3151 (greenish blue HdNIR) has a larger molecular mass, 50 kDa per monomer, and exhibits different spectroscopic and functional features (11, 12). The electronic absorption, circular dichroism, and electron paramagnetic resonance spectra of HdNIR suggest that the monomer has two type 1 Cu atoms and one type 2 Cu atom. We have recently determined the amino acid sequence and prepared wild-type HdNIR and two mutants, C114A and C260A, replacing the Cys ligand of each type 1 Cu with Ala (13). Full-length (50 kDa) HdNIR is composed of a 15-kDa N-terminal region with a type 1 Cu-binding motif, like the cupredoxins, and a 35-kDa C-terminal region with a type 1 Cu-binding motif and a type 2 Cu-binding motif, like the well characterized CuNIRs. The amino acid sequence of the C-terminal region shows a significant similarity (48% identity) to that of class II CuNIR AniA from Neisseria gonorrhoeae (10). Moreover, according to the mutation of HdnIR, C114A and C260A possessing one type 1 Cu and one type 2 Cu demonstrated that the N-terminal and C-terminal regions have blue and green type 1 Cu sites, respectively (13, 14).

Author contributions: M.N. and Y.X. contributed equally to this work; M.N., Y.X., T.I., Y.K., and S.S. designed research; M.N., Y.X., T.I., Y.K., D.I., and K.Y. performed research; M.N., Y.X., T.I., T.Y., and H.M. analyzed data; and M.N. and S.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: NIR, nitrite reductase; CuNIR, Cu-containing NIR; AcNIR, CuNIR from Achromobacter cycloclastes; AfNIR, CuNIR from Alcaligenes xylosoxidans; AniA, anaerobically induced outer membrane Cu protein from pathogenic Neisseria gonorrhoeae; HdNIR, CuNIR from Hyphomicrobium denitriticans; ET, electron transfer; eSd, estimated SD; Cyt c₁₅₃₀, cytochrome c₁₅₃₀.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2DV6).

 présent article is a PNAS direct submission.

© 2007 by The National Academy of Sciences of the USA

PNAS: Volume 104, Number 11, 4315–4320

www.pnas.org/cgi/doi/10.1073/pnas.0609195104

PNAS | March 13, 2007 | vol. 104 | no. 11 | 4315–4320
Here we describe the crystal structure of HdNIR at a resolution of 2.2 Å and the functions of the Cu centers. The whole structure shows that HdNIR folds a trigonal prism-shaped hexameric structure containing 12 type 1 Cu and 6 type 2 Cu atoms (total of 18 Cu atoms per enzyme). This supramolecular assembly provides an example of CuNIRs, although the structures of the N-terminal and C-terminal regions are similar to those of blue Cu proteins and well characterized CuNIRs, respectively. Based on the three-dimensional arrangement of the Cu centers, we discuss the functions of the type 1 Cu and type 2 Cu in the intermolecular and intramolecular electron transfer (ET) processes of HdNIR.

Results and Discussion

Overall Structure. The overall structure of HdNIR reveals a trigonal prism-shaped hexamer of which the triangular edge length and the height are \( \approx 85 \) and 125 Å, respectively (Fig. 1a and b). Six noncrystallographic related monomers exist in the asymmetric unit for a total of 2,532 amino acid residues [the crystal volume per unit of protein molecular weight (\( V_{c} \)) of 3.4 Å\(^3\)/Da and a solvent content (\( V_{sol} \)) of 63.7%]. The monomers are organized into a homohexamer with one threefold axis and three twofold axes in the molecule. Moreover, the monomer is structurally divided into three components, which are the N-terminal (residues 24–131) and C-terminal (residues 163–445) regions and a long linker between their regions (residues 132–162) (Fig. 1c). In the whole molecule, the two trimeric C-terminal regions are stacked on each other, and a pseudo threefold axis exists at the center of the trimers. The two trimers are tightly bound by three “head-to-head” interactions between the N-terminal regions, and three twofold axes are present at each interface. This hexameric architecture is a unique feature of the HdNIR monomers with the extra cupredoxin domain (\( \approx 15 \) kDa) at the N termini of well-characterized CuNIRs.

According to the domain organization analysis of blue multi-Cu proteins (15–17), the monomer structure of HdNIR is further characterized as three consecutive cupredoxin domains (I, II, and III) folded into a Greek key β-barrel. The N-terminal region (cupredoxin domain I) is comprised of 108 amino acid residues, and the C-terminal region exhibits a typical CuNIR-folding fashion (cupredoxin domain I) is comprised of 108 amino acid residues, and the C-terminal region is positioned \( \approx 5 \) Å below the interface between the N-terminal regions. Three ligands (Cys-114, His-119, and Met-124) originate in a small loop (a1) and its neighborhood. Two His nitrogen atoms, His-77 (N\(_{\alpha}\)) and His-119 (N\(_{\alpha}\)), and one Cys sulfur atom, Cys-114 (S\(_{\alpha}\)), are positioned in a trigonal His/His/Cys (NNS) arrangement of 12 type 1 Cu and 6 type 2 Cu atoms is shown in Fig. 2a in the same orientation as in Fig. 1a. Type 1 Cu\(_{N}\) is localized at the “northern” end of cupredoxin domain I and is positioned \( \approx 5 \) Å below the interface between the N-terminal regions. Three ligands (Cys-114, His-119, and Met-124) originate in a small loop (a1) and its neighborhood. Two His nitrogen atoms, His-77 (N\(_{\alpha}\)) and His-119 (N\(_{\alpha}\)), and one Cys sulfur atom, Cys-114 (S\(_{\alpha}\)), are positioned in a trigonal His/His/Cys (NNS) ligand plane at distances of 2.11 Å (estimated SD (eSD) of 0.034 Å), 2.11 Å (eSD of 0.012 Å), and 2.24 Å (eSD of 0.026 Å), respectively, whereas the Met-124 S\(_{\alpha}\) atom and backbone carbonyl O atom of Gln-76 are in axial positions at distances of 2.91 Å (eSD of 0.036 Å) and 3.23 Å (eSD of 0.053 Å), respectively (Fig. 2b). Therefore, the type 1 CuN has a distorted trigonal bipyramidal geometry like the Cu sites of azurins, being displaced by 0.24 Å from the NNS ligand plane toward the Met sulfur atom, although the displacement of the Cu atom from the ligand plane is larger than that (\( \approx 0.1 \) Å) in azurins (18, 19).

The type 1 Cu\(_{C}\) in the C-terminal region is ligated by two His nitrogen atoms [His-219 (N\(_{\alpha}\)) – Cu, 2.13 Å (eSD of 0.056 Å), and His-268 (N\(_{\alpha}\)) – Cu, 2.13 Å (eSD of 0.058 Å)], one Cys sulfur atom [Cys-260 (S\(_{\alpha}\)) – Cu, 2.18 Å (eSD of 0.053 Å)], and one Met sulfur atom [Met-273 (S\(_{\alpha}\)) – Cu, 2.53 Å (eSD of 0.029 Å)] in a flattened tetrahedral geometry (Fig. 2c). The two His and Cys residues form the strong planar ligands, whereas the Met sulfur atom forms a weaker axial ligand. The Cu atom is displaced by 0.46 Å from the NNS ligand plane toward the Met sulfur atom. The type 1 Cu\(_{C}\) and the structural comparison of the C-terminal region with the crystal structure of the recombinant soluble domain of AniA (sAniA) (PDB ID code 1KDW) indicates that their structures are quite similar with an rmsd of 1.1 Å for the C\(_{\alpha}\) atoms (SI Fig. 8). The characteristic “tower loop” structure in class II CuNIR (10) is also conserved in the C-terminal region of HdNIR.

Type 1 and 2 Cu Centers. The arrangement of 12 type 1 Cu and 6 type 2 Cu atoms is shown in Fig. 2a in the same orientation as in Fig. 1a. Type 1 Cu\(_{N}\) is localized at the “northern” end of cupredoxin domain I and is positioned \( \approx 5 \) Å below the interface between the N-terminal regions. Three ligands (Cys-114, His-119, and Met-124) originate in a small loop (a1) and its neighborhood. Two His nitrogen atoms, His-77 (N\(_{\alpha}\)) and His-119 (N\(_{\alpha}\)), and one Cys sulfur atom, Cys-114 (S\(_{\alpha}\)), are positioned in a trigonal His/His/Cys (NNS) ligand plane at distances of 2.11 Å (estimated SD (eSD) of 0.034 Å), 2.11 Å (eSD of 0.012 Å), and 2.24 Å (eSD of 0.026 Å), respectively, whereas the Met-124 S\(_{\alpha}\) atom and backbone carbonyl O atom of Gln-76 are in axial positions at distances of 2.91 Å (eSD of 0.036 Å) and 3.23 Å (eSD of 0.053 Å), respectively (Fig. 2b). Therefore, the type 1 CuN has a distorted trigonal bipyramidal geometry like the Cu sites of azurins, being displaced by 0.24 Å from the NNS ligand plane toward the Met sulfur atom, although the displacement of the Cu atom from the ligand plane is larger than that (\( \approx 0.1 \) Å) in azurins (18, 19).

The type 1 Cu\(_{C}\) in the C-terminal region is ligated by two His nitrogen atoms [His-219 (N\(_{\alpha}\)) – Cu, 2.13 Å (eSD of 0.056 Å), and His-268 (N\(_{\alpha}\)) – Cu, 2.13 Å (eSD of 0.058 Å)], one Cys sulfur atom [Cys-260 (S\(_{\alpha}\)) – Cu, 2.18 Å (eSD of 0.053 Å)], and one Met sulfur atom [Met-273 (S\(_{\alpha}\)) – Cu, 2.53 Å (eSD of 0.029 Å)] in a flattened tetrahedral geometry (Fig. 2c). The two His and Cys residues form the strong planar ligands, whereas the Met sulfur atom forms a weaker axial ligand. The Cu atom is displaced by 0.46 Å from the NNS ligand plane toward the Met sulfur atom. The type 1 Cu\(_{C}\) and
Two Unique Interactions. The unique hexameric architecture of HdNIR is defined as a couple of the usual trimeric CuNIR molecules. The structure is basically formed by the interactions between cupredoxin domains. One is an interaction between the N-terminal region (cupredoxin domain I) of one monomer and the C-terminal region (cupredoxin domains II and III) of an adjacent monomer, and another is an interaction between the two N-terminal regions like a cupredoxin dimer (Fig. 3). The side surface of cupredoxin domain I interacts with the surface positioned ~6 Å above the type 1 CuC in the adjacent cupredoxin domain II (Fig. 3a). Specifically, a variable loop region (residues 88–96, β-strands 4 and 7, and cupredoxin domain I interacts with the attenuated tower loop region (residues 300–324) in domain III. The molecular surfaces of both domains involved in the interaction are complementary in shape [shape complementarity value (21) of 0.67]. The interface is continuous, burying ~1,300 Å² of molecular surface, and is bound by several hydrogen bonds. Four direct hydrogen bonds are clearly observed: Pro-263 (O)–Ser-101 (O), 2.63 Å; Glu-312 (O)–Asn-92 (N), 2.93 Å; Met-313 (O)–Ser-97 (N), 2.77 Å; and Tyr-315 (O)–Asn-68 (N), 3.14 Å. Several water molecules are also located at the interface and contribute to stabilize the domain–domain interaction by mediation of some hydrogen bond networks. A head-to-head interaction between the cupredoxin domains I as in dimeric azurin (19) is observed in the hexameric formation (Figs. 1a and 3b). The interface occupies an area of ~500 Å². The two domains I are associated with a noncrystallographic twofold sym-

---

**Fig. 2.** Cu centers in HdNIR. (a) Arrangement of the type 1 CuN, type 1 CuC, and type 2 Cu in the HdNIR molecule shown in the same orientation as in Fig. 1a. Blue, green, and gray spheres represent the type 1 CuN, type 1 CuC, and type 2 Cu, respectively. The figure was created by using PyMOL. (b and c Left) 2Fo – Fc difference maps for the Cu sites contoured at the 1.0σ (light blue) and 6.5σ levels (dark blue). (Right) Ball-and-stick representation of the same sites. The His-416 residue comes from the adjacent cupredoxin domain III. The figures were created by using TURBO-FRODO (www_afmb_univ-mrs_fr-TURBO). Wat, Water molecule.
metry through contact of their hydrophobic patches at the northern part of the domain. The nonpolar patch surrounding His-119 is comprised of Ile-35, Met-40, Ala-75, Ile-116, Ala-117, Gly-118, and Ala-122 (Fig. 3b). The interface is dominated by van der Waals interactions between nonpolar side chains (fraction of nonpolar atoms \( f_{NP} \) per total atoms in the interface, \( f_{NP} = 0.64 \)). One direct hydrogen bond between Gln-121 (N2) and Gln-121 (O1) also connects the two domains. The N2 atom of the solvent-exposed His-119 ligand forms a hydrogen bond with a water molecule (His-119 (Ne2) O H2O, 2.66 Å), which also hydrogen-bonds to the backbone carbonyl oxygen atom of Ala-122 in the opposite cupredoxin domain I. The azurin dimer also has the two water molecules as bridging groups between the azurin molecules and shows an area of 540 Å2 and a \( f_{NP} \) of 0.78 (19), which are similar to those of the dimeric cupredoxin domains I.

The hexameric structure is also maintained in a solution, because the molecular mass of HdNIR has been determined to be 304 kDa (hexamer) by gel filtration chromatography (Fig. 4). Interestingly, C260A (13) depleting the type 1 CuC was also estimated to be a hexamer (309 kDa), whereas C114A (13) depleting the type 1 CuN was calculated to be a trimer (162 kDa). These findings indicate that HdNIR retains hexameric architecture not only in crystals but also in solutions, and the type 1 CuN is essential for dimerization of the trimers. A triple interaction between the apocupredoxin domains I is perhaps insufficient to stabilize the hexameric structure in solutions.

Intramolecular ET Processes. Judging from the Cu–Cu distances, there are at least three possible intramolecular ET pathways in the hexamer (Fig. 2a). The first ET process from the type 1 CuC to the type 2 Cu (the closest Cu–Cu distance, 12.6 Å) is of importance for the following NO2 reduction at the type 2 Cu. The apparent intramolecular first-order ET rate constant (\( k_{ET} \)) of C114A is 1.0 \( \times 10^3 \) s\(^{-1} \) at pH 6.0 in the presence of NO2 by pulse radiolysis, being comparable with those (1.9 to 2.0 \( \times 10^3 \) s\(^{-1} \)) of class I AxNIR and AcNIR (22). On the other hand, the \( k_{ET} \) value (2.4 \( \times 10^{-2} \) s\(^{-1} \)) of HdNIR at same conditions is much smaller than those of C114A, AxNIR, and AcNIR (12). These findings suggest that an electron generated by pulse radiolysis perhaps attacks the type 1 CuN, but not the type 1 CuC, and then the reduced type 1 CuN gives an electron to the type 2 Cu through the type 1 CuC in the adjacent monomer (Fig. 2c). In general, the ET process is known to be strongly dependent on the distance between the electron donor and acceptor groups (23). The distance dependence on ET along the \( \beta \)-strands by measurement of Cu(I) \( \rightarrow \) Ru(III) ET reactions of several Ru-modified azurins has been previously reported (24, 25). Therefore, the long-range ET process from the type 1 CuN to the type 1 CuC (the second ET process) is likely to be very slow, because the distance between the type 1 CuN and the type 1 CuC is approximately twice as long as that between the type 1 CuC and the type 2 Cu. That is to say, reduction of the type 1 CuN is unfavorable for NO2 reduction at the type 2 Cu.

![Fig. 4. Analytical gel filtration. (a) Elution profile of marker proteins (glutamate dehydrogenase, 290 kDa; lactate dehydrogenase, 142 kDa; enolase, 67 kDa; myokinase, 32 kDa; and cytochrome c, 12.4 kDa) made by using a Superose 12 HR 10/30 (Amersham, Little Chalfont, U.K.). The elution buffer was 100 mM potassium phosphate (pH 7.0) containing 200 mM sodium sulfate, and the flow-rate was 0.2 ml/min. (b) Elution profiles of HdNIR (red) and C260A (blue) and C114A (green) mutants. Each applied protein was ~0.5 mg. The loading conditions were the same as those of the marker proteins. (c) Molecular weight (on a logarithmic scale) versus elution time for HdNIR, C260A, C114A, and the marker proteins. The dashed line represents the logarithmic relationship for the marker proteins.](https://www.pnas.org/cgi/doi/10.1073/pnas.0609195104)

![Fig. 5. Stopped-flow kinetics. Reduction kinetics of HdNIR (a), C114A (b), and C260A (c) by reduced Cyt c550 at 25°C. The reactions were monitored at 415 nm. Cyt c550 (2.5 \( \mu \)M) and the enzyme (50 \( \mu \)M) were prepared in 0.1 M Mes-NaOH buffer (pH 6.0) under N2 atmosphere. Abs, Absorbance.](https://www.pnas.org/cgi/doi/10.1073/pnas.0609195104)
fast and slow phases were calculated as follows:

\[ R_{\text{sym}}^\alpha = \frac{\sum_i I_i h_i}{\sum_i h_i^2} \]

where \( h_i \) is the mean intensity of the \( i \)th observation of symmetry-related reflections of \( h \).

The third ET process is the exchange ET between the type 1 CuC, which is a possible Cyt \( c_5 \) binding site located on the protein surface, and the type 1 CuN atoms of Cyt \( c_5 \). Unusual \( R_{\text{sym}}^\alpha \) values were calculated with 5% of the observations of symmetry-related reflections of \( h \).

Table 1. Summary of crystallographic analysis for HdNIR

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalllographic statistics</td>
<td></td>
</tr>
<tr>
<td>Wavelength, Å</td>
<td>0.9</td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>48.6–2.2</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>195,178</td>
</tr>
<tr>
<td>Completeness, % (outer shell)</td>
<td>99.7 (100)</td>
</tr>
<tr>
<td>( R_{\text{sym}}^\alpha ) (outer shell)</td>
<td>0.09 (0.50)</td>
</tr>
<tr>
<td>Data redundancy (outer shell)</td>
<td>3.8 (3.7)</td>
</tr>
<tr>
<td>Average ( I/s ) (outer shell)</td>
<td>10.9 (2.46)</td>
</tr>
<tr>
<td>Refinement statistics</td>
<td></td>
</tr>
<tr>
<td>Resolution range, Å</td>
<td>8.0–2.2</td>
</tr>
<tr>
<td>No. of reflections ( F &gt; 0 )</td>
<td>180,940</td>
</tr>
<tr>
<td>Total no. of atoms (water)</td>
<td>21,087 (1,803)</td>
</tr>
<tr>
<td>Completeness of data, %</td>
<td>99.8</td>
</tr>
<tr>
<td>( R_{\text{free}}^\alpha )</td>
<td>0.152±0.165</td>
</tr>
<tr>
<td>rms deviation(^{\alpha})</td>
<td></td>
</tr>
<tr>
<td>Bond, Å</td>
<td>0.017</td>
</tr>
<tr>
<td>Angle, °</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\(^{\alpha}\) rms deviations in bond lengths and angles are the deviations from ideal values.

The negatively and positively charged surfaces are shown in red and blue, respectively. (b) The green, blue, and gray spheres represent the type 1 CuN, type 1 CuC, and type 2 Cu, respectively. The Gln-76 and Glu-172 residues are located on the molecular surfaces of the N-terminal and C-terminal regions, respectively, which belong in the different monomers. The figure was created using PyMOL APBS (http://apbs.sourceforge.net) tools.

The third ET process is the exchange ET between the type 1 CuN atoms, like the electron self-exchange (ESE) processes of blue Cu proteins (19). The distance between the type 1 CuN atoms in the N-terminal regions that interact head-to-head is 14.0 Å, being comparable with those of dimeric cupredoxins. However, the ESE of HdNIR has not yet been observed.

Intermolecular ET Processes. The reduction kinetics of HdNIR, C114A, and C260A by a physiological electron donor protein, cytochrome \( c_5 \) (Cyt \( c_5 \)), is shown in Fig. 5. These reactions were monitored by recording a change in the Soret band of Cyt \( c_5 \). The kinetics of HdNIR by Cyt \( c_5 \) (Fig. 5a) was studied under the pseudo first-order conditions with \([\text{HdNIR}] > [\text{Cyt } c_5]\). Unusual kinetics was observed because the reaction exhibited two phases. The initial fast phase was associated with an amplitude of \( \approx 80\% \) of the total absorbance change. The second-order rate constants of the fast and slow phases were calculated as follows: \( R_{\text{sym}}^\alpha \) for the fast phase was \( 1.1 \pm 0.28 \times 10^5 \text{M}^{-1}\text{s}^{-1} \) and \( R_{\text{sym}}^\alpha \) for the slow phase was \( 9.4 \pm 1.9 \times 10^3 \text{M}^{-1}\text{s}^{-1} \), respectively. On the other hand, reductions of C114A and C260A followed monophasic kinetics with second-order rate constants as follows: \( R_{\text{sym}}^\alpha \) for C114A was \( 8.4 \pm 1.0 \times 10^5 \text{M}^{-1}\text{s}^{-1} \) and \( R_{\text{sym}}^\alpha \) for C260A was \( 3.5 \pm 0.21 \times 10^5 \text{M}^{-1}\text{s}^{-1} \), respectively (Fig. 5b and c). Because both C114A and C260A are obviously reduced by Cyt \( c_5 \), the type 1 CuN and type 1 CuC in these mutants can accept an electron from the electron donor. Therefore, both type 1 Cu sites of native HdNIR will also accept an electron. The fast and slow phases of reduction of HdNIR might correspond to the reduction processes of the type 1 CuC and type 1 CuN, respectively, but further kinetic measurement is necessary to discuss the detailed ET mechanism.

For the reduction of the type 1 CuC by Cyt \( c_5 \), we propose a possible Cyt \( c_5 \) binding site located on the protein surface \( \approx 15 \AA \) from the type 1 CuC, which is a \( \approx 500-\text{Å}^2 \) cleft formed by the cupredoxin domains I and II (Fig. 6a). There are four hydrophobic amino acids, Ala-89 and Ile-90 (domain I) and Thr-184 and Leu-216 (domain II), at the center of the cleft surrounded by the polar residues Gln-76, Arg-87, Asn-92, and Ser-115 (domain I); Glu-172, Lys-174, Trp-186, and Asp-214 (domain II); and Glu-310 (domain III). The Glu-172 residue is included in the cleft surface and its carboxylate \( \text{O}^\text{e1} \) atom forms a hydrogen bond with the \( \text{Ne}_{2} \) atom of the His-219 ligand [Glu-172 (O\( \text{e1} \))--His-219 (Ne\( \text{e} \)) (2.6 Å)] (Fig. 6b). This hydrogen bond provides direct surface accessibility to the type 1 CuC, because the distance between the Cu site and the carboxylate group of Glu-172 is \( \approx 7 \AA \). Therefore, reduced Cyt \( c_5 \), bound to the cleft probably donates an electron to the type 1 CuC through Glu-172 in the initial fast phase, and the intramolecular ET occurs from the type 1 CuC to the type 2 Cu to reduce the substrate. Moreover, the amide side chain of Gln-76 lies on the surface of cupredoxin domain I, and the distance between the Cu center and the amide group is \( \approx 8 \AA \). The electron donor protein will also give an electron to the type 1 CuN through Gln-76. However, the reduction of the type 1 CuN is unfavorable to the catalytic reaction, as described in the previous section.

Methods

Preparation, Crystallization, and Data Collection. Native HdNIR was purified from \( \text{Hyphomicrobium denitrificans} \), A3151 cells, as described previously (12). Crystals of native HdNIR were grown by the hanging-drop vapor-diffusion method at 16°C by using 40 mM Tris-HCl buffer (pH 7.0), 16% (wt/vol) PEG 3350, and 170 mM potassium thiocyanate as a reservoir solution. A 1-μl aliquot of protein [20 mg/ml in 20 mM Tris-HCl buffer (pH 7.5)] was mixed with an equal volume of the reservoir solution as a precipitant. The crystal was cryoprotected by transfer to reservoir solution supplemented with 20% (vol/vol) 2-methyl-2,4-pentanediol and was flash-frozen in liquid nitrogen for data collection at 100 K. X-ray diffraction data were collected on a Bruker AXS (Yokohama, Japan) DIP-6040 detector at beamline BL44XU of SPring-8 (Japan Synchrotron Radiation Research Institute, Hyogo, Japan).
differences images were processed and scaled with the HKL package (26). The crystal belongs to the space group P4₁ and the unit cell has dimensions of a₁ = 162.55 Å and c₁ = 148.97 Å. The data processing statistics are summarized in Table 1.

**Structure Determination and Refinement.** At the early stage, the structure of the C-terminal region (cupredoxin domains II and III) of HdNIR was determined by molecular replacement. The molecular structure of the soluble domain of AniA (sAniA) (PDZ ID code 1KBW) (10) was used as the search model after first omitting Cu₁, water, and side chains of amino acid residues, which differ in the two proteins. MOLREP (27) gave a unique molecular replacement solution, which was then rigid body-refined by using CNS (28). Two trimers were found in the asymmetric unit, and the resulting initial model gave an R-factor of 39% at a resolution of 3.0 Å after rigid body refinement and minimization. On the basis of this model, an electron density map was used for fitting the omitted residues to generate the start model for refinement. After density modification with CNS, an electron density map of sufficient quality was produced to allow cupredoxin domain I and the long linker structure model to be built into the density by using O (29). Additionally, noncrystallographic symmetry (NCS) restraints were used with CNS during the early refinement of the overall HdNIR model, and then the model was manually rebuilt and adjusted by using XtalView (30). At a later stage, refmac (31) was used for refinement without NCS, and solvent molecules were gradually included in the model. Only solvent molecules with thermal parameters <60 Å² and a reasonable hydrogen-bonding environment were included in the final model. A Ramachandran plot of the Refined HdNIR structure was generated by PROCHECK (32). Although only four residues, Gln-83 (chain B), Gln-83 (chain C), Gln-83 (chain E), and Arg-159 (chain F), were slightly outside the allowed regions (≤5°), they were kept untouched. The overall structure, including the surface loops, was well ordered. Each averaged B-factor for all atoms of the N-terminal, C-terminal, and long linker regions exhibited 30.2, 26.0, and 34.5 Å², respectively. The N-terminal and C-terminal residues of the model showed slightly elevated B-factors and somewhat diffuse electron density. In the final model, there were 23 residues (DAPAMKDK-SKSHHEEKTDPPTAGA) at the N terminus and two residues (KQ) at the C terminus that remained undefined due to disorder. The refinement statistics are also summarized in Table 1.

**Stopped-Flow Kinetics.** The reduction of HdNIR with Cyt c₅₅₀ as a reducing agent was recorded at 415 nm by monitoring the decay of the Soret band of Cyt c₅₅₀. The kinetic traces were acquired at 25°C by using an RA-2000 stopped-flow spectrophotometer (Otsuka Electronics, Osaka, Japan) in the single-wavelength mode of the machine. Pseudo-first-order rate constants were calculated by nonlinear regression with a Guggenheim algorithm available with the RA system (Otsuka Electronics) or IgorPro version 4.02 (WaveMetrics, Portland, OR). Typically, under N₂ atmosphere, syringe A of the stopped-flow apparatus contained 2.5 μM Cyt c₅₅₀ in 0.1 M Mes-NaOH buffer (pH 6.0), and syringe B contained 25–100 μM HdNIR, C114A, or C260A in the same buffer.

We thank Prof. A. Nakagawa and Drs. M. Suzuki, M. Yoshimura, E. Yamashita (BL41XU), M. Kawamoto, H. Sakai, N. Shimizu, and K. Hasegawa (BL38B1 and BL41XU) for kind help with structural refinement and kinetic measurement, respectively. Synchrotron radiation experiments were performed at the BL44XU [Proposal No. 2005B6718 (to M.N.)], BL38B1, and BL41XU [Proposal No. 2003A0442-NL1-np (to T.I.)] beamlines at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute. This work was supported in part by Grants-in-Aid for Encouragement of Young Scientist 16710156 (to T.I.) and 16750144 (to M.N.), Scientific Research in Priority Area 18540419 (to T.I.), National Project on Protein Structural and Functional Analyses (to T.I.), and by the 21st Century Center of Excellence Program “Creation of Integrated Chemistry” of Osaka University (to Y.K. and S.S) from the Ministry of Education, Culture, Sport, Science, and Technology, Japan.