X-ray Structure and Site-Directed Mutagenesis of a Nitrite Reductase from *Alcaligenes faecalis* S-6: Roles of Two Copper Atoms in Nitrite Reduction[†,‡]

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Received December 13, 1993; Revised Manuscript Received February 25, 1994[⊛]

ABSTRACT: Nitrite reductase (NIR) from the denitrifying bacterium *Alcaligenes faecalis* S-6 is a copper-containing enzyme which requires pseudoazurin, a low molecular weight protein containing a single type I copper atom, as a direct electron donor *in vivo*. Crystallographic analysis shows that NIR is a trimer composed of three identical subunits, each of which contains one atom of type I copper and one atom of type II copper, and that the ligands to the type I and type II copper atoms are the same as those of the *Achromobacter cycloclastes* NIR. An efficient NIR expression-secretion system in *Escherichia coli* was constructed and used for site-directed mutagenesis. An NIR mutant with a replacement of the type II copper ligand, His135, by Lys still retained a type II copper site as well as a type I copper atom, but it completely lost nitrite-reducing activity as measured with methyl viologen as an electron donor. On the other hand, another mutant with a replacement of the type I copper ligand, Met150, by Glu contained only a type II copper atom, but it still retained significant nitrite-reducing activity with methyl viologen. When pseudoazurin was used as an electron donor for the reaction, however, Met150Glu failed to catalyze the reduction of nitrite. Kinetic analysis of the electron transfer between NIR and pseudoazurin revealed that the electron-transfer rate between Met150Glu and pseudoazurin was reduced 1000-fold relative to that of wild-type NIR. These results clearly indicate that the type I copper site in NIR plays a crucial role for electron transfer from pseudoazurin to the type II copper site of NIR, which comprises the catalytic center of NIR for the reduction of nitrite.

A copper-containing nitrite reductase (NIR) from the denitrifying bacterium *Alcaligenes faecalis* S-6 catalyzes reduction of NO_2^- to NO under anaerobic conditions (Kakutani et al., 1981a,b). Pseudoazurin, a member of the cupredoxin family of electron-transfer proteins with copper centers, serves as a direct electron donor to NIR in the same bacterial cells (Kakutani et al., 1981c; Hormel et al., 1986). A similar nitrite-reducing system involving electron transfer between the copper-containing proteins is also reported in *Achromobacter cycloclastes* (Liu et al., 1986). The 3-D structure of *A. faecalis* pseudoazurin has been shown to be a typical β-barrel binding a type I copper atom (Adman et al., 1989). Site-directed mutagenesis has provided the first clues as to the required structural elements for the electron-transferring ability to NIR (Nishiyama et al., 1992).

Recent X-ray crystallographic analysis of a copper-containing NIR from *A. cycloclastes* revealed its structure to be a trimer composed of three identical subunits, each of which is formed from two β-barrels with a cupredoxin fold and two copper atoms (Godden et al., 1991; Petratos et al., 1986). The type I copper site is embedded within one of the β-barrels, while the type II copper atom is bound at the interface between the subunits. Four amino acid residues (His95, Cys136, His145, and Met150) serve as ligands for the type I copper atom, and three histidine residues (His100, His135, and His306) serve as ligands for the type II copper atom (Figure 1). Site-directed mutagenesis of NIR can now reveal the electron-transferring mechanism between the copper-containing proteins.

Our recent cloning and sequencing of the NIR gene from *A. faecalis* revealed that its amino acid sequence has significant identity with that of *Achromobacter* NIR (81%) (Nishiyama et al., 1993), which suggests that *A. faecalis* NIR adopts a similar 3-D structure. We have constructed an efficient expression system for the NIR gene and carried out substitutions of several amino acid residues involved in the binding of type I and type II copper atoms. This paper describes the results of crystallographic and site-directed mutagenesis studies of *A. faecalis* NIR that confirm the predicted structural similarity and characterize the roles of two copper atoms in nitrite reduction.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *Escherichia coli* JM105 [Δ(*lac pro*) *thi strA endA sbc-15 hsdR4* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*] was used as a host for production of wild-type and mutant NIRs and for propagation of M13 phage. Several plasmids were constructed for efficient expression of the NIR genes as follows. The construction of pNIR601, which carries the truncated NIR gene lacking an N-terminal signal sequence for secretion, was carried out by using the

[†] This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan (to M.N.), NIH Grant GM31770 (to E.T.A.), and an MRC of Canada Fellowship (to M.E.P.M.).

[‡] Coordinates for *Alcaligenes faecalis* NIR have been deposited in the Protein Data Bank (1AFN).

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[⊛] Abstract published in *Advance ACS Abstracts,* April 1, 1994.

0006-2960/94/0433-5246$04.50/0

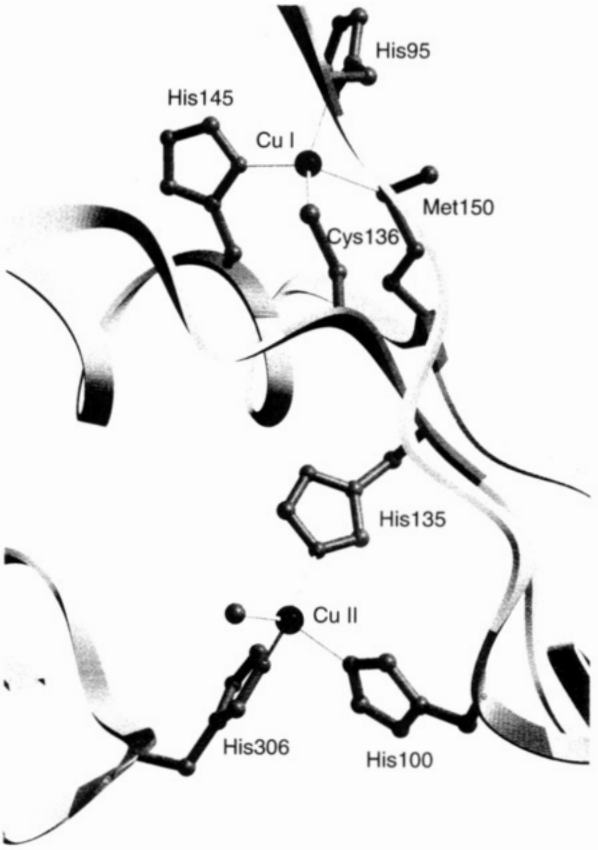

FIGURE 1: Structure around two copper sites in NIR. The main-chain atoms are represented as a ribbon. The copper ligands are drawn as balls and sticks. The Cu II ligand, His306, is derived from a different subunit than the other Cu ligands. Residues His135 and Cys136 provide a covalent link between the two copper atoms.

polymerase chain reaction with pNIR501 (Nishiyama et al., 1993) as a template and using two synthetic oligonucleotides [5′TATCGATGCAAGGCGCGGTGCGGAAGGCA3′, corresponding to the region for translational initiation, and 5′GGTCGAATTCGACCACCTTGGG3′, containing an *Eco*RI site in the coding sequence (Nishiyama et al., 1993)]. Thermal cycling (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) was repeated 10 times. The amplified DNA fragment of 130 base pairs (bp) was digested with *Cla*I and *Eco*RI, purified by agarose gel electrophoresis, and ligated with the 1.4-kilobase (kb) *Eco*RI–*Pst*I fragment and the 2.7-kb *Cla*I–*Pst*I fragment from pNIR501. The resulting plasmid, which allows the production of NIR in the cytoplasm of *E. coli* cells, was named pNIR601. A series of other expression plasmids for NIR which have signal sequences showing, to various extents, similarity to that of azurin at the N-terminus were constructed as follows. To construct pNIR700, the 250-bp *Hin*dIII–*Eco*RI fragment from pNIR501 encoding the N-terminal portion of NIR was cloned in M13mp18, and site-directed mutagenesis was performed with its single-stranded DNA and two synthetic primers, 5′TAAAGGAGAGTATCGATGCGCCGCACCATATTGGCAG3′ and 5′CTTGCGCCGGTTCTGGCACAAGGCGCGGTGCGGAAGGC3′, according to the method of Kunkel (1985). A mutagenized fragment was introduced at the corresponding portion of pNIR501. The resulting plasmid, named pNIR700, lacks seven and six amino acid residues of the N- and C-terminal portions of the signal sequence, respectively. Further site-directed mutagenesis with the synthetic primer 5′GCCGCAAGATCTTGGCAGTAGCCGCCCTGGCTTTGGCGCT3′ was carried out to exchange the fourth,

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        -33                                        -1 1
pNIR501   MAEQMQISRRTILAGAALAGALAPVLATTSAWG QGAVRKAT
                    S1   S5   S10  S15  S20
pNIR700             MRRTILAGAALAGALAPVLA QGAVRKAT
pNIR701             MRRKILAVAALALALAPVLA QGAVRKAT
pNIR702             MLRKLAAVSLLSLALAPVLA QGAVRKAT
pNIR703             MLRKLAAVSLLSLLSAPLLA AEQGAVRK
azurin              MLRKLAAVSLLSLLSAPLLA AECSVDIQ

pNIR601                                  MQGAVRKAT
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FIGURE 2: Amino acid sequences of the N-terminal regions of NIRs directed by expression plasmids pNIR501, pNIR601, pNIR700, pNIR701, pNIR702, and pNIR703. The sequence labeled "azurin" is the signal sequence and the N-terminal portion of the cupredoxin azurin from *P. aeruginosa*. Bold letters indicate that the amino acid is identical to that of NIR.

eighth, and thirteenth amino acid residues in the signal peptide encoded on pNIR700, Thr(S4), Gly(S8), and Gly(S13), for Lys, Val, and Leu, respectively. The mutagenized *Hin*dIII–*Eco*RI fragment was similarly introduced into pNIR501. The resulting plasmid was designated pNIR701. Next we carried out further site-directed mutagenesis by using the corresponding portion of pNIR701 as a mutagenic template to exchange Arg(S2), Ile(S5), Leu(S6), Ala(S9), Ala(S10), and Ala(S12) for Leu, Leu, Ala, Ser, Leu, and Ser, respectively. The oligonucleotide 5′TCGATGCTCCGCAAGCTCGCGGCAGTATCCCTCCTGTCTTTGGCG3′ was synthesized and used for the mutagenesis, and the resulting plasmid was named pNIR702. The oligonucleotide 5′CTGTCTTTGCTGTCTGCGCCGCTTCTGGCAGCGGAGCAAGGCGCGGTG3′ was used to construct pNIR703, which contains the signal sequence and the first two amino acid residues of azurin at the N-terminus of NIR. The plasmids pNIR700, pNIR701, pNIR702, and pNIR703 may allow the secretion of NIR in the periplasmic space of *E. coli* cells. Amino acid sequences of the N-terminal portions of these plasmids are shown in Figure 2.

Construction of NIR Mutants. Site-directed mutagenesis of NIR was carried out using synthetic oligonucleotides 5′GTATCGGGCGAGAATGGCGCCATCAT3′ and 5′TCGTCTACAAATGCGCACCTCCCGGGATG3′ to introduce the mutations Met150Glu and His135Lys, respectively. The nucleotide sequences of mutation points were confirmed by the M13 dideoxy chain termination method (Sanger et al., 1977; Messing & Vieira, 1982) with a DSQ-1 (Shimadzu Co., Kyoto) DNA sequencer. The mutated gene was introduced into the expression plasmid pNIR701. The resulting plasmid was introduced into *E. coli* JM105 by the method of Norgard et al. (1978).

Expression of NIR Proteins in E. coli. E. coli cells harboring pNIR501, -601, -700, -701, -702, or -703 were precultured in 10 mL of 2× YT medium (Yanisch-Perron et al., 1985) containing 50 μg/mL ampicillin at 37 °C for 12 h. One-tenth of one milliliter of the preculture was transferred to 10 mL of the same medium and cultured at 26.5 °C for 2 h. Isopropyl β-D-thiogalactopyranoside (IPTG) and $CuSO_4$ were then added to give concentrations of 1 mM and 100 μM, respectively. Ten hours later, cells were harvested and suspended in 0.85% NaCl in 10 mM Tris-HCl buffer, pH 8.0. Cell fractionation was performed according to the method of Cornelis et al. (1982). Each fraction (cytoplasmic, periplasmic, extracellular, or insoluble fraction) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970), and NIR production was analyzed by Western blotting (Burnett, 1981) with anti-NIR antiserum. The N-terminal amino acid sequence was deter-

mined with an automated protein sequencer (Applied Biosystems, Model 477A) as described previously (Nishiyama et al., 1993).

Purification of NIR and Pseudoazurin. *E. coli* cells carrying pNIR701 were precultured in 10 mL of 2× YT medium containing 50 μg/mL ampicillin at 37 °C for 8 h. The cells were then transferred into 1 L of the same medium and cultured aerobically at 30 °C for 2 h. IPTG was then added to 1 mM, and the cultivation was continued for 12 h. The cells were harvested and subjected to cell fractionation to prepare the periplasmic fraction. One molar potassium phosphate buffer, pH 6.5, was added to the periplasmic fraction to give a concentration of 20 mM. This enzyme solution was applied to a DEAE-Toyopearl column (30 × 70 mm) equilibrated with 20 mM potassium phosphate buffer, pH 6.5 (buffer A) and then eluted with a linear gradient of 0–400 mM KCl in buffer A. The active fractions were pooled, dialyzed against buffer A, and applied to a MonoQ HR10/10 FPLC column (Pharmacia). Elution was performed with a linear gradient of 200–400 mM KCl in buffer A, and the active fractions were collected and concentrated in a Centricon 30 tube (Amicon). The concentrated sample was applied to a Superose 12 FPLC column equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. The active fractions were resubjected to MonoQ chromatography in the same way. The NIR preparations thus obtained gave a single band on SDS–PAGE.

Native NIR produced by *A. faecalis* S-6 was purified as reported previously (Nishiyama et al., 1993) and used for crystallographic analysis.

Pseudoazurin of *A. faecalis* S-6 was purified from *E. coli* cells harboring pUB1 which carried the pseudoazurin gene under the control of the *lac–tac* tandem promoter by the method described previously (Nishiyama et al., 1992).

Spectral Analysis. Absorption spectra of oxidized NIRs were recorded with a Spectronic 3000 Array (Milton Roy) spectrophotometer. ESR spectra of NIRs were determined with JEOL JES-RE2X ESR spectrometer. Conditions used were as follows: 70 μM NIR in 40 mM potassium phosphate buffer, pH 7.0; temperature, 80 K; microwave frequency, 9.157 GHz; microwave power, 10 mW; modulation amplitude, 6.3 G.

Kinetic Analysis. Nitrite reductase activities using methyl viologen as an electron donor were determined by the method of Kakutani et al. (1981b). One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NO_2^-/min. Kinetic analysis with pseudoazurin as an electron donor was carried out as follows. The reaction mixture contained variable concentrations of reduced-form pseudoazurin and 2 mM KNO_2 in 20 mM potassium phosphate buffer, pH 7.0. The reaction was started by adding appropriate amounts of wild-type NIR or Met150Glu mutant NIR to the reaction mixture, and an increase in the absorption at 593 nm due to an increase in oxidized pseudoazurin was measured. Although NIR shows optical absorption around 590 nm in its oxidized form, this absorption did not affect the rate analysis because the concentration of NIR in the reaction mixture was extremely low in comparison with that of pseudoazurin. The molar extinction constant of pseudoazurin ($\epsilon = 2.9 \times 10^3$ M^{-1} cm^{-1} at 593 nm) was used to calculate kinetic parameters. Proteins were determined by the method of Bradford (1976).

Measurement Of Copper Content. Since type II copper is easily depleted from NIR, reconstitution of the copper for wild-type and mutated NIRs was carried out prior to measurement of copper content by the method of Libby and Averill (1992). Thirty picomoles of NIR was dissolved in 1 mL of 1 mM $CuSO_4$ in 10 mM Tris-HCl, pH 7.5, and stored at 4 °C for 3 days. After removal of excess copper using a Centricon tube, NIR was diluted with 20 mM potassium phosphate buffer, pH 7.0, to give concentrations of 10–20 μM and analyzed with an atomic absorption spectrophotometer (Seiko, SAS-725).

Table 1: Data-Collection Statistics for Native NIR

parameter	20–2.6 Å	2.8–2.6 Å
unique reflections		
number	29 071	5219
total possible (%)	94	86
redundancy	2.2	1.8
I/σ_I	17.7	3.7
R_{merge}[a]	0.054	0.190

[a] $R_{merge} = \sum_{h,k,l} \sum_{i=1}^{n} |I_i(h,k,l) - \bar{I}(h,k,l)| / \sum_{h,k,l} I(h,k,l)$.

Crystallographic Analysis. Crystals were grown from 10% PEG 4000 and 0.1 M sodium acetate, pH 4.6, at room temperature. X-ray diffraction data were collected at room temperature from a 1.25 × 0.25 × 0.2 mm crystal mounted in a glass capillary on a Siemens X100 area detector using a graphite monochromator and a 0.3-mm collimator. Copper radiation from an RU200 rotating anode operated at 50 kV and 80 mA was used. The crystal-to-detector distance was 11.5 cm. Data were processed using XENGEN software (Howard et al., 1987).

The structure solution was carried out by using molecular replacement programs from MERLOT (Fitzgerald, 1988). Refinement was carried out by using X-PLOR version 3.1 (Brünger, 1990), and electron density maps were inspected by using the program "O" on an Evans and Sutherland ESV workstation (Jones et al., 1991).

RESULTS

Crystallographic Analysis of Native A. faecalis NIR. NIR was purified from *A. faecalis* and crystallized for X-ray analysis. Table 1 summarizes the data collection statistics. The space group is $P2_12_12_1$, with cell dimensions $a = 63.49$, $b = 103.52$, and $c = 147.52$ Å. A trimer constructed from the partially refined model of *A. cycloclastes* NIR with side chains unchanged was used as the search model for molecular replacement. Data between 10 and 6 Å were used for the fast rotation function, and vectors to 36 Å were included in the search. A single peak 6σ above the mean was observed in the rotation function. The translation search was done with 8–4-Å data and revealed a single peak in each Harker section 6–8σ above the mean. An initial model of *A. faecalis* NIR was constructed from the coordinates of *A. cycloclastes* NIR by using the program MUTATE (R. Read, unpublished). Rigid body refinement of the initial trimer model reduced the *R*-factor from 0.41 to 0.39, using data from 8- to 2.8-Å resolution. No rigid body refinement of the individual subunits was performed. The model was subjected to positional and individual thermal factor refinement while extending the data to 2.6 Å resolution. Thirty-four solvent molecules were added to the refinement model by examining peaks in $F_o - F_c$ maps, including a putative water ligand at each copper II site. The R-factor of the current refined model is 17.6%, using 23 688 reflections in the resolution range of 10–2.6-Å resolution. The rms deviation from ideal bond lengths is 0.026 Å.

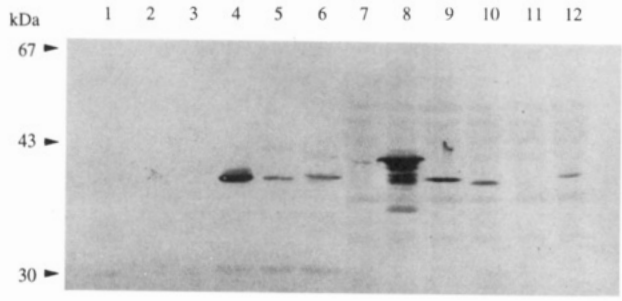

FIGURE 3: Production and localization of NIR in *E. coli* cells analyzed by Western blotting. Lanes 1–6, periplasmic fractions; lanes 7–12, cytoplasmic fractions. Lanes 1 and 7, *E. coli* JM105, as control; lanes 2 and 8, *E. coli* JM105 (pNIR501); lanes 3 and 9, *E. coli* JM105 (pNIR601); lanes 4 and 10, *E. coli* JM105 (pNIR701); lanes 5 and 11, *E. coli* JM105 (pNIR702); lanes 6 and 12, *E. coli* JM105 (pNIR703).

The structure confirms that *A. faecalis* NIR not only is a trimer but also has four amino acid residues (His95, Cys136, His145, and Met150) that serve as ligands for the type I copper atom and three histidine residues (His100, His135, and His306) that serve as ligands for the type II copper atom, exactly as in *A. cycloclastes* NIR (Godden et al., 1991). We chose Met150 and His135 as residues for site-directed mutagenesis to reveal the role of each copper atom in the reduction of nitrite.

Expression of Wild-Type and Mutant NIRs in E. coli. We have previously reported cloning of the NIR gene from *A. faecalis*, which includes an N-terminal signal sequence for secretion along with its expression in *E. coli* (Nishiyama et al., 1993). The expression plasmid pNIR501 allowed the production of NIR precursor protein with its N-terminal signal peptide in the form of cytoplasmic inclusion bodies at 37 °C. Cultivation at 26.5 °C allowed the production of NIR in the cytoplasm in a soluble form with no secretion into the periplasm (Figure 3). However, the NIR produced at the lower temperature showed multiple bands on SDS–PAGE probably due to random proteolytic cleavage in the cytoplasm. The plasmid pNIR601 in which the N-terminal signal sequence was deleted directed production of the mature NIR in the cytoplasm with low yields (4.0 units of activity per 10 mL of culture).

To produce the correctly processed NIR with better yields, we constructed several plasmids in which the N-terminus of NIR was altered. For this purpose, a part of the signal sequence of azurin from *Pseudomonas aeruginosa* was introduced by replacing a part of the original NIR signal sequence, since the azurin sequence is known to cause efficient secretion of several heterologous proteins from *E. coli* (Karlsson et al., 1989; Chang et al., 1991). We constructed a set of plasmids, pNIR700, -701, -702, and -703. pNIR700 contained a part (20 amino acids) of the original signal sequence of NIR, while pNIR703 possessed the azurin sequence of the identical length in place of the NIR signal. pNIR701 and pNIR702 are the derivatives with the hybrid sequences between those of pNIR700 and pNIR703. The production of NIR protein directed by these plasmids was analyzed by Western blotting using anti-NIR antiserum (Figure 3). pNIR703 and pNIR702 were found to show only slight or negligible production of NIR in both the cytoplasm and the periplasm. pNIR700 directed the production of NIR precursor protein in the cytoplasm as well as pNIR501 (data not shown). However, pNIR701 containing a signal sequence similar to that of pNIR700 but with replacements of Thr(S4)Lys, Gly(S8)Val, and Gly(S13)Leu directed the significantly improved production of the mature NIR, which was detected as a single band in SDS–PAGE in both the periplasm (12.1 units per 10 mL of culture) and the cytoplasm (3.5 units per 10 mL of culture). The NIR recovered from the cytoplasmic fraction showed a molecular size (37 kDa) by SDS–PAGE identical to that of the mature NIR recovered from the periplasm. We assume that a part of the processed NIR in the periplasm was not extracted by the cold osmotic shock treatment possibly due to its large molecular size in trimer (111 kDa) and was apparently recognized as cytoplasmic.

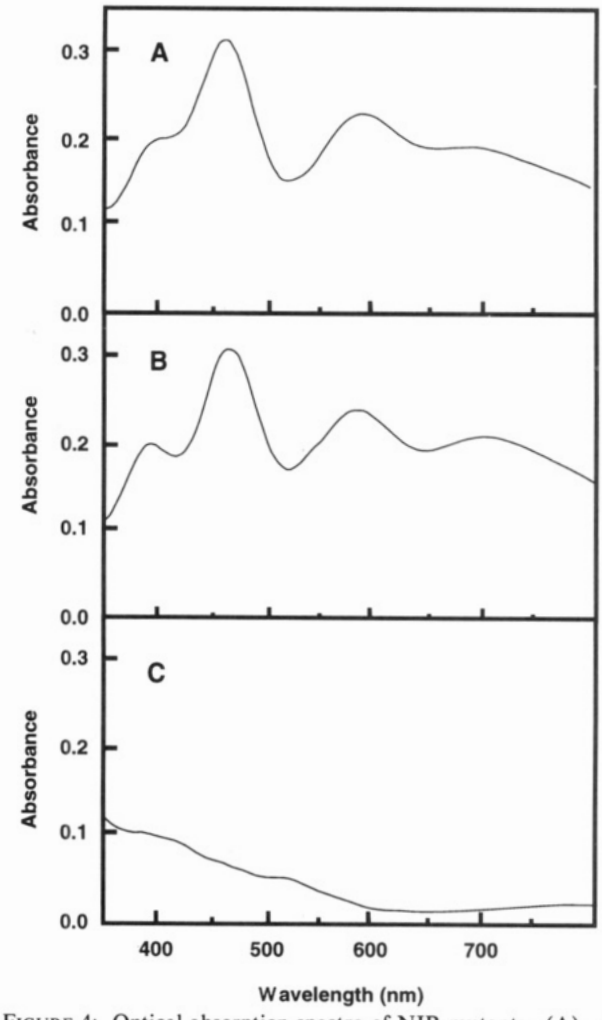

FIGURE 4: Optical absorption spectra of NIR mutants: (A) wild-type NIR, (B) His135Lys mutant, and (C) Met150Glu mutant.

The NIR purified from the periplasmic fraction of *E. coli* cells carrying pNIR701 was subjected to automated amino acid sequencing. The N-terminal sequence was determined to be AVRKAT, which corresponds to the amino acid sequence from the third to the eighth position of the native NIR purified from *A. faecalis* (Nishiyama et al., 1993). The specific activity of the NIR purified from *E. coli* is 350 units/(mg of protein), which is identical to that from *A. faecalis* [348 units/(mg of protein)], indicating that the loss of the first two amino acids has no effect on the enzyme activity. We used pNIR701 for the production of the mutated NIR proteins.

Site-directed mutagenesis was conducted to exchange one copper ligand of type I and type II copper, respectively, for the amino acid that cannot act as a ligand (Met150Glu and His135Lys). The NIR genes containing these mutations were expressed using the *E. coli* expression system described above. With this system, approximately 1.5–2 mg of purified NIR protein was obtained from a 1-L culture.

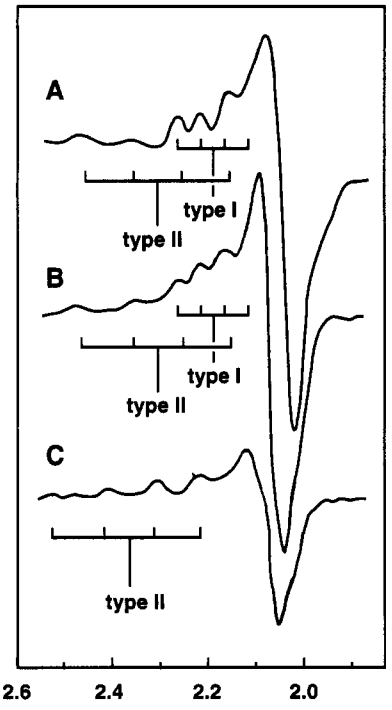

FIGURE 5: ESR spectra of NIR mutants: (A) wild-type NIR, (B) His135Lys mutant, and (C) Met150Glu mutant.

Table 2: ESR Parameters of Wild-Type and Mutant NIRs[a]

enzyme	copper type	$g_{\parallel}$	$A_{\parallel}$ (cm^{-1})
wild type	type I	2.19	0.0066
	type II	2.30	0.0151
His135Lys	type I	2.19	0.0061
	type II	2.30	0.0157
Met150Glu	type II	2.37	0.0156

[a] Conditions: 70 mM NIR in 40 mM potassium phosphate buffer, pH 7.0; temperature, 80 K.

Spectroscopic Properties of Mutated NIRs. The wild-type NIR in the oxidized form shows three distinct peaks around 460, 590, and 700 nm in the optical absorption spectrum (Figure 4). This is a characteristic spectrum of a type I copper, although the relative intensities differ from those seen in cupredoxin (Han et al., 1993). The optical absorption spectrum of His135Lys mutant, in which one of the ligands for a type II copper atom was replaced, was similar to that of wild-type NIR, suggesting that the arrangement of ligands for binding a type I copper atom is similar to that of the wild-type enzyme. On the other hand, Met150Glu NIR, where one of the four ligands to the type I copper atom is replaced, showed no optical absorption signal, suggesting the loss of type I copper in this mutant.

For further clarification of the environment of the copper atoms, ESR spectra of wild-type and mutant NIRs were determined (Figure 5). The parameters obtained from the ESR spectra are given in Table 2. Wild-type NIR showed signals for both type I and type II copper atoms, while Met150Glu mutant showed only a signal for a type II copper atom and not for type I copper. On the other hand, no significant difference in ESR spectra between the His135Lys and wild-type NIRs was observed, indicating the presence of both type I and type II copper atoms in this mutant. These observations agree well with the results of optical absorption spectra of these mutants.

Table 3: Specific Activities and Copper Contents of Wild-Type and Mutant NIRs

enzyme	specific activity [units/(mg of protein)]	copper content per monomer
wild type	350	1.90
His135Lys	0	1.44
Met150Glu	15	0.83

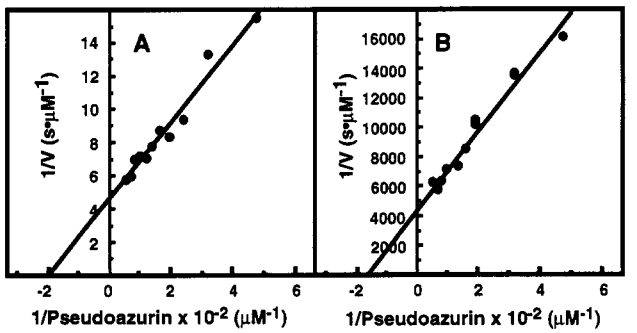

FIGURE 6: Double-reciprocal plots of the type I site mutant in pseudoazurin oxidation: (A) wild-type NIR and (B) Met150Glu mutant.

Copper Contents of NIRs. To confirm the above results, we measured the copper contents of wild-type and mutant NIRs by atomic absorption spectroscopy (Table 3). Wild-type NIR contains approximately 2 copper atoms per monomer, as observed in the crystal structure. The copper content of His135Lys NIR is observed to be 1.44 copper atoms per monomer, which is less than the theoretical value of 2 for wild-type NIR. In the ESR spectrum of this mutant, signals for both type I and type II copper were detected. Furthermore, the intensity of the optical absorption signals in this mutant are typical of a type I copper and almost the same as that of the wild-type enzyme (see Figure 4). Therefore, we assume that type II copper may be retained but partially depleted in His135Lys. On the other hand, the copper content of Met150Glu was determined to be approximately 1 copper per monomer. Considering the results of the absorption and ESR spectra described above, we conclude that the Met150Glu mutant has only type II copper atoms, while His135Lys contains both type I and type II copper.

Activities of NIR Mutants. We measured the enzyme activities of His135Lys and Met150Glu NIRs using methyl viologen as an electron donor (Table 3). As described above, His135Lys seems to possess a type I copper environment similar to that of the wild-type enzyme. However, no nitrite-reducing activity was found for this mutant. This result strongly suggests that type II copper with three His ligands is required for the reduction of nitrite. When a similar measurement was carried out with Met150Glu NIR, a low but measurable nitrite-reducing activity was observed, although this mutant contains only type II copper (see Figure 5).

Pseudoazurin is known to be a direct electron donor to NIR in *A. faecalis* cells. We therefore measured the nitrite-reducing activity of Met150Glu NIR by using reduced pseudoazurin as an electron donor. In contrast to the results with methyl viologen as an electron donor, no activity was detected. These results indicate that type I copper is not directly involved in nitrite reduction, but probably plays the role of an electron acceptor from pseudoazurin.

To confirm this hypothesis, we measured the rate of electron-transfer between pseudoazurin and NIR at variable concentrations of reduced pseudoazurins (Figure 6). Since oxidized pseudoazurin shows an intense optical absorption maximum at 593 nm, electron transfer from reduced pseudoazurin to

Table 4: Kinetic Parameters for Electron Transfer between Pseudoazurin and Wild-Type and Met150Glu NIRs[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)
wild type	396	50
Met150Glu	0.43	66

[a] Conditions: 2 mM KNO_2 in 20 mM potassium phosphate buffer, pH 7.0.

NIR can be observed by measuring an increase in absorption. This analysis revealed that Met150Glu NIR possesses a k_{cat} value of 0.43 s^{-1} which is 1000-fold lower than that of the wild-type enzyme (396 s^{-1}), while no significant change was observed in K_m for pseudoazurin (66 and 50 μM in Met150Glu and wild-type NIR, respectively) (Table 4). Therefore, we conclude that the type I copper atom plays the role of an electron acceptor from pseudoazurin and transfers the electron to a type II copper atom, where nitrite reduction occurs.

DISCUSSION

We developed an improved expression system for the NIR gene which allowed the efficient secretion of the correctly processed NIR and its mutants. Although the wild-type NIR gene directs a precursor with a signal peptide for secretion, the signal did not work efficiently in *E. coli*. The signal sequence of NIR is longer than the typical signals of the Gram-negative bacteria and seems to contain several residues perturbing the secretion through cytoplasmic membranes of *E. coli* cells. Considering the results with a set of derivatives of the plasmids with modified signal sequences, it may be concluded that the lack of an Arg in the N-terminal portion (pNIR702 and pNIR703) and the presence of Gly or Ser residues in the hydrophobic stretch (pNIR700, pNIR702, and pNIR703) were responsible for the decreased secretion. In contrast, pNIR701 possesses increases in both positive charge at the N-terminus and hydrophobicity in the central part of the signal sequence, which caused the efficient secretion of NIR.

Sequence information is now available for nitrite reductases from four sources: *A. cycloclastes* (Fenderson et al., 1991), *A. faecalis* S-6 (81% identity) (Nishiyama et al., 1993), *Pseudomonas aureofaciens* (64% identity) (Glockner et al., 1993), and *Pseudomonas* sp. strain G-179 (78% identity) (Ye et al., 1993). The combination of this sequence similarity with the X-ray crystal structures and low-angle X-ray scattering measurements for a fifth NIR, that from *Achromobacter xylosoxidans* (Grossman et al., 1993), suggests that all of these conform to the same trimeric structure. The first two NIRs listed above show green color, whereas the last two show blue. The green versus blue color is believed to be a consequence of the degree of distortion of the tetrahedral copper-binding site (Han et al., 1993), but it is still not understood what causes this distortion, particularly when one compares the NIR sequences.

The mechanism of electron transfer along with the role of the copper atoms in these nitrite reductases has still not been clarified. Several NIRs such as those from *A. xylosoxidans* (Masuko et al., 1984) and *P. aureofaciens* (Zumft et al., 1987) had been assumed to contain only type I copper, which might play a central role in electron transfer as seen in many cupredoxins. However, demonstration of both type I and type II copper atoms in the trimeric structures of *A. cycloclastes* and *A. faecalis* NIRs has placed a totally different light on the function of the copper-containing NIRs. Recent crystallographic analysis of *A. cycloclastes* NIR with an excess of nitrite suggests that a water molecule, which binds to the type II copper in the absence of nitrite, may be displaced, probably by nitrite (Godden et al., 1991). This implies possible involvement of the type II copper in the catalysis of nitrite reduction. Furthermore, on the basis of the observation that NIR activity was proportional to type II copper content, Libby and Averill (1992) concluded that the type II copper atom of *A. cycloclastes* NIR is involved in nitrite reduction. Recent studies on the *A. xylosoxidans* NIR show that it contains both type I and type II copper as well (Grossman et al., 1993). Site-directed mutagenesis in the present study definitively demonstrates the role of each copper atom in nitrite reduction, type I copper as an electron acceptor from pseudoazurin and type II copper as a component of the active site for nitrite reduction.

The His135Lys mutant, in which one of the ligands for type II copper is replaced, still binds type II copper as well as type I copper. Lysine is a rather large side-chain replacement for a histidine, and one could suspect that it prevents trimer formation, causing a loss in activity. However, analysis by gel filtration showed that this mutant still possessed the molecular size of 106 kDa, which is almost identical to the theoretical value (111 kDa) for that of NIR in trimer. Therefore, an altered type II environment is the cause of the loss of activity and the lower affinity for copper. The determination of the 3-D structure of His135Lys, however, will provide important information on the relationship between the copper environment and nitrite-reducing activity. Preliminary efforts to crystallize this mutant have been unsuccessful, in part due to decreased stability of this mutant with respect to the wild type.

The type I copper environment is lost in the Met150Glu mutant, indicating the essential involvement of Met150 in binding of the type I copper atom. Similar mutation of the Met ligand of a type I copper atom to Glu was carried out with azurin, a cupredoxin from *P. aeruginosa*, by Karlsson et al. (1991) and Pascher et al. (1993), and their analysis showed that this mutation did not cause large structural perturbation but did cause the low occupancy of the copper atom in this mutant and, at pH 7, an increase in $g_{\|}$ to nearly that of a type II copper. Our X-ray studies of NIR have shown that the type I copper binding site is constructed within the cupredoxin-like β-barrel in each subunit, in which Met150 is involved as one of the copper-binding ligands (Godden et al., 1991; this work). Met150Glu NIR was found to retain significant nitrite-reducing activity. Optical and ESR spectra of Met150Glu revealed the absence of type I copper as well as the presence of type II copper. Therefore, we may conclude that the electron for the reduction of nitrite is transferred probably directly to type II copper from the small electron donor methyl viologen in this mutant. When pseudoazurin was used as an electron donor, the electron-transferring rate between pseudoazurin and NIR was markedly decreased in the Met150Glu mutant in which the type I copper is absent. This result clearly indicates that the type I copper of NIR serves as an electron acceptor from the type I copper of pseudoazurin. Met150Glu mutant showed a signal for type II copper with a somewhat larger g-value (Table 2). We assume that this may reflect the change in the environment of type II copper induced by a conformational change in the adjacent type I copper site due to mutation of the type I ligand.

We propose here a model of the electron-transfer pathway in the NIR–pseudoazurin system (Figure 7). The type I copper of NIR accepts an electron from the type I copper of pseudoazurin, and then donates it *via* a Cys136–His135

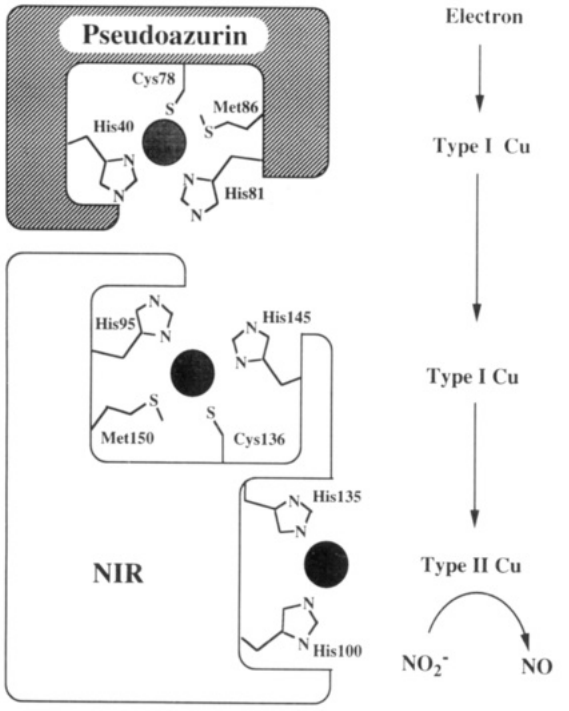

FIGURE 7: Proposed model for electron transfer between NIR and pseudoazurin.

intramolecular electron transferring pathway, analogous to that proposed in ascorbate oxidase by Messerschmidt et al. (1989), to the type II copper in the same subunit where nitrite is reduced to nitric oxide. Further studies of the protein–protein interaction between pseudoazurin and NIR, as well as studies of the roles of other amino acids in NIR activity, are now in progress.

ACKNOWLEDGMENT

We thank K. Okamoto (Department of Agricultural Chemistry, The University of Tokyo) for assistance in amino acid sequencing. We are grateful to K. Sonoike (Department of Biology, The University of Tokyo) for his kind help in ESR analysis. We also thank A. Okubo (Department of Agricultural Chemistry, The University of Tokyo) for his help in atomic absorption spectroscopy. We also thank J. Sanders-Loehr (Department of Chemical and Biological Science, Oregon Graduate Institute of Science and Technology) and G. W. Canters (Department of Chemistry, Gorlaeus Laboratories, Leiden University) for their helpful discussion on ESR spectra of NIR mutants.

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