Studies on Protein-Protein Interaction between Copper-containing Nitrite Reductase and Pseudoazurin from Alcaligenes faecalis S-6*

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Site-directed mutagenesis of a copper-containing nitrite reductase (NIR) from Alcaligenes faecalis S-6 was carried out to identify the amino acid residues involved in interaction with its redox partner, pseudoazurin, in which four positively charged residues were previously shown to be important in the interaction. Ten negatively charged residues located on the surface of NIH were replaced independently by alanine or serine. All the altered NIRs showed CD spectra and optical spectra identical to those of wild-type NIR, suggesting that all the replacements caused no gross change in the overall structure or in the environment of type I copper site.

Kinetic analysis of electron transfer between pseudoazurin and altered NIRs revealed that the replacement of Glu-118, Glu-197, and Asp-201 caused a significant increase in the $K_m$ value for pseudoazurin compared with that of wild-type NIR. Furthermore, the simultaneous replacement of three of these residues (Glu-118, Glu-197, and Asp-201) caused a further increase in the $K_m$ value. These results suggested that the negatively charged residues are involved in electrostatic interaction with pseudoazurin. Kinetic analyses of the altered NIRs (E118A, E197A, or D201A) with altered pseudoazurins (K10A, K57A, or K77A) implicated specific pairs of the charged residues that are involved in electrostatic interaction between NIR and pseudoazurin.

Nitrite reductase (NIR) from Alcaligenes faecalis S-6 is a copper-containing enzyme that catalyzes the reduction of NO$_2^-$ to NO in the denitrifying pathway in this bacterium (1, 2). Pseudoazurin, a 14-kDa protein containing a type 1 (blue) copper atom, has been isolated as a direct electron donor to NIR in vivo (3, 4). We have cloned genes of both pseudoazurin and NIR from A. faecalis S-6 and developed expression systems in Escherichia coli for site-directed mutagenesis of these proteins (5, 6). X-ray crystallographic analysis of pseudoazurin revealed its typical $\beta$-barrel structure followed by $\omega$-helices at the C terminus, binding a single type 1 copper atom at the top of the $\beta$-barrel (7, 8). Our recent site-directed mutagenesis studies suggested that a ring of lysine residues close to the type I copper site of pseudoazurin is involved in the interaction with its redox partner, NIR (9). To further clarify the electron transfer between pseudoazurin and NIR, identification of amino acid residues of NIR that are involved in the interaction with pseudoazurin is now required.

Three-dimensional structures of NIRs from two sources, Achromobacter cycloclastes and A. faecalis S-6, were determined by x-ray crystallographic analyses (10, 11). The analyses revealed that both NIRs have the same trimeric structure with a type 1 copper atom and a type 2 copper atom in each subunit. The type 1 copper is bound inside domain 1 of the two $\beta$-barrel subunits, and the type 2 copper is bound between two adjacent subunits. Using site-directed mutagenesis of selected ligands to these two types of copper atoms, we have shown that the type 1 copper plays a role as an efficient electron acceptor from pseudoazurin and the type 2 copper is involved in a catalytic mechanism of this enzyme (11). We therefore expected that the surface near the type 1 copper was involved in interaction with the lysine residues of pseudoazurin. In the present study, 10 negatively charged residues on the surface of NIR were chosen as candidates for site-directed mutagenesis and were replaced independently by alanine or serine. Glu-46, Glu-58, Glu-89, Glu-113, Glu-118, Glu-197, Asp-201, Glu-204, and Asp-205 are located on the surface close to the type 1 copper site, and Glu-160 is located on the opposite side of the molecule (Fig. 1). We report here the involvement of electrostatic interactions in forming an electron transfer complex composed of NIR and pseudoazurin and the identification of pairs of the amino acid residues responsible for the electrostatic interaction between these two copper-containing proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strain J M105 (lac pro thi strA endA sdcB15 hisD4 F' traD36 proAB lacI4 lac2M15) was used as a host for the production of wild-type and altered NIRs. pNIR701 is a plasmid that was designed to give efficient production of NIR in the periplasmic space of E. coli as described previously (11).

Construction of Altered NIRs—Site-directed mutagenesis of Glu-46 to Ser, Glu-58 to Ala, Glu-89 to Ser, Glu-113 to Ala, Glu-118 to Ala, Glu-160 to Ala, Glu-197 to Ala, Asp-201 to Ala, Glu-204 to Ala, and Asp-205 to Ala of NIR was carried out by the Kunkel method (12) using oligonucleotides 5'-ATGCGATACGGCGAAAGAAG-3', 5'-GATGCCGGTACCGCAATGAC-3', 5'-GCGACGCGTATGAAGCCACCGTC-3', 5'-GGTGCTGCCCCGGGCGGGTCTGCAT-3', 5'-CGGCGGGTTAACCGCAATCAATCCG-3', 5'-ATCAATCCCGTCGACCAACA-3', 5'-CGGCCGGTTAACCCGGGAACT-3', 5'-GGGCCGGTGCAAAGACCATC-3', 5'-GCTCATCAACCCGTCGACCAACA-3', 5'-AAATACGCGGCGCCCGGGGACGCTTAT-3', 5'-GCGCCGGGGGGGGGCGCCCGGTATCA-3', 5'-GCGCCGGCGCTGACGACAGCACCAGC-3', and 5'-CGGCGACGCGTATGAAGCCACCGTC-3', respectively. Hereafter, these altered NIRs will be abbreviated as follows: E46S, E58A, E89S, E113A, E118A, E160A, E197A, D201A, E204A, and D205A, respectively. We also constructed another NIR, E118A/E197A/D201A, in which Glu-118, Glu-197, and Asp-201 were simultaneously replaced by Ala. DNA sequencing of all the mutated genes was carried out by the M13 dye exon chain termination method (13, 14) to confirm the mutations introduced by the mutagenesis.
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ENZYME ACTIVITIES OF ALTERED NIRs

The enzyme activities were determined by the method of Kakutani et al. (2) as described under "Experimental Procedures."

| NIRs        | Specific activities | Relative activities |
|-------------|---------------------|---------------------|
| Wild type   | 372 ± 10            | 100                 |
| E46S        | 299 ± 6             | 80                  |
| E58A        | 217 ± 10            | 58                  |
| E89S        | 206 ± 14            | 55                  |
| E113A       | 46 ± 2              | 12                  |
| E118A       | 95 ± 6              | 26                  |
| E160A       | 123 ± 1             | 33                  |
| E197A       | 174 ± 8             | 47                  |
| D201A       | 176 ± 10            | 47                  |
| E204A       | 116 ± 1             | 31                  |
| D205A       | 139 ± 2             | 37                  |
| E118A/E197A/D201A | 63 ± 8       | 17                  |

Electron Transfer between Altered NIRs and Wild-type Pseudoazurin—We determined next the apparent kinetic parameters of electron transfer between pseudoazurin and wild-type or altered NIRs by steady-state kinetics according to the method described under "Experimental Procedures." As shown in Table II, the \( k_{\text{cat}} \) values of several altered NIRs for pseudoazurin were significantly increased, suggesting decreased affinity in these altered NIRs; E113A, E197A, D201A, E204A, and D205A showed a 2.0-, 2.2-, 2.1-, and 2.3-fold increase in the \( k_{\text{cat}} \) value, respectively. E113A showed a small increase in the \( k_{\text{cat}} \) value, while no change in the \( k_{\text{cat}} \) value was observed in the other altered NIRs (E160A, E46S, E58A, and E89S). Furthermore, multiple replacement of the three residues, Glu-113, Glu-197, and Asp-201, in the single subunit caused a further increase in the \( k_{\text{cat}} \) value (13.9-fold). These results suggested that the negatively charged residues around the type 1 copper site, Glu-118, Glu-197, and Asp-201, in the single subunit caused a further increase in the \( k_{\text{cat}} \) value. This increase in the \( k_{\text{cat}} \) value suggested the possibility that Glu-113 may also be involved in interaction with pseudoazurin.

As described above, most of the replacements caused a decrease in \( \text{NO}_2^- \) reducing activity, suggesting that the replacements had some effect on the type 2 copper site, where \( \text{NO}_2^- \) reduction occurs. Since the \( \text{NO}_2^- \) reduction is the rate-limiting step in the steady-state assay system monitoring the overall reaction (20), a decrease in the \( k_{\text{cat}} \) value in the altered NIRs was anticipated in this study. As shown in Table II, the \( k_{\text{cat}} \) values of the altered NIRs with pseudoazurin were reduced by the replacements, similar to those in the assay measuring directly nitrite reduction as described above.

Identification of Electrostatic Pairs in the Electron Transfer

RESULTS

Spectroscopic Properties of Altered NIRs—All the altered NIRs were expressed in E. coli and purified to homogeneity on SDS-polyacrylamide gel electrophoresis (data not shown). In the oxidized state, wild-type NIR shows two intense absorption peaks at 462 and 583 nm along with a broad one at 690 nm in the optical spectrum, which is characteristic of the type 1 copper of this enzyme (11). When optical spectra of the altered NIRs were analyzed, no significant changes in the spectra were observed. This indicated that the environment of the type 1 copper site of each altered NIR was similar to that of wild-type NIR. To examine the structure of altered NIRs, we next measured CD spectra of each of the altered NIRs. All the altered NIRs showed CD spectra identical to that of wild-type NIR (data not shown), suggesting that overall structure was not distorted by the replacements.

Enzyme Activities of Altered NIRs—Specific activities of the altered NIRs were determined using dithionite/methyl viologen as an electron donor to NIR. As described above, spectral analysis of these altered NIRs suggests that no gross change in both overall structure and environment of the type 1 copper site was caused by the replacements. However, most of the altered NIRs had reduced activities (Table I). E46S showed about 80% activity compared with wild-type NIR. E58A, E89S, E197A, and D201A possessed 50–60% activities, while E118A, E160A, E204A, and D205A showed 30–40% activities. The largest decrease in the enzyme activity was observed with E113A and E118A/E197A/D201A, which showed only 10–20% enzyme activities. In addition, E113A seemed to be unstable since the activity was further reduced during long storage of the enzyme at 4 °C (data not shown).

Electron Transfer between Altered NIRs and Wild-type Pseudoazurin—We determined next the apparent kinetic parameters of electron transfer between pseudoazurin and wild-type or altered NIRs by steady-state kinetics according to the method described under "Experimental Procedures." As shown in Table II, the \( k_{\text{cat}} \) values of several altered NIRs for pseudoazurin were significantly increased, suggesting decreased affinity in these altered NIRs; E113A, E197A, D201A, E204A, and D205A showed a 2.0-, 2.2-, 2.1-, and 2.3-fold increase in the \( k_{\text{cat}} \) value, respectively. E113A showed a small increase in the \( k_{\text{cat}} \) value, while no change in the \( k_{\text{cat}} \) value was observed in the other altered NIRs (E160A, E46S, E58A, and E89S). Furthermore, multiple replacement of the three residues, Glu-113, Glu-197, and Asp-201, in the single subunit caused a further increase in the \( k_{\text{cat}} \) value (13.9-fold). These results suggested that the negatively charged residues around the type 1 copper site, Glu-118, Glu-197, and Asp-201, in the single subunit caused a further increase in the \( k_{\text{cat}} \) value. This increase in the \( k_{\text{cat}} \) value suggested the possibility that Glu-113 may also be involved in interaction with pseudoazurin.
Complex between NIR and Pseudoazurin—Our previous study revealed that several lysine residues surrounding the type 1 copper site of pseudoazurin are involved in the interaction with NIR (9). Furthermore, the involvement of glutamic and aspartic acid residues of NIR in the interaction with pseudoazurin has been shown in the present study. These observations suggest an electrostatic interaction between NIR and pseudoazurin. To identify specific electrostatic pairs between the two proteins, we next carried out the same kinetic analysis using the altered pseudoazurins, K10A, K57A, or K77A, which were already shown to possess increased \( K_m \) values compared with wild-type pseudoazurin (9). Although replacement of Lys-38 of pseudoazurin was also shown to cause an increase in the \( K_m \) value, we could not use K38A pseudoazurin for the kinetic analysis due to the low yield of this protein. Among the altered NIRs possessing the replacements that caused increases in the \( K_m \) values for pseudoazurin, three NIRs, E118A, E197A, and D201A, were chosen; and electron transfer between each of the altered pseudoazurins and the altered NIRs was analyzed in the same way as described above (Table III). In this assay system, it is possible to show putative pairs of charged residues that are involved in the electrostatic interaction by comparing the \( K_m \) values. When a replacement of two residues that do not interact with each other is introduced, a cumulative effect of the replacements is expected. On the other hand, simultaneous replacement of a pair of residues interacting with each other would cause no such additive effect on the apparent affinity. For example, the \( K_m \) value of NIR for pseudoazurin was increased 2.2-fold by the replacement of K10A pseudoazurin and was increased 2.0-fold by the introduction of E118A to NIR. When the kinetic analysis was carried out with E118A NIR and K10A pseudoazurin, a 3.9-fold increase in the \( K_m \) value was observed. This suggested no interaction between the two residues. On the other hand, the replacement of Glu-197 of NIR by Ala did not cause a further increase in the \( K_m \) value for K10A pseudoazurin, and the \( K_m \) value of E197A NIR for K10A pseudoazurin was almost the same as that of wild-type NIR for K10A pseudoazurin (2.0- and 2.2-fold increases, respectively). This suggests that Glu-197 of NIR could interact with Lys-10 of pseudoazurin in the electron transfer complex. By similar analysis, a possible interaction of Glu-118 of NIR with Lys-77 and/or Lys-57 is suggested.

**DISCUSSION**

In a previous study, we showed that replacement of four lysine residues (Lys-10, Lys-38, Lys-57, and Lys-77) that form a ring surrounding the type 1 copper site of pseudoazurin increased the \( K_m \) value of NIR, suggesting that these residues of pseudoazurin are necessary in docking to NIR and subsequent efficient electron transfer. These results suggest the involvement of electrostatic interaction between pseudoazurin and NIR. We previously showed that the type 1 copper atom of NIR served as a recipient of electrons from pseudoazurin (11). Therefore, residues around the type 1 copper site of NIR were deemed good candidates for mutagenesis. We chose 10 negatively charged residues (8 glutamic acids and 2 aspartic acids) located mainly on the surface close to the type 1 copper site of NIR, replaced each independently by a non-charged residue (alanine or serine), and analyzed their apparent kinetic parameters for pseudoazurin. When the effects of the replacement of the negatively charged residues were examined, apparent increases in the \( K_m \) value for pseudoazurin were observed for replacement by alanine of Glu-118, Glu-197, Asp-201, Glu-204, or Asp-205, which are close to the type 1 copper site of NIR. These results confirm that both copper proteins interact with each other through the regions close to their type 1 copper sites and strongly suggest electrostatic interactions in an electron transfer complex of the two copper-containing proteins.

When the amino acid sequences of copper-containing NIRs from A. cycloclastes (21) and A. faecalis S-6 (6) are compared, they show that Glu-118, Glu-197, Glu-204, and Asp-205 are conserved, and the other position, 201, is occupied by an acidic residue, Glu or Asp. This suggests that the recognition of positively charged residues of pseudoazurin by negatively charged ones of NIR may be a common feature for the nitrite-reducing complex. In Pseudomonas aureofaciens NIR, however, only Asp-205 is conserved, and the other residues are occupied by non-polar residues (22). It is notable that the NIRs from A. cycloclastes (23) and A. faecalis S-6 (2) are green in color, whereas the NIRs from P. aureofaciens (24) and Alcaligenes xylosoxidans (25) are blue. For the former NIRs, pseudoazurins were found as the redox partners (3, 26), while azurin serves as a redox partner for the latter NIRs (24, 27). Both pseudoazurins from A. faecalis S-6 and A. cycloclastes have a lysine ring around their type 1 copper sites, and all the lysine residues that were shown to be involved in the interaction with NIR are conserved in both pseudoazurins (8), whereas azurin has no such ring on the surface (28). Although both NIR with pseudoazurin and NIR with azurin pairs function in comparable dissimilatory nitrite reduction systems, the interaction of blue NIR with its redox partner may be different from the interaction of green NIR with pseudoazurin.

Each replacement of 10 negatively charged residues on the surface of the molecule caused reduced specific activity for nitrite reduction, although no gross change in the overall structure was observed by spectral analyses. Since it is found that depletion of the type 2 copper of NIR from A. cycloclastes causes loss of the enzyme activity (29), one might suppose that the low

### Table II

| NIRs   | \( k_{cat} \) | \( K_m \) |
|--------|---------------|-----------|
| Wild type | 387 ± 8       | 50 ± 3    |
| E160A  | 121 ± 2       | 56 ± 3    |
| E46S   | 221 ± 7       | 41 ± 3    |
| E58A   | 223 ± 10      | 43 ± 5    |
| E895   | 93 ± 2        | 49 ± 2    |
| E112A  | 26 ± 3        | 73 ± 22   |
| E204A  | 163 ± 7       | 106 ± 9   |
| D205A  | 195 ± 12      | 115 ± 14  |
| E118A  | 111 ± 3       | 101 ± 5   |
| E197A  | 238 ± 16      | 108 ± 14  |
| D201A  | 217 ± 19      | 104 ± 18  |
| E118A/E197A/D201A | 29 ± 5 | 692 ± 140 |

### Table III

| Pseudoazurins | NIRs  | \( k_{cat} \) | \( K_m \) |
|---------------|-------|---------------|-----------|
| Wild type     | E118A | 111 ± 3       | 101 ± 5   |
|               | E197A | 238 ± 16      | 108 ± 14  |
|               | D201A | 217 ± 19      | 104 ± 18  |
|               | E118A | 56 ± 2        | 140 ± 9   |
| K10A          | E118A | 49 ± 5        | 196 ± 33  |
|               | E197A | 131 ± 13      | 101 ± 11  |
|               | D201A | 135 ± 6       | 156 ± 13  |
| K57A          | E118A | 65 ± 2        | 122 ± 7   |
|               | E197A | 106 ± 5       | 149 ± 13  |
|               | D201A | 99 ± 5        | 221 ± 17  |
| K77A          | E118A | 128 ± 5       | 183 ± 12  |
|               | E197A | 106 ± 8       | 268 ± 33  |

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capable of interacting with Glu-204 and/or Asp-205. The suggested orientation of pseudoazurin relative to NIR positions the molecule so that the type 1 copper in each would be 14–15 Å apart. However, the northern histidines of each are not directly apposed as we once thought. Intriguingly, His-81 (pseudoazurin) is within hydrogen-bonding distance of the carbonyl oxygen of residue Thr-92 (NIR), three residues away from the copper ligand His-95. This is similar to a pathway seen in the cupredoxin-like subunit II of Paracoccus cytochrome oxidase, the initial recipient of electrons from cytochrome c. In this subunit, His-224, a ligand to the binuclear Cu₅ site, lies within hydrogen-bonding distance of a carbonyl oxygen (Arg-473) of subunit I. Arg-473 is in turn hydrogen-bonded to the propionate of heme a₃, and the peptide containing the carbonyl is hydrogen-bonded to a propionate of heme a, thus providing electron transfer pathways to both of the other redox centers in this protein (30). Both our proposed model and the cytochrome oxidase subunit II structure are consistent with a recent study providing direct evidence that hydrogen bonds are more important than previously believed in electron transfer pathways (31). Clearly, a structure of a complex of NIR and pseudoazurin would clarify our model. Site-directed mutagenesis in this study revealed involvement of electrostatic interaction between these two copper-containing proteins for the optimal electron transfer. The complex presented here will be a good guide, for the next site-directed mutagenesis, to understand the mechanism of intermolecular electron transfer.

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