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Type 1 Cu Structure of Blue Nitrite Reductase from *Alcaligenes xylosoxidans* GIFU 1051 at 2.05 Å Resolution: Comparison of Blue and Green Nitrite Reductases[1]

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The crystal structure of the blue nitrite reductase from *Alcaligenes xylosoxidans* GIFU 1051 (AxgNIR) has been determined at 2.05 Å resolution. AxgNIR contains both type 1 and 2 Cu sites, the geometry of the former being distorted tetrahedral. The superpositioning of the type 1 Cu sites in the blue enzyme and a green nitrite reductase revealed that the orientation of the Met150 side chain differed. The deviation of the S_δ(Met150) atom from the axial position of the NNS plane formed by two N_δ(His95 and His145) and one S_β(Cys136) atom caused the difference in the colors of the enzymes, *i.e.* blue and green.

Key words: nitrite reductase, *Alcaligenes xylosoxidans* GIFU, type 1 copper, three-dimensional structure, X-ray crystal analysis.

Denitrification comprise the dissimilatory reduction on a nitrate or nitrite ion to produce usually dinitrogen by prokaryotic organisms (*1*). Nitrite reductase (NIR) is a key enzyme in the anaerobic respiratory pathway of denitrifying bacteria, in which nitrate is reduced to gaseous products (NO, N_2O, and N_2). There are two classes of NIRs, one containing two hemes (heme c, d_1) and the other containing two types of copper as redox-active centers (*2, 3*). About ten Cu-containing NIRs from a variety of denitrifying bacteria have been characterized up to now. The first Cu NIR was found in *Alcaligenes xylosoxidans* NCIB 11015 by Iwasaki *et al.* (*4, 5*). The blue NIRs from *A. xylosoxidans* GIFU 1051 [AxgNIR (*6*)], *A. xylosoxidans* NCIB 11015 [AxnNIR (*7, 8*)], and *Pseudomonas aureofaciens* [PsaNIR (*9*)] exhibit an intense *ca.* 590 nm absorption band. They show EPR signals with an axial symmetry like those of plastocyanin and azurin (*10, 11*). On the other hand, the NIRs from *Achromobacter cycloclastes* IAM 1013 [AciNIR (*12, 13*)], *Alcaligenes faecalis* S-6 [AfsNIR (*14*)], *Rhodobacter sphaeroides* forma sp. *denitrificans* (*15, 16*), and *R. sphaeroides* 2.4.3 (*17*) display two strong absorption bands near 460 and 600 nm, and rhombic EPR signals due to the type 1 Cu site. These NIRs are green or bluish green.

Comparison of the primary structures of four NIRs (blue AxgNIR and PsaNIR, and green AfsNIR and AciNIR) showed that AxgNIR exhibits *ca.* 80% sequence identity with PsaNIR, and *ca.* 70% sequence identity with AfsNIR and AciNIR (Kataoka, K. and Suzuki, S., unpublished results). X-ray crystal structure analyses of Cu NIRs demonstrated that AciNIR (*18, 19*), AfsNIR (*20*), and AxnNIR (*21*) are similar trimers with one type 1 Cu (blue copper) in each subunit and one type 2 Cu (nonblue copper) between two subunits (*18-21*). Although high resolution X-ray studies have been performed on green AciNIR and AfsNIR at 1.6 and 1.85 Å resolution, respectively, the structure of blue AxnNIR was determined at 3.0 Å resolution (*21*). In this report, we describe the 2.05-Å crystal structure of blue AxgNIR, focusing on the type 1 Cu site different from in green AfsNIR.

The isolation and purification of AxgNIR were carried out by the methods reported previously (*6*). Single crystals of AxgNIR were grown by the hanging-drop vapor-diffusion method with PEG4000 as the precipitant at 20°C. A 4 μl droplet of a 5 mg/ml protein solution comprising 0.1 M Tris-HCl (pH 8.5), 0.2 M sodium acetate, and 12% PEG-4000, was set as a hanging-drop, against 500 μl of a reservoir solution comprising the same buffer and 24% PEG4000. Blue hexagonal-shaped crystals grew up to the size of $0.25 \times 0.25 \times 0.4$ mm at 293 K within 1 week. The X-ray diffraction data for AxgNIR were collected at room temperature with an RAXIS-IIc imaging-plate system (Rigaku) using CuK_α radiation, $\lambda = 1.5418$ Å. A single crystal was used for data collection with the crystal-to-detector distance of 100 mm. The AxgNIR crystal diffracted up to at least 2.0 Å resolution and was stable in the X-ray beam. The space group was determined to be hexagonal $P6_3$ with unit cell parameters of $a = b = 106.56$ Å, and $c = 63.58$ Å. With one molecule of AxgNIR per asymmetric unit, the crystal density (Vm) was calculated to be 2.77 Å^3/Da,

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Abbreviations: NIR, nitrite reductase; AxgNIR, NIR from *Alcaligenes xylosoxidans* GIFU 1051; AxnNIR, NIR from *Alcaligenes xylosoxidans* NCIB 11015; PsaNIR, NIR from *Pseudomonas aureofaciens*; AciNIR, NIR from *Achromobacter cycloclastes* IAM 1013; AfsNIR, NIR from *Alcaligenes faecalis* S-6.

which is close to the average for proteins (22). A data set up to 2.05 Å resolution has been collected with 87.3% completeness. The total 89,691 reflections collected were reduced to 23,309 independent reflections with an R_{merge} of 5.4%. The last resolutional shell (2.25-2.05 Å) has a completeness of 78.7% and an R_{merge} of 18.2%. Image data were processed using *DENZO & SCALEPACK* (*23*) software.

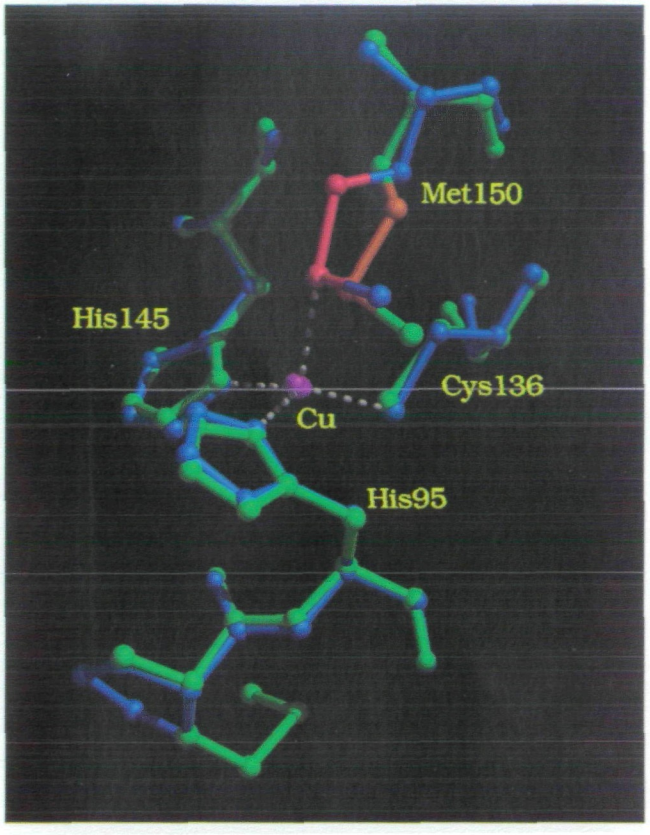

Fig. 1. **The superposed structures of the type 1 Cu sites in AxgNIR (blue) and AfsNIR (green) drawn with *MIDAS* (*30*, *31*).** The C_γ and S_δ atoms in AxgNIR are shown in red, and the corresponding atoms in AfsNIR are shown in orange. The r.m.s. deviation of the three strong ligands, N_δ(His95), S_γ(Cys136), and N_δ(His145), is only 0.04 Å.

The crystal structure of AxgNIR was solved by the molecular replacement method using *AMoRe* (*24*) in the *CCP4* program suite (*25*). The molecular structure of AciNIR refined at 2.0 Å resolution was used as the starting model. AxgNIR and AciNIR exhibit 70% sequence identity. The rotation function parameters were found to be $\alpha = 12.06°$, $\beta = 83.77°$, and $\gamma = 122.43°$, and the translation parameters were $x = 0.136$, $y = 0.5586$, and $z = 0$. The *R*-factor for the model structure was calculated to be 33.5% for 8-4 Å data. The molecular dynamics program of *XPLOR* (*26*) and the least-squares refinement program of *REFMAC* (*27*) were applied to the AxgNIR structure. The current structure of AxgNIR includes 2,549 protein atoms (non-hydrogen), two metal ions, and 111 water molecules. The final *R*-factor for 20,280 reflections between 10.0 and 2.05 Å was 18.0% with an R_{free} factor (*28*) of 22.6% for 5% of the total data within the same resolution range. The last cycle of the refinement gave a reasonable stereochemistry. The model stereochemistry was analyzed using the *PROCHECK* suite (*29*). A Ramachandran plot of the conformational angles of φ/ψ for the molecule was also reasonable. The root mean-square (r.m.s.) deviations from standard values are 0.009 Å for bond distances (1-2 distance), 0.030 Å for angle distances (1-3 distance), and 0.028 Å for dihedral angles (planar 1-4 distance). The expected atomic coordinate error was estimated to be about 0.23 Å from a Luzzati plot.

The overall structure of AxgNIR is trimeric, being the same as those found on structural analyses of AciNIR, AfsNIR, and AxnNIR (*18-21*). The r.m.s. deviation for the superposed backbone structures of AxgNIR and AciNIR, AfsNIR, or AxnNIR is 0.79, 0.68, or 0.53 Å, respectively.

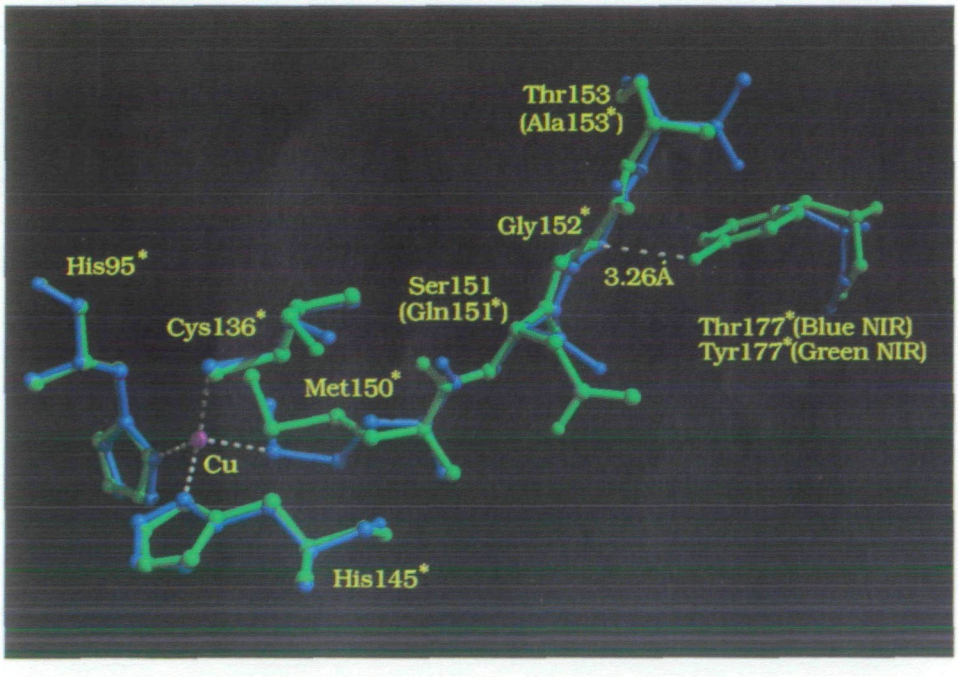

Fig. 2. **The superimposed structures around the loop containing the Met150 ligand in AxgNIR (blue) and AfsNIR (green).** The amino acid residues are shown on the upper and lower sides for AxgNIR and AfsNIR, respectively. The amino acid residues with asterisks are the conserved ones in blue or green NIRs. The OH(Tyr177) atom is located at a distance of 3.26 Å from the C_α(Gly152) atom in green AfsNIR. The corresponding distance of $O_{\gamma 1}$(Thr177)-C_α(Gly152) was calculated to be 6.65 Å in blue AxgNIR.

While the backbone structure of AxgNIR is quite similar to those of other green NIRs, the difference of the type 1 Cu site is quite remarkable. The type 1 Cu site lies *ca.* 8 Å from the protein surface. Residues His95, Cys136, His145, and Met150 in AxgNIR form a distorted tetrahedron, as shown in Fig. 1. The distance parameters are 2.11 Å for Cu-N_δ(His95), 2.18 Å for Cu-S_γ(Cys136), 2.00 Å for N_δ(His145), and 2.62 Å for Cu-S_δ(Met121). It was presumed that the Cu-S_δ(Met) bond length might be correlated with the difference in color between blue AxnNIR and green AciNIR (*19*), but the distances of Cu-S_δ(Met150) in blue AxgNIR and AxnNIR are quite similar to those (2.59-2.64 Å) in green NIRs. The type 1 Cu sites of green NIRs are flattened tetrahedral and the displacement of Cu from the NNS plane formed by strong 2N(His) and S(Cys) ligands is *ca.* 0.5 Å (*19, 20*). Dodd *et al.* suggested that the small displacement ($\leq$0.2 Å) of Cu from the NNS plane in AxnNIR at 3.0 Å resolution is perhaps the cause of the difference in color observed for an otherwise "classical" blue Cu center (*21*). However, the distance of displacement of Cu from the NNS plane was calculated to be 0.6 Å in the same blue NIR, AxgNIR, at 2.05 Å resolution.

When the type 1 Cu site of AxgNIR is superposed on that of green AfsNIR, the r.m.s. deviation of three strong ligands, N_δ(His95), S_γ(Cys136), and N_δ(His145), is only 0.04 Å. However, the orientation of the Met150 side chain in AxgNIR appears to be different from that observed in AfsNIR. The dihedral angle between the two planes defined by the C_β-C_γ-S_δ side chain was estimated to be 77°. On the other hand, the superposed structure of AciNIR shows that the conformation of Met150 is almost the same as that of AfsNIR and the dihedral angle was estimated to be 87°.

The superpositioning of the backbone structures around the Met loops revealed that the main-chain from Met150 to Gly152 in blue AxgNIR is shifted compared with in green AfsNIR (Fig. 2). The distance between the C_α(Met150) atoms was estimated to be 0.4 Å, while the corresponding distance was estimated to be 0.6 Å between AxgNIR and AciNIR. This displacement of the main-chain might be due to the difference in the 177th amino acid residue, Thr and Tyr, respectively, conserved in all blue and green NIRs reported so far. That is to say, the C_α(Met150) atom of AxgNIR is remote from the type 1 Cu center compared with that of AfsNIR or AciNIR containing a bulky Tyr residue, and the ligand, S_δ(Met150), is located in a nearly axial position as to the NNS plane to form the distorted tetrahedral geometry of the type 1 Cu site. The bulkiness of the 177th amino acid residue might regulate the color of NIRs, *i.e.* blue or green.

The type 2 Cu site in AxgNIR is located at the interface between two subunits like those in other NIRs (*18-21*). Three histidine residues provide three ligands *via* $N_{\epsilon 2}$ atoms of the imidazole rings. The fourth ligand is an oxygen from either a water or hydroxyl ion. The geometry of the site in AxgNIR is distorted tetrahedral like those of green AfsNIR and AciNIR. The copper-to-His ligand distances are 2.07 (His100), 2.18 (His135), and 2.27 (His306) Å. However, the solvent copper ligand is located at a distance of 2.55 Å from the type 2 Cu, which is relatively longer than in other NIRs (1.70-1.90 Å). The angle parameters for N(His100)-Cu-O, N(His135)-Cu-O, and N(His306)-Cu-O are 164, 81, and 94°, respectively. Since the contribution of the type 2 Cu site to the visible absorption spectrum is quite low (*6*), the difference in the coordination of the fourth ligand is not concerned with the enzyme color.

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