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Crystal structure of a NO-forming nitrite reductase mutant: an analog of a transition state in enzymatic reaction

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Abstract

I257E was obtained by site directed mutagenesis of nitrite reductase from *Achromobacter cycloclastes*. The mutant has no enzyme activity. Its crystal structure determined at 1.65 Å resolution shows that the side-chain carboxyl group of the mutated residue, Glu257, coordinates with the type 2 copper in the mutant and blocks the contact between the type 2 copper and its solvent channel, indicating that the accessibility of the type 2 copper is essential for maintaining the activity of nitrite reductase. The carboxylate is an analog of the substrate, nitrite, but the distances between the type 2 copper and the two oxygen atoms of the side-chain carboxyl group are reversed in comparison to the binding of nitrite to the native enzyme. In the mutant, both the type 2 copper and the Nε atom on the imidazole ring of its coordinated residue His135 move in the substrate binding direction relative to the native enzyme. In addition, an EPR study showed that the type 2 copper in the mutant is in a reduced state. We propose that mutant I257E is in a state corresponding to a transition state in the enzymatic reaction.

Keywords: Anolog of transition state; Copper-containing nitrite reductase; Crystal structure; Enzyme activity; Site-directed mutagenesis

NONIRs (NO-forming nitrite reductases) are involved in the terrestrial nitrogen cycle by reducing nitrite ions to nitric oxide in the dissimilatory metabolism pathway of denitrifying bacteria [1]. There are two kinds of NONIRs containing either heme (heme-containing nitrite reductase, cd_1NIR) or copper (copper-containing nitrite reductase, CuNIR) as cofactors. These two enzymes play the same role but differ from each other in amino acid composition and electron-transfer pathway [2]. *Ac*NIR is a CuNIR purified from *Achromobacter cycloclastes*. Structural studies show [3] that the functional unit of the enzyme is a trimer with a total of six copper atoms. It is similar to CuNIR purified from *Alcaligenes faecalis* (*Af*NIR) [4] and *Alcaligenes xylosoxidans* (*Ax*NIR) [5]. Copper atoms in the trimer can be further classified into two types: the type 1 copper is buried in each monomer and lies ~4 Å from the Connolly surface in *Ac*NIR [3], 6–8 Å away in others [6,7]. The type 2 copper lies between the monomers, sitting at the bottom of a 12 Å deep solvent channel. The type 1 copper accepts electrons donated by other copper proteins such as pseudoazurin or azurin. The type 2 copper has been considered to be the catalytic center of the enzyme, since the enzyme activity is closely related to its content [8,9], and the substrate, nitrite, can bind to it to become its fourth ligand [3,10]. According to the substrate binding forms in crystal structures of the native enzyme [7,11,12], it is generally considered that the transitional complex of the CuNIR reaction is a pentacoordinate complex with a reduced type 2 copper at the center with nitrite coordinated to the copper via its oxygen atoms in an asymmetric bidentate manner [2,13]. However, such a complex seems to contradict the high stability [14,15] of complex Cu^+–NO^+ in the reaction. The electron transfer between the type 1 copper and its

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donor is accomplished through protein interaction. The distance between the type 1 copper and the copper atom in the donor is much larger than the range of ordinary chemical bonds and they need no direct contact. It is still not clear if such long-distance interaction also exists between the substrate and the type 2 copper. In order to clarify if the direct binding of substrate to the type 2 copper is essential for CuNIR activity and to further explore the intramolecular electron-transfer mechanism after substrate binding, a mutant I257E was constructed and its crystal structure was determined at 1.65 Å resolution.

Materials and methods

Molecular biology. Site-directed mutagenesis was used to synthesize an oligonucleotide primer containing the desired nucleotide mutation: 5′-CCGCACCTGGAAGGCGGGCATTGG-3′ to construct the mutant, I257E. The original isoleucine at position 257, which is the hydrophobic residue nearest to the type 2 copper [11], was mutated to glutamic acid. The mutant gene was obtained using Gene-Editor (Promega) together with the plasmid containing the *Ac*NIR gene constructed as reported earlier [16]. The expression was performed using the 6xHis tag system (Qiagen) [17]. A precast SDS–PAGE system (Novagen) was used to identify the expressed refolded protein. The mutated protein was prepared and purified as before [18] and the enzyme activity was determined as reported earlier [16].

Biochemical properties. The expressed mutant protein showed no obvious differences to the wild type protein during purification. Its molecular weight determined by mass spectrometry was exactly as predicted (data not shown). The refolded protein migrated as a trimer in non-reducing Tris–tricine buffered SDS–PAGE just as the native *Ac*NIR (data not shown). In addition, the absorption spectrum of the mutated protein in the range of 400–700 nm did not change significantly relative to that of the native protein, indicating that the microenvironment around the copper atoms did not change significantly. However, the mutant enzyme showed no nitrite reductase activity (data not shown).

Crystallization and data collection. Crystals of mutant I257E suitable for X-ray studies were obtained in about 2–4 weeks at 15 °C by the hanging drop vapor-diffusion method used for wild type *Ac*NIR [11,19] with modification. The drop was composed of 2 μl of protein solution (9 mg ml^{-1}) and an equal volume of precipitant (0.1 M phosphate buffer at pH 4.0, 33% saturated ammonium sulfate, and 5% MPD) hanging over 1 ml of precipitant.

Diffraction data were collected using a synchrotron radiation plate-Weissenberg camera system [20] at the BL6B station in the Photon Factory at room temperature. The X-ray wavelength was 1.0 Å. The oscillation range was 3.5° per frame. The distance between the detector and crystal was 573 mm. The diffraction capability of the crystal was greater than 1.5 Å. The data processing was done using the HKL program package (DENZO and SCALEPACK) [21]. The crystal belongs to the cubic system, space group $P2_13$ with cell parameters: $a = b = c = 98.16$ Å, $\alpha = \beta = \gamma = 90°$. It is isomorphous with wild type *Ac*NIR. The data processing results are summarized in Table 1.

Structure refinement. The wild type enzyme structure at pH 5.0 (PDB code 1nie) was used as the starting model, using only the protein part and with Ile257 replaced by Ala. The phase problem was solved using the Fourier differential method. The X-plor program [22] was used to refine the structure, search for the water molecules, and calculate the electron density maps. The TURBO-FRODO program [23] was repeatedly used for model building, residue replacement, and the assignment of water molecules. Ten percent reflections were selected randomly for the calculation of free *R*-values. The refinement results were examined using the free *R*-values and the PROCHECK program [24].

Table 1
Crystallographic data for mutant I257E structure

Data statistics	
Space group and unit	$P2_13$, $\alpha = \beta = \gamma = 90°$
Cell dimensions	$a = b = c = 98.16$ Å
Temperature	Room temperature
Number of reflections	393,521
Number of unique reflections	37,954
Resolution, Å (last shell)	1.65 (1.69–1.65)
Completeness, %, $I \geqslant 2\sigma(I)$ (last shell)	86.1 (50.4)
$I/\sigma(I)$ (last shell)	48.2 (3.0)
R_{merge}, %, $I \geqslant 2\sigma(I)$ (last shell)	5.2 (25.0)
Completeness (%)	99.9
Refinement statistics	
R-value/R_{free} (%)	17.0/20.4
Resolution range of data (Å)	10–1.65
Number of reflections ($I \geqslant 2\sigma(I)$)	32,700
Number of residues	333
Number of Cu atoms	2
Number of water	167
RMS deviations from ideal	
Bond lengths (Å)	0.006
Bond angles (deg)	1.520

There are 340 residues in *Ac*NIR, but no coordinates of the first seven residues of the N-terminal can be found in all the *Ac*NIR structures of the same space group in PDB. We also did not find any electron densities of these residues in the structure of I257E. In the Ramachandran plot of the final model of I257E using program PROCHECK [24], over 90% of the residues (except Pro and Gly) were located in the most favored regions, with all others located in additional allowed regions. The nearest non-bonded distances between intermolecular and intramolecular atoms were all in the reasonable range. The statistical data for the structure refinement are summarized in Table 1. The code number of the atomic coordinates in PDB is 1KCB.

Determination of EPR spectrum. Protein solutions were prepared at pH 7.1, to a concentration of 15 mg ml^{-1} in 0.05 M phosphate buffer. EPR spectra were recorded at 77 K with a Bruker ESP 300E spectrometer at X-band frequencies. The data collection parameters were: microwave power 20 mW, microwave frequency 9.48 GHz, modulation frequency 100.000 kHz, and modulation amplitude 2.024 G.

Results and discussion

Overall structure

The replacement of Ile257 in the native enzyme by Glu has little influence on the overall protein structure. The mutant still forms trimers (Fig. 1A) in agreement with the electrophoresis result. Superposition of the main chain of I257E with that of the native protein (Fig. 1B) resulted in no significant differences in position including the position of the mutated site.

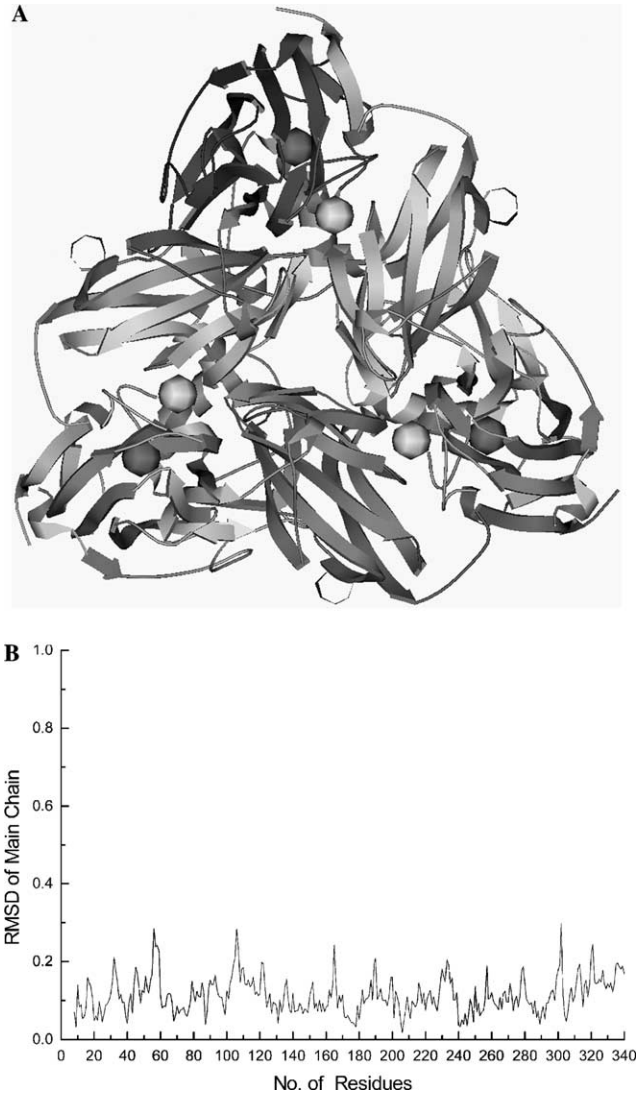

Fig. 1. Overall structure of mutant I257. (A) Schematic plot showing the mutant I257E trimer. Darker balls represent the type 1 copper and lighter balls the type 2. (B) RMSD of main chain superposition of mutant I257E with native enzyme 1nie from PDB. The value for residue 257 is 0.189.

Coordination of Glu257 to the type 2 copper

The mutant residue Glu257 has good electron density distribution in the original, final, and omit electron density maps (Figs. 2A and B). These maps show that the Glu257 side-chain densities are connected with those of the type 2 copper. The distances between the type 2 copper and the oxygen atoms of the carboxyl group of Glu257 are 2.20 and 2.63 Å, forming Cu–O coordinated linkages. No water molecule densities were found to be coordinated to the type 2 copper, indicating that in I257E the side-chain carboxyl group of Glu257 occupies the fourth ligand position of the type 2 copper, which was occupied by a water molecule in the native enzyme.

In the native enzyme, the type 2 copper is easily lost in solution [8], probably because it is directly connected to the solvent channel. Therefore, in *Ac*NIR crystal structures [11], the densities of the type 2 copper are always less than those of the type 1 copper. This becomes more pronounced at lower pH. For *Ac*NIR crystallized at pH 5.0, the type 2 copper occupancy is only 80% [11] in contrast to an occupancy of 100% in I257E crystallized at pH 4.0, in the absence of copper salt. The temperature factors of the type 1 and type 2 copper in I257E are 18.62 and 13.31, respectively. The densities of the two types of copper are almost the same, with that of type 2 being a little higher (Fig. 2C) since when Glu257 coordinates with the type 2 copper, it enhances the ability of the protein to bind the type 2 copper and blocks the contact of the type 2 copper with the solvent. The coordination buries the type 2 copper in a closed environment as it is bound more tightly to the protein. The energy barrier for replacing the coordinated side-chain of Glu257 by nitrite is then much higher than that needed to replace a water molecule as in the native enzyme. The binding of the substrate nitrite with the type 2 copper actually becomes impossible. The lack of enzymatic activity in I257E shows that the accessibility of the type 2 copper for the binding of nitrite is essential for CuNIR activity. Therefore, the interaction between the substrate and the enzyme catalytic center is more similar to a classical inorganic process instead of the long-distance electron-transfer interaction between the metal centers of proteins.

Changed coordinate distances of the type 2 copper

EXAFS studies on *Ax*NIR showed [25] that upon nitrite binding, the average Cu–His distance of the type 2 copper increased by about 0.1 Å. It was suggested that this change probably provided a trigger mechanism for electron transfer from the type 1 copper. Following EPR studies on CuNIR from *Rhodobacter sphaeroides* (*Rs* NIR), it was proposed [26] that the binding of nitrite might increase the redox potential of the type 2 copper, to favor electron transfer from the type 1 copper. ENDOR studies on *Rs*NIR showed [27] that nitrite binding would weaken the coordinating strength between the type 2 copper and its His residue ligands. However, none of these changes have been found in the native enzyme crystal structure [7,11,12]. Note that the ENDOR studies were carried out at 2.1 K and the EPR and EXAFS at 77 K, whereas the crystal structure was determined at 160 K or room temperature, and the detection time in the crystallographic method is much longer than that needed for the other physical methods. Possibly the changed coordination sphere of the type 2 copper is an unstable transition state which can only be captured at ultralow temperatures in relatively short times.

Comparison of the structure of mutant I257E with several kinds of native *Ac*NIR showed that the average

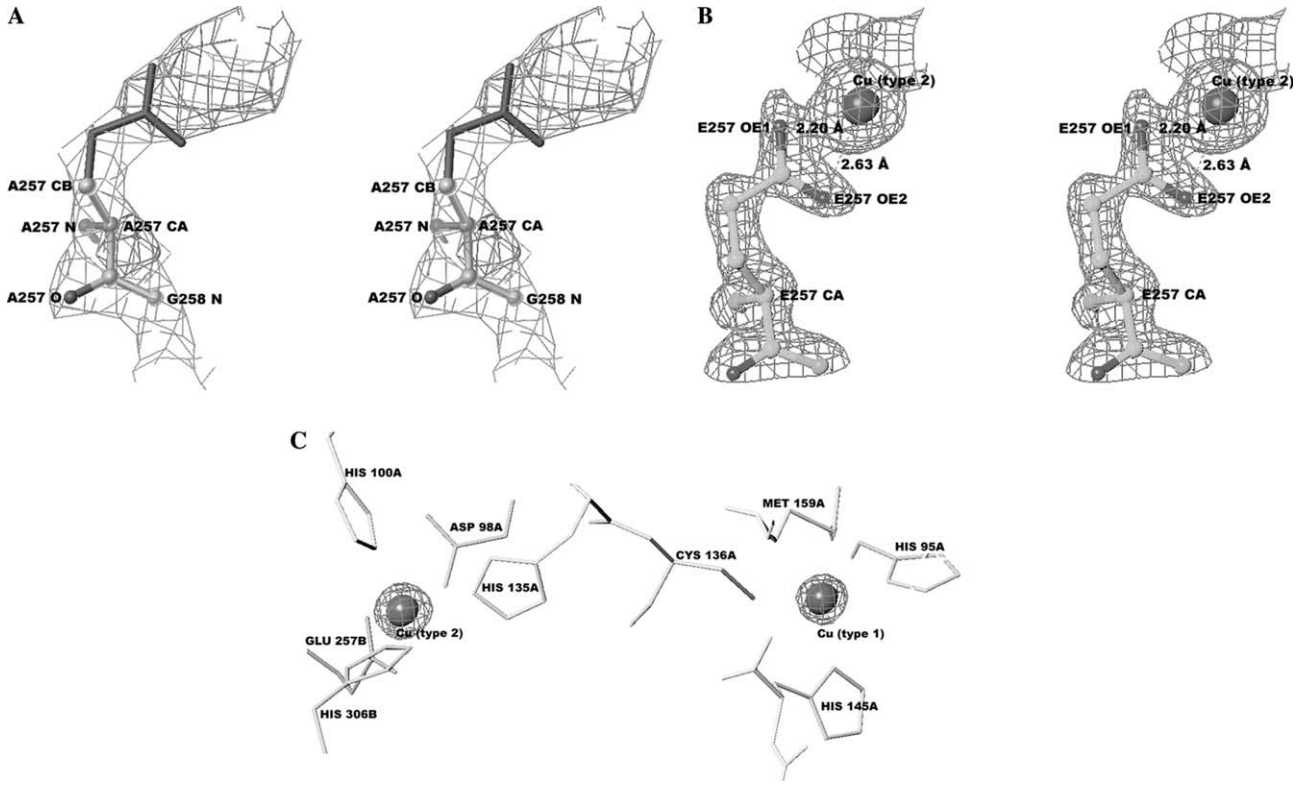

Fig. 2. Detailed structures around the type 2 copper in I257E. The 2Fo-Fc map is in lighter lines and the Fo-Fc map is in darker lines. (A) Electron density map of starting model at 2.0 Å resolution. Darker sticks show the potential side-chain of the Glu residue at mutant site 257 where it is Ala in the model. (B) Omit map of mutant I257E. The type 2 copper and all its ligands were omitted. (C) Electron density map of the type 1 and type 2 copper of mutant I257E contoured at 9σ.

Cu-His coordinate distance in the type 2 copper was obviously greater than that in the native enzyme (Table 2). Carboxylate is very similar to the substrate, nitrite. When carboxylate is coordinated with the type 2 copper, the binding of the two oxygen atoms of Glu257 is likely to be similar to the binding of the two oxygen atoms in nitrite. The results obtained at ultralow temperatures suggest that I257E may represent a relatively stable analog of the transition state in the catalytic reaction. The average coordinate distances of the type 2 copper are all greater than in the native enzyme in the structure of mutant H255N of *Af*NIR [28] and the recently reported structures [29] of the oxidized and reduced mutant with nitrite binding (Table 2).

EPR studies on I257E

Although the densities of both types of copper atoms are clearly visible in the I257E crystal structure and the content of copper is totally 5.84 atoms per trimer in I257E according to the plasma emission study, the EPR spectrum has only signals from one type of copper (Fig. 3). The EPR parameter $g_{\|} = 2.19$ ($A_{\|} = 73$G) is identical with the value for the type 1 copper [28] and I257E is

Table 2
Distances between the type 2 copper and the coordinated Nε atoms in coordinated histidine residues in different structures (Å)

	1nid[a]	2nrd[a]	1nie[a]	I257E	1as7[a]	1et8[a]	1j9s[a]	1j9t[a]
Cu2[b]–H100 Nε2	2.07	2.00	2.01	2.13	1.91	1.98	2.04	2.01
Cu2[b]–H135 Nε2	2.10	2.04	2.05	2.22	2.07	2.13	2.19	2.10
Cu2[b]–H306 Nε2	2.17	2.19	2.22	2.19	2.09	2.19	2.19	2.16
Averaged value	2.11	2.08	2.09	2.18	2.02	2.10	2.14	2.09
Resolution	2.2	2.1	1.9	1.65	2.0	1.8	1.9	1.95

[a] PDB Accession Code. 1nie is the model of the mutant I257E structure crystallized at pH 5.0, without nitrite, 1nid is *Ac*NIR with nitrite at pH 5.4, and 2nrd is without nitrite at the same pH; 1as7 is the structure of *Af*NIR, 1et8 is the mutant H255N of *Af*NIR, 1j9s is oxidized H255N with nitrite, and 1j9t is reduced H255N with nitrite.

[b] Cu2 stands for the type 2 copper.

green in color, so the EPR signals must be from the type 1 copper, indicating that in I257E, the type 1 copper is in the oxidized state and most of the type 2 copper is in the reduced state. The carboxyl group has almost no redox capability, so the reduced type 2 copper can exist stably in mutant I257E. In the native enzyme, such a valency combination is unlikely to occur, because the redox potential of the type 1 copper is higher than that of the type 2 copper [2]. Only in the transition state of the catalytic reaction just after the intramolecular electron transfer can such valency states exist. Therefore, the mutant I257E is very likely an analog of the transition state. By the way, EPR signals for the type 2 copper in mutant H255N are quite weaker than those in native *Af*NIR [28].

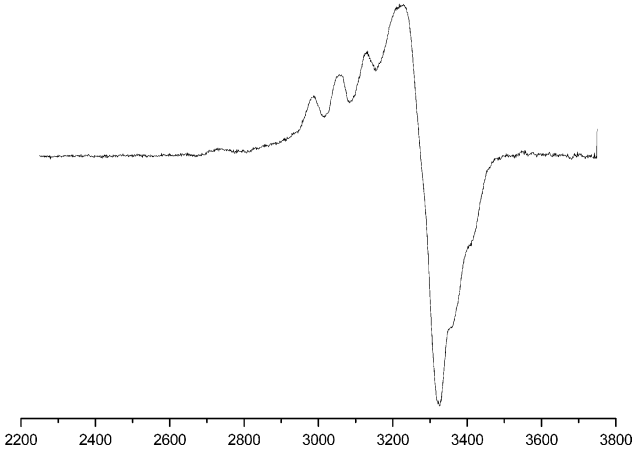

Fig. 3. EPR spectroscopy of mutant I257E.

Shift of the type 2 copper and residue His135

The structures of the type 2 copper and its surroundings in different structures are usually compared by superposition based on the copper and its ligands. This kind of superposition will average the various changes in the coordinate sphere of the type 2 copper, so it is unsuitable for examining small movements of individual parts of the coordinate sphere. Instead, the type 2 copper and its surroundings were superposed based on the Cα atoms in I257E and in two native *Ac*NIR, 1nid and 1nie (PDB Accession Code) (Fig. 4A).

No obvious differences between 1nid and 1nie were found by superposition. However, the position of the type 2 copper in I257E clearly differed from those in 1nid and 1nie (Fig. 4B). The type 2 copper of I257E moved toward the fourth ligand in the substrate binding

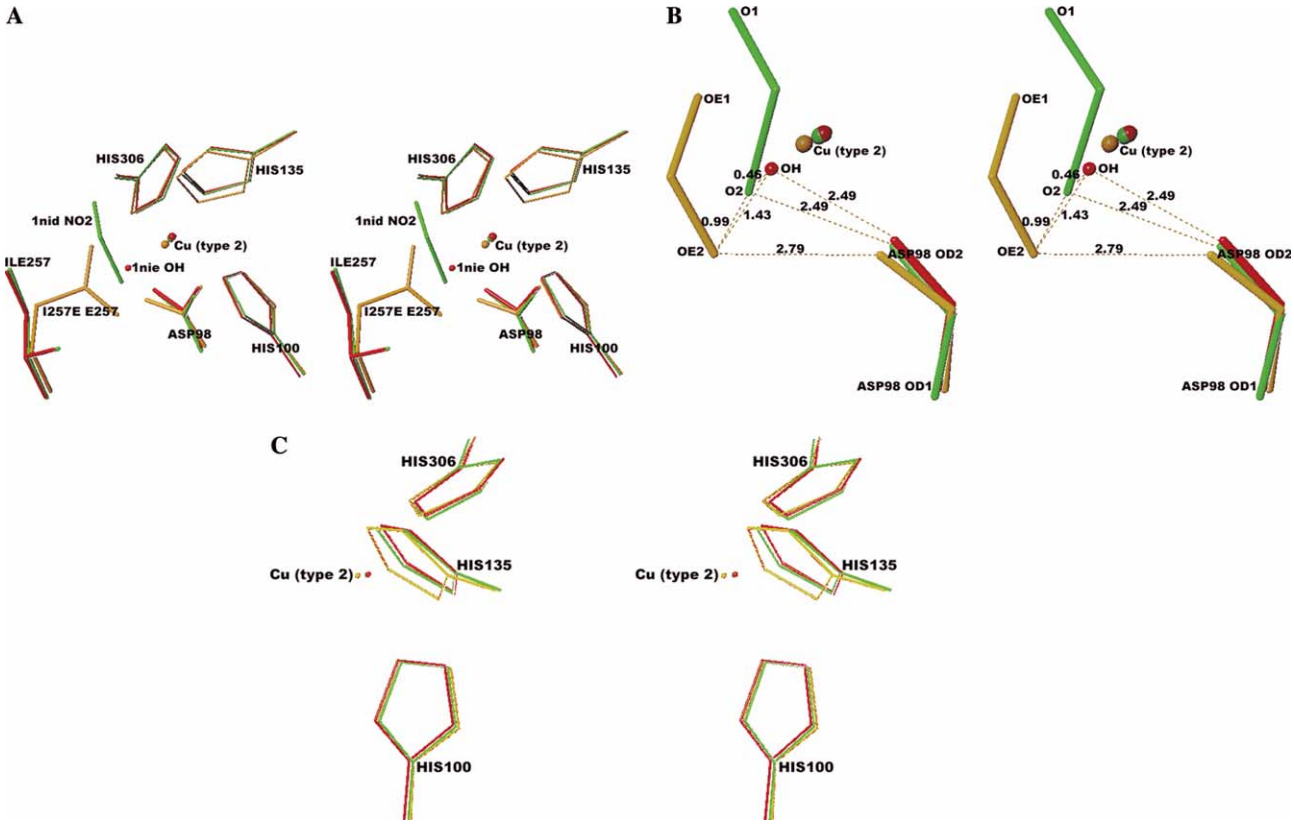

Fig. 4. Superposition of the type 2 copper and its surroundings in 1nid, 1nie, and I257E. Green stands for 1nid, red for 1nie, and yellow for I257E. (A) Overall view of the superposition. (B) Superposition of the type 2 copper, its 'fourth ligand' and Asp98. (C) Superposition of the type 2 copper and three His residues.

orientation. This shift probably represents the movement of the type 2 copper in the enzyme reaction under the influence of the substrate. The changes of the average distances between the type 2 copper and its His ligands during substrate binding [25] may well be the result of the movement of the type 2 copper.

Of the three coordinated His residues, the positions of His100 and His306 in I257E scarcely differ from those in 1nid and 1nie, but the shift of the imidazole ring of His135 is significant. The movement of its coordinated Nε atom is in the same direction as that of the type 2 copper (Fig. 4C). The Nε atom movement can be considered to be caused directly by the type 2 copper movement. The movement of the residue His135 side-chain will certainly affect the type 1 copper through residue Cys136. The intramolecular electron transfer in the catalytic reaction of CuNIR may be triggered by the substrate binding through the pathway: the type 2 copper movement, shift of the His135 side-chain, movement of the His135 main-chain, movement of residue Cys136, and the state of the type 1 copper change.

Shift of oxygen atoms in substrate

Distinct positional variances of the different fourth ligands of the type 2 copper in the three proteins were found by superposition. Surprisingly, the three oxygen atoms, namely O2 in 1nid, OH in 1nie, and Oε2 of Glu257 in I257E, form approximately a straight line. Each oxygen atom is also connected with a nearby oxygen atom, Oδ2 of Asp98 by a hydrogen bond (Fig. 4B). The side-chain carboxyl group of Asp98 is involved in the nitrite binding by providing the proton required for the reaction [11]. After the reaction, the O2 of the nitrite will become OH [2,13]. Therefore, this line probably represents the relationship among Oε2, O2, and OH which are grouped as type A. In 1nid and I257E, the fourth ligand has another oxygen atom not hydrogen bonded to Asp98. These oxygen atoms are grouped as type B. The distance of the type 2 copper from the type A oxygen atom is less than that from the type B oxygen atom in high resolution crystal structures of native enzyme [7,11,12], including the 1nid structure. However, opposite results were found in I257E, where the distances between the reduced type 2 copper and the two types oxygen atoms are 2.63 Å for Cu2–Oε2 (type A) and 2.20 Å for Cu2–Oε1 (type B). If the nitrite ion binds to the type 2 copper in this way, a stable Cu^{+}–NO^{+} complex will be favored in the reaction. It is more interesting that the oxygen atoms in nitrites which do not coordinate to the type 2 copper all belong to type A in the pentacoordinate complexes formed by the nitrite and a solvent molecule coordinated to the oxidized and reduced mutant H255N of *Af*NIR [29], corresponding to the increased coordinate distances (Table 2) and weak EPR signals [28] of the type 2 copper. There were no differences between another mutant D98N and native *Af*NIR in these three aspects [28,29].

We propose that in the enzyme reaction, there is probably a transition state in which the structure of the coordinate sphere of the type 2 copper is just like the one in mutant I257E, especially the relative position of the two oxygen atoms of the substrate and the reduced copper atom during the approach of nitrite to the type 2 copper. The transitional complex Cu^{+}–NO^{+} is formed directly from this transition state. The substrate binding forms found in crystal structures of the native enzyme [7,11,12] are probably just a stable state between the substrate nitrite and the oxidized type 2 copper.

Acknowledgments

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