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Characterization of Azide-Binding Type 2 Cu(II) Site of Nitrite Reductase

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The azide-binding type 2 Cu sites of nitrite reductases (NIRs) from denitrifying bacteria, *Alcaligenes xylosoxidans* GIFU 1051 and *Achromobacter cycloclastes* IAM 1013, have been spectroscopically characterized. The titration of two NIRs with azide indicates that two monodentate azide ions are coordinated to type 2 Cu(II). The difference between azide-treated and native NIR absorption spectra shows one absorption band near 450 nm due to N_3^- → type 2 Cu(II) charge-transfer transitions.

Nitrite reductase (NIR) is a key enzyme in the alternative respiration system of denitrifying bacteria. In the denitrifying pathway (NO_3^- → NO_2^- → NO → N_2O → N_2), NIR accepts one electron from an electron donor and catalyzes the reduction of NO_2^- to NO.[1,2] The structures of Cu-containing NIRs isolated from *Achromobacter cycloclastes* IAM 1013 (AciNIR)[3,4] and *Alcaligenes xylosoxidans* GIFU 1051 (AxgNIR),[5,6] have been determined by X-ray crystal analyses. These enzymes exhibit a high degree of amino acid sequence homology and their three-dimensional structures are very similar to each other. Each enzyme is a trimer, in which monomer (36 kDa) contains one type 1 Cu and one type 2 Cu. The type 1 Cu site is an electron acceptor from cytochrome c_{551}[7] or pseudoazurin,[1] and mediates the electron transfer to the catalytic site of type 2 Cu. The type 1 Cu site having four ligands (2His, Cys, and Met) form a flattened tetrahedron in AciNIR[3,4] and a distorted tetrahedron in AxgNIR.[5,6] The type 2 Cu site binds, with a distorted tetrahedral geometry, 3 His residues and a solvent molecule in both enzymes.

In this work, we describe the spectroscopic properties of azide-treated AxgNIR and AciNIR. Although the binding of azide to the type 2 Cu(II) center has already been reported,[8,9] the detailed characterization of the azide-binding type 2 Cu(II) site is not yet carried out.

Figure 1 shows the visible absorption spectra for AxgNIR in the absence and in the presence of azide. These spectra are completely superimposable in the range of 550–900 nm, but a difference is observed in the 400–500 nm region. This finding implies that azide dose not bind to type 1 Cu but to type 2 Cu.

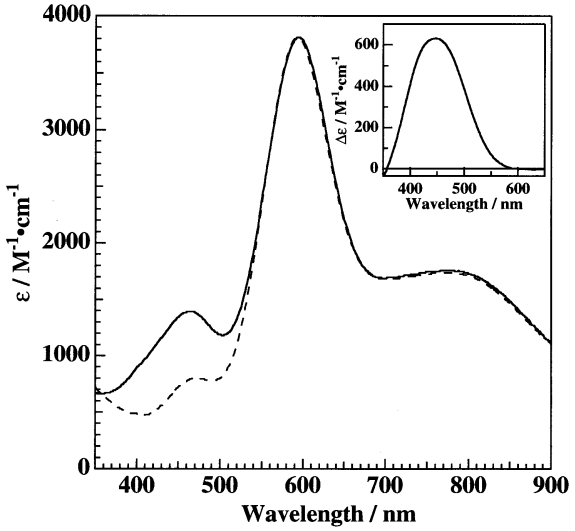

Fig. 1. Visible absorption spectra of native (broken line) and 3 equivalent azide-treated (continuous line) AxgNIRs in 0.1 M Tris-HCl buffer (pH 7.0) at 25 °C. The inset shows the difference spectrum.

The spectrum of type 2 Cu-depleted AxgNIR (type 1 Cu only) is unchanged by the addition of excess azide, as described in the previous paper.[9] The difference spectrum in Fig. 1 shows a 445-nm absorption band (ε = 630 M^{-1} cm^{-1}; M = mol dm^{-3}), which is assigned to N_3^- → type 2 Cu(II) charge transfer transitions. Azide-treated AciNIR also gives the difference absorption spectrum showing a 425-nm maximum (ε = 610 M^{-1} cm^{-1}; The ε value for AciNIR reported previously[8] should be corrected to this value). The molecular coefficients at 445 and 425 nm can be employed for the determination of the amount of type 2 Cu in these NIRs. Moreover, the catalytic activities of azide derivatives of AxgNIR and AciNIR are 3- and 1.5-fold lower than those of the corresponding native NIRs, respectively, indicating that azide binds to type 2 Cu and inhibits the catalytic reaction.

Figure 2 depicts the titration curves of AxgNIR and AciNIR with azide. The increasing absorbances of AxgNIR and AciNIR reach the constant values at the ratio of 2.5, which is explained by the 1:2 complex formation between type 2 Cu and azide. In the presence of excess azide ([azide]/[type 2 Cu] > 2.5), the type 2 Cu(II) site changes from a distorted tetrahedral geometry to a pentacoordinate geometry having three histidyl imidazoles and two monodentate azide ligands. The pentacoordinate type 2 Cu(II) sites have been reported on nitrite-treated NIRs,[4,6,10] in which the type 2 Cu(II) sites bind three histidyl nitrogen atoms and two oxygen atoms of the substrate. Moreover, on the basis of the titration curves, the formation constants (K = [type 2 Cu(II)•2N_3^-]/[type 2 Cu(II)][N_3^-]^2) were determined to be 2.4×10^4 (AxgNIR) and 2.6×10^3 (AciNIR) at pH 7.0. The binding constant of azide to type 2 Cu(II) of AxgNIR is about 10-fold larger than that for AciNIR. Such findings imply that the geometries of the pentacoordinate azide-binding type 2 Cu(II) sites in AxgNIR and AciNIR are somewhat different from each other. The difference between

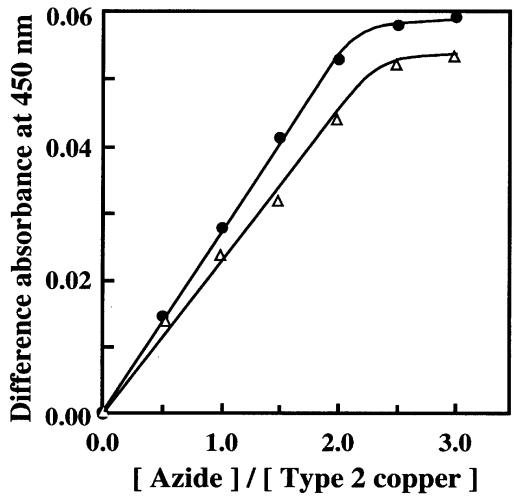

Fig. 2. Titration curves of AxgNIR (●) and AciNIR (△) with azide monitored by difference absorption at 450 nm in 0.1 M Tris-HCl buffer (pH 7.0) at 25 °C.

the absorption maxima of the N_3^- → type 2 Cu(II) charge-transfer bands also suggests the different geometries of the pentacoordinate Cu sites.

Experimental

Purification and Characterization of AxgNIR and AciNIR. The isolation and purification of AxgNIR and AciNIR were carried out by the previous methods.[8] The ratios of type 1 Cu:type 2 Cu in AxgNIR and AciNIR were estimated to be 1:0.98 and 1:0.5, respectively. Nitrite reduction activities of AxgNIR and AciNIR in the absence and in the presence of azide were spectroscopically measured at 25 °C by the previous method.[11]

Spectroscopic Measurement. Electronic absorption spectra were measured with a Shimadzu UV-2200 spectrophotometer at 25 °C.

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