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COPPER SITE OF NITRITE REDUCTASE FROM ALCALIGENES SP. STRUCTURAL EVIDENCE FROM X-RAY ABSORPTION SPECTROSCOPY

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The EXAFS study has been carried out for the copper site of nitrite reductase from *Alcaligenes* sp. The curve-fitting results showed that the copper site has N_2S with Cu-N=1.97 and Cu-S=2.17 Å.

A nitrite reductase (NiR) of a denitrifying bacterium, *Alcaligenes* sp. is a copper-containing metalloprotein which catalyzes the reaction, $NO_2^- \longrightarrow NO$. The oxidized form of the enzyme shows intense blue color with an absorption maximum at 594 nm. The esr parameters are $g_\perp$=2.05 and $g_{//}$=2.22 with smaller hyperfine constant ($A_{//}$=65 G).[1] These features suggest that the protein contains the "blue" (or type I) copper site.[2] Typical "blue" copper proteins are azurin and plastocyanin, of which function is known as the electron-transfer with the redox of copper. The structures of those two blue proteins have been solved by the X-ray diffraction analysis; the copper ion is coordinated by one cysteine, two imidazoles, and one methionine (N_2SS^*).[3,4] As the function of NiR is the terminal oxidoreductase for nitrite reduction, it is interesting to study the structure of NiR in comparison with that of azurin. The technique of the X-ray absoprtion spectroscopy has proven to be a powerful method for investigating the structure around a specific metal even in non-crystalline materials. In this work, we have studied the copper site of NiR by the EXAFS.

Nitrite reductase and azurin were isolated from *Alcaligenes* sp. NCIB 11015. The known purification procedures[1] were modified, in which we used the ammonium sulfate precipitation (40-90% saturation) and DEAE-cellulose ion-exchange chromatography. Purity indexes of A_{280}/A_{594} for NiR and A_{280}/A_{625} for azurin showed 13.4-14.3 and 2.1, respectively. Purified NiR and azurin were freeze-dried and used for the EXAFS. Following reference compounds, $[Cu(Im)_4]SO_4$ (Im =

imidazole),[5] $[(n\text{-}C_4H_9)_4N]_2[Cu(mnt)_2]$, (mnt = maleonitriledithiolate),[6] and $K_2[Cu(ox)_2]$ (ox = oxalate)[7] were prepared according to the cited references and were diluted by 20% with boron nitride.

The X-ray absorption measurements have been carried out on the storage ring of the Photon Factory at National Laboratory of High-energy Physics (2.5 GeV, 70-120 mA).[8] Spectra were registered in a transmission mode at room temperature. Energy was calibrated relative to a copper foil by assigning 8978.8 eV to the first inflection point on the copper K-absorption edge.

The EXAFS spectra have been analyzed following standard procedures. The absorption background before the edge was subtracted from the experimental absorption spectrum, and the resulting spectrum $\mu(k)$ was normalized by using the atomic absorption coefficient $\mu_0(k)$, in order to give the EXAFS defined by $\chi(k)=[\mu(k)-\mu_0(k)]/\mu(k)$. The photoelectron wave vector was given by $k=[(E-E_0)2m\hbar^2]^{1/2}$, where E is the photon energy and E_0 is some reference energy which may not coincide with the energy of the absorption edge.

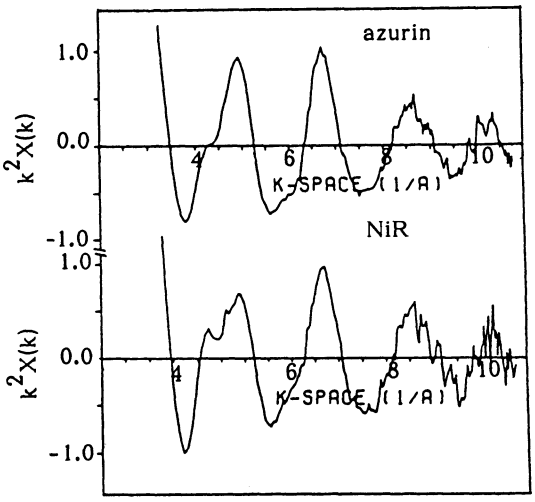

Fig. 1. Experimental EXAFS spectra near the Cu edge.

Figure 1 shows the EXAFS spectra $k^2\chi(k)$ of azurin and NiR. The transformation was applied to the $k^2\chi(k)$ values in the k range of 3.6-10.5 Å^{-1}. Figure 2 shows the radial distributions from Cu atom. Both distribution functins show a main peak centered at about 1.8 Å and two satellite peaks at 2.9 and 3.8 Å. The main peak of azurin should correspond to the N and S atoms of the coordinated imdazole and cysteine ligands.[3,4] The satellite peaks are due to the imidazole ring.[9] The similarities between azurin and

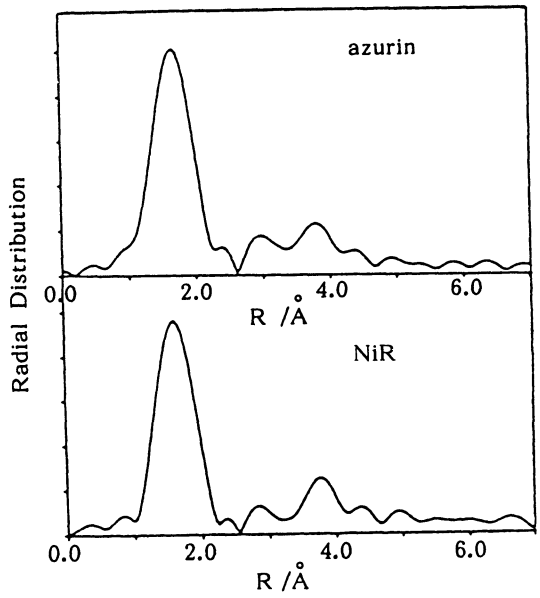

Fig. 2. Radial distribution functions for Fig. 1 (not corrected the phase-shift).

NiR are evident.

Figure 3 shows the XANES spectra of azurin and NiR with the model compounds. The spectra show the pre-edge, the mid-edge, and the main peaks. The spectrum of azurin is similar to that of NiR. EXAFS and XANES spectra suggest that the first coordination shell around Cu atom for NiR resembles that for azurin.

The curve-fitting analysis was performed according to the method of Cramer et al.[10] using the following formula;

$$k^2\chi(k) = \Sigma\, N/R^2 c_0 \exp(-c_1 k^2)\cdot k^{-c_2}\cdot\sin[a_0+(a_1+2R)k+a_2k^2],$$

where N and R represent the coordination number and the interatomic distance, respectively. The c_0, c_1, and c_2 and a_0, a_1, and a_2 are the amplitude and phase-shift parameters, respectively. These parameters were determined by fitting to the EXAFS spectrum of a model compound with a known structure and transferred to the analysis of the spectrum of the enzyme. We determined the parameter values for Cu-N, Cu-S, and Cu-O from the EXAFS of $[Cu(Im)_4]SO_4$, $[(n\text{-}C_4H_9)_4N]_2[Cu(mnt)_2]$, and $K_2[Cu(ox)_2]$.

Table 1 shows the results of the curve-fitting analysis. Obtained values (N_{Cu-N}=2.3 and N_{Cu-S}=0.9) for azurin give N_2S in agreement with the previous EXAFS result.[11] This is not inconsistent with the X-ray diffraction result which shows distorted tetrahedral N_2SS^* for the copper site, because the EXAFS from the fluctuating and distant atom is known to show the dubious signal. Since NiR of

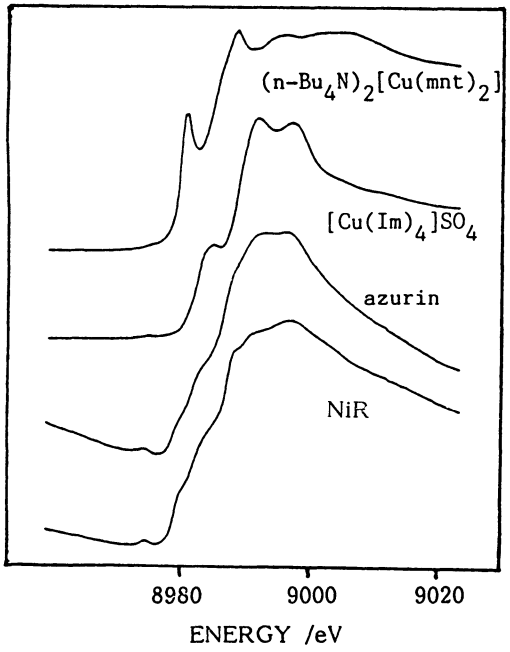

Fig. 3. XANES spectra for the Cu edge.

Table 1. Results of two shell (nitrogen and sulfur) fits to the filtered EXAFS

Sample	Cu-N		Cu-S	
	R/Å	N	R/Å	N
Azurin	1.95	2.3	2.16	0.9
NiR	1.98	3.2	2.14	0.4
	1.98	3[a)]	2.15	0.6
	1.98	2[a)]	2.17	1.0

a) Curve-fitting analysis was performed for the fixed value of N_{Cu-N}.

Alcaligenes sp. contains only one type of copper atom,[1] the coordination number should have integer value. However, the coordination numbers of NiR showed 3.3 for N and 0.4 for S, respectively.[12] This may come from the strong correlation with N_{Cu-N} and N_{Cu-S} on the fitting procedure, in which this odd values for N_{Cu-S} may be an artifact. Curve-fitting analyses were also performed for the two fixed values of N_{Cu-N}. The N_{Cu-N} value of 2 is obtained for the integer N_{Cu-S} value. Though there is uncertainty of the intensity for the satellite peaks owing to the low signal-to-noise ratio in the EXAFS spectra, their intensities from the imidazole ring of histidine in NiR are close to those in azurin. Thus the curve-fitting analysis shows that the copper site of NiR has N_2S with Cu-N=1.95 and Cu-S=2.17 Å. The result of EXAFS does not exclude the tetrahedral N_2SS^* structure for the copper site of NiR because of the reason described above.

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