Isolation and Characterization of Two Distinct Azurins from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* NCIB11015 or GIFU1051

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Two distinct azurins isolated from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* NCIB11015 or GIFU1051 have been characterized by electronic absorption, circular dichroism (CD), and electronic paramagnetic resonance (EPR) spectroscopies. On the basis of CD spectra, two azurins from bacteria are clearly distinguishable.

Azurin is a bacterial blue copper protein, which is considered to play a role of electron transfer in biological systems.[1] It have been known that only one azurin is obtained from one species of bacteria, except for the case of *Methylomonas J.*[2] However, we report here that two kinds of azurins were isolated from *Alcaligenes xylosoxidans* NCIB11015 or GIFU1051.[3]

The extracts containing two azurins were obtained from the cells of *A. xylosoxidans* subsp. *xylosoxidans* NCIB11015 and GIFU1051, according to the methods reported already.[4] Two azurins were separated on linear gradient ion-exchange chromatography of CM-Sephadex.[5] The first and the second blue fractions were termed azurin-I (Az-I) and azurin-II (Az-II), respectively. The amounts of azurins (Az-I and Az-II) were almost equal in each species. SDS-polyacrylamide gel electrophoresis of each protein showed a single band at the same position (molecular weight, 16kDa). The copper content of the azurins was estimated to be one atom per protein by use of an atomic absorption spectrometer.

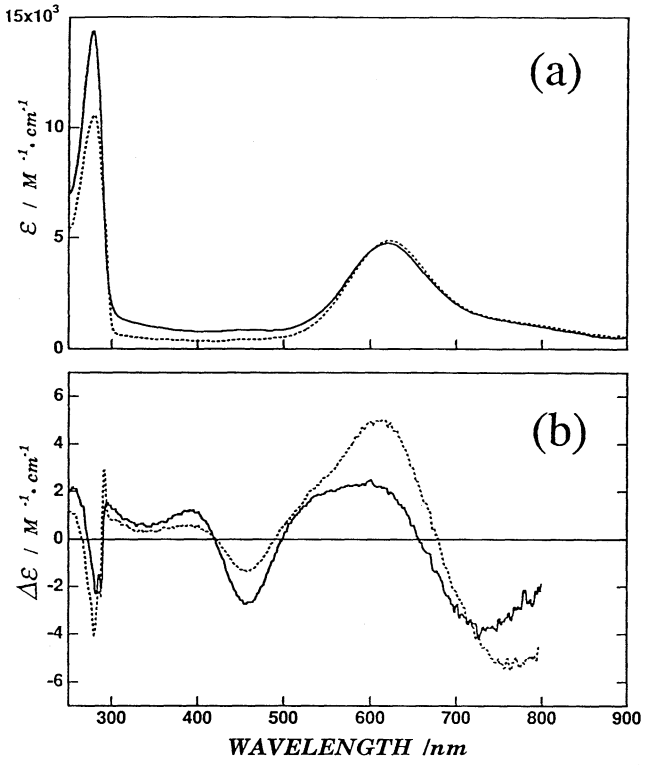

Figure 1. The electronic (a) and CD (b) spectra of Az-I (-----) and Az-II (——) from *A. xylosoxidans* NCIB11015 in 0.1 M potassium phosphate buffer (pH 6.0) at room temperature.

The electronic and CD spectra of azurins from *A. xylosoxidans* NCIB11015 in 0.1 M phosphate buffer (pH 6.0) are represented in Figure 1(a) and (b), respectively. Both the absorption spectra of Az-I and Az-II display two peaks at 279 and 620 nm. However, the molar absorption coefficient (ε_{279}=10,700 $M^{-1}cm^{-1}$) at 279 nm of Az-I is considerably smaller than that (ε_{279}=14,500 $M^{-1}cm^{-1}$) of Az-II. The composition of aromatic amino acid residues in Az-II would be different from that of Az-I, since the band around 280 nm is assigned to the electronic transitions of aromatic amino acid residues in the protein. Moreover, the CD spectrum of Az-I is clearly distinguishable from that of Az-II (Fig. 1(b)). The positive sharp CD band at 291 nm of Az-I is not observed in Az-II, and the CD spectrum of Az-I in the 250-300 nm region is different from that of Az-II. This would be also attributed to the difference between Az-I and Az-II in the amounts of aromatic amino acids. This result agrees with that of the electronic spectra. In the visible region, the CD spectrum of Az-I exhibits two positive peaks at 393 and 610 nm, one positive shoulder band around 545 nm, and two negative peaks at 458 and 766 nm. The pattern of CD spectrum for Az-I is almost identical with that of azurin from *Pseudomonas aeruginosa* reported previously.[4] Accordingly, the Cu in Az-I is considered to have an extremely similar coordination geometry to that in *P. aeruginosa* azurin. It is now generally

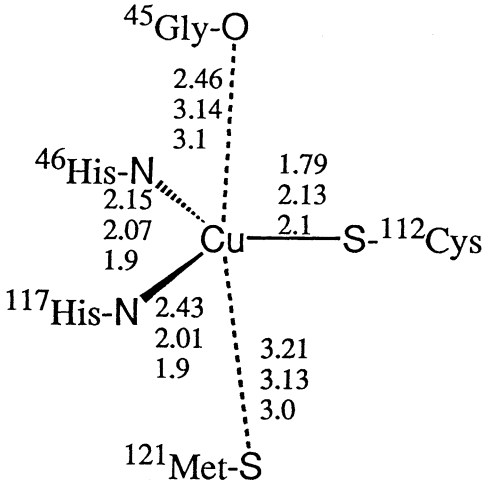

Figure 2. The schematic structure of the copper binding site of azurin, and the distances (Å) between copper atom and its ligands (top: *P. aeruginosa*, middle: *A. denitrificans*, bottom: *A. xylosoxidans* NCIB11015 AzII).

Table 1. Spectral and electrochemical data of azurins from *A. xylosoxidans* NCIB11015 and GIFU1051

	NCIB11015		GIFU1051	
	Az-I	Az-II	Az-I	Az-II
electronic spectrum				
λ_{max} /nm	620, 279	620, 279	620, 279	620, 279
(ε_{max} /$M^{-1}cm^{-1}$)	(4800, 10700)	(4700, 14500)	(5000, 10800)	(4500, 14800)
CD spectrum				
λ /nm ($\Delta\varepsilon$ /$M^{-1}cm^{-1}$)	766 (-5.4), 610 (+5.1), 545 (+2.6), 458 (-1.4), 393 (+0.6), 291 (+3.0), 278 (-4.2)	735 (-3.9), 610 (+2.2), 545 (+2.1), 458 (-2.8), 393 (+1.2), 280 (-4.2)	766 (-5.4), 609 (+5.0), 538 (+2.2), 454 (-1.3), 390 (+0.6), 292 (+2.7), 279 (-4.2)	735 (-4.0), 609 (+2.3), 538 (+2.0), 454 (-2.9), 390 (+1.3), 280 (-2.5)
ESR spectrum				
$g_{//}$ ($A_{//}$ /mT), $g_{\perp}$	2.26 (60), 2.06	2.26 (60), 2.06	2.26 (60), 2.06	2.26 (60), 2.06
electrochemistry				
$E_{1/2}$ /mV (ΔE /mV)	289 (68)	275 (63)	267 (72)	274 (63)
D /cm^2s^{-1}	$8.7X10^{-7}$	$9.9X10^{-7}$	$1.79X10^{-6}$	$9.7X10^{-7}$

accepted that the bands at 610 and 766 nm are associated with S(Cys)→Cu charge transfer transitions.[6] However, it has been proposed that the 458-nm band has S(Cys)→Cu or N(His)→Cu charge transfer contribution.[6,7] On the other hand, Az-II exhibits the weaker band at 610 nm and the 735-nm band shifted to the shorter wavelength compared to those of Az-I (Fig. 1(b)). The binding of cysteine sulfur to Cu in Az-II might be weaker than that in Az-I.

The copper centers of azurins, generally, have been established by X-ray analyses to a distorted trigonal-planar geometry (two imidazole nitrogens of His and one thiol sulfur of Cys) with two weakly interacting groups (the thioether sulfur of Met and the peptide carbonyl oxygen of Gly) in axial positions, completing an axially elongated trigonal bipyramid (Fig.2). Recently, the three-dimensional structure of Az-II at 2.5 Å resolution have been reported.[8] As represented in Fig. 2, the distances between Cu atom and its ligands of Az-II are close to the data[9] of azurin from *Alcaligenes denitrificans* NCTC8582 rather than those from *P. aeruginosa*. Az-II has the shorter distances between Cu and nitrogens (His-46 and His-117) and the longer one between Cu and sulfur (Cys-112) than the corresponding bond distances of *P. aeruginosa* azurin. The structure of copper site in Az-I will resemble to that of *P. aeruginosa* azurin, because of the CD spectral similarity between them. Additionally, on the partial amino acid sequence experiment (data not shown), Az-II was found to be quite similar to that from *A. denitrificans* NCTC8582, while Az-I is the same protein as the azurin of *A. xylosoxidans* NCIB11015 reported already.[10]

The spectral and electrochemical data of four azurins from *A. xylosoxidans* NCIB11015 and GIFU1051 are summarized in Table 1. Az-I and Az-II from *A. xylosoxidans* GIFU1051 are spectroscopically similar to the corresponding azurins from *A. xylosoxidans* NCIB11015. Both Az-I and Az-II from *A. xylosoxidans* NCIB11015 and GIFU1051 exhibit the same EPR parameters ($g_{//}$=2.26, $g_{\perp}$=2.06, and $A_{//}$=60mT) and the slightly different redox potentials. Moreover, we tried to examine the electron transfer reaction between azurin (Az-I or Az-II) and nitrite reductase from *A. xylosoxidans* NCIB11015 or GIFU1051 in the presence of nitrite ion by cyclic voltammetry.[11,12] However, the catalytic currents were hardly detected, and hence no electron transfer reactions occur. The finding suggests that the azurins are not electron donors to nitrite reductase in these bacteria.

Finding of two azurin from one strain will give a insight into a structure-function relation of type 1 copper protein. More details of structures and functions of these azurins are under investigation.

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References and Notes

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