Bull. Chem. Soc. Jpn., 73, 1839—1840 (2000) 1839

Spectroscopic and Electrochemical Properties of Cytochrome c_{551} from *Alcaligenes xylosoxidans* GIFU 1051

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(Received March 15, 2000)

Spectroscopic and electrochemical characterizations of cytochrome c_{551} from a denitrifying bacterium, *Alcaligenes xylosoxidans* GIFU 1051, have been performed. The spectral data indicate that the oxidized and reduced proteins have a six-coordinate low-spin heme. The fast electron-transfer process from cytochrome c_{551} to cognate Cu-containing nitrite reductase was electrochemically observed.

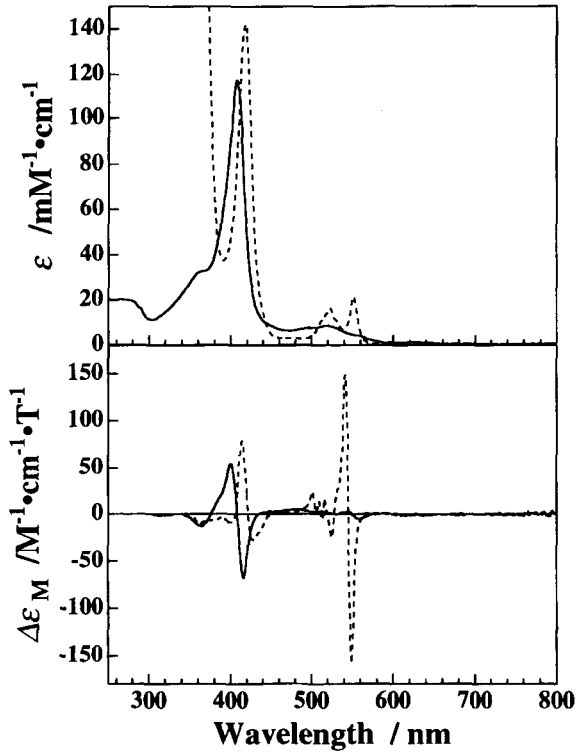

Fig. 1. Absorbtion (top) and MCD (bottom) spectra of oxidized (solid line) and reduced (dashed line) Cyt c_{551} (80 μM) in 0.1 M phosphate buffer (pH 6.0) at room temperature.

Nitrite reductases (NIR) from denitrifying bacteria catalyze the one-electron reduction of nitrite ion to nitrogen oxide as a part of the anaerobic respiration of nitrate to dinitrogen.[1,2] A blue copper protein, the pseudoazurin from *Achromobacter cycloclastes* IAM 1013 (pAz), donates one electron to cognate green copper-containing NIR (AciNIR)[3,4] with an electron-transfer rate constant of 7.3×10^5 M^{-1} s^{-1} at pH 7.0.[5] The other denitrifying bacteria, *Alcaligenes xylosoxidans* GIFU 1051 (Axg) and *Alcaligenes xylosoxidans* NCIB 11015 (Axn), give respectively two azurins (Az-I-Axg and Az-II-Axg; Az-I-Axn and Az-II-Axn) instead of pAz.[2] Az-I-Axn and Az-II-Axn have been reported as electron donors for blue AxnNIR.[6] However, the cyclic voltammetric responses of Az-I-Axg and Az-II-Axg were slightly changed in the presence of AxgNIR and nitrite, which indicate the very slow electron-transfer processes.[2] Moreover, according to an in vivo approach with the mutant strains of *Pseudomonas aeruginosa* deficient in either cytochrome c_{551} (Cyt c_{551}) or Az or both, it was shown that Cyt c_{551}, not Az, is functional as an electron donor for hemes *c* and d_1-containing NIR.[7] Therefore, the electron donor protein for copper-containing NIR might not be Az but cytochrome *c*.

In this work, we report the spectroscopic and electrochemical properties of Cyt c_{551} isolated from the Axg strain with two Az's. Moreover, the intermolecular electron transfer rate constant from this heme-protein to AxgNIR have been estimated by cyclic voltammetry.

Figure 1 shows the electronic absorption and magnetic circular dichroism (MCD) spectra of oxidized and reduced Cyt c_{551} in 0.1 M (M = mol dm^{-3}) phosphate buffer (pH 6.0). In the absorption spectrum of oxidized Cyt c_{551}, there are two maximum peaks at 408 (Soret band, ε = 118 mM^{-1} cm^{-1}) and 521 nm. Reduced Cyt c_{551} gives the intense Soret, β, and α bands at 417, 522, and 551 nm, respectively. These absorption spectra are quite similar to those of typical *c*-type heme proteins. The MCD spectrum of the oxidized form shows an intense "S-shaped" signal having the extrema at 400(+) and 415(−) nm, while that of the reduced form exhibits two intense "S-shaped" signals (Soret, 414(+) and 427(−) nm ; α, 541(+) and 550(−) nm). These findings indicate that both the oxidized and reduced forms have a six-coordinate low-spin heme like horse heart Cyt *c*.[8,9]

The cyclic voltammograms of (a) Cyt c_{551}, (b) Cyt c_{551} in the presence of nitrite, and (c) Cyt c_{551} in the presence of AxgNIR and nitrite are shown in Fig. 2. In Fig. 2 (a), the voltammetric response of Cyt c_{551} at a 6-mercaptopurine-modified gold electrode[10] shows the quasi-reversible electron transfer process (ΔE = 74 mV), an observed half-wave peak potential ($E_{1/2}$) of +241 mV vs. NHE, and a diffusion coefficient[11] of 6.0×10^{-7} cm^2 s^{-1} in 0.1 M phosphate buffer (pH 6.0), but not at a bare gold electrode. After the addition of nitrite to the Cyt c_{551} solution, the voltammetric response is hardly changed (Fig. 2 (b)), suggesting that no electron-transfer from reduced Cyt c_{551} to nitrite occurs. However, by the addition of AxgNIR (λ_{max} = 593 nm, ε = 3800 M^{-1} cm^{-1}) to the Cyt c_{551} solution containing nitrite, the shape of the voltammogram is dramatically changed and an enhanced sigmoidal cathodic current-potential curve is observed (Fig. 2

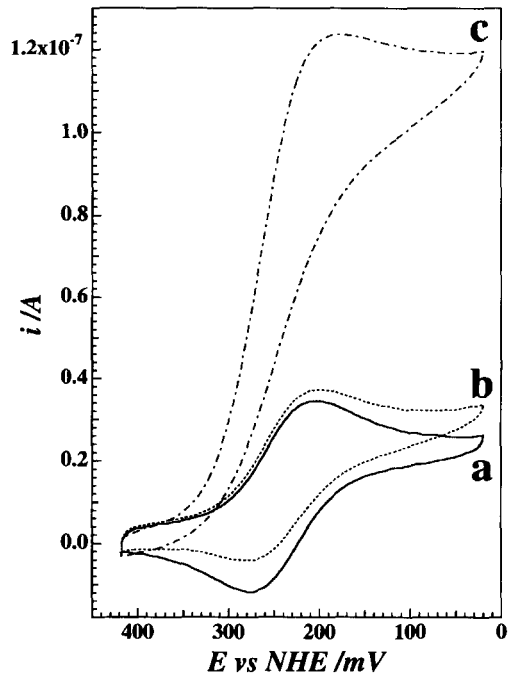

Fig. 2. Cyclic voltammograms of (a) Cyt c_{551} (100 μM) in 0.1 M phosphate buffer (pH 6.0), (b) Cyt c_{551} after the addition potassium nitrite (50 mM), and (c) Cyt c_{551} after the addition of AxgNIR (1 μM) and potassium nitrite (50 mM). All voltammograms were recorded at 25 °C and a potential sweep rate of 2 mV s^{-1}.

(c)). The appearance of the catalytic current (increased cathodic current) implies the regeneration of oxidized Cyt c_{551} by the electron-transfer to AxgNIR in the diffusion layer. The second-order rate constant of the intermolecular electron-transfer process from Cyt c_{551} to AxgNIR was estimated to be 4.0×10^5 M^{-1} s^{-1} at pH 6.0 and 25 °C.[12,13] The addition of apo AxgNIR instead of the native enzyme does not change the voltammetric response of Cyt c_{551}. The large electron-transfer rate constant between Cyt c_{551} and AxgNIR is similar to that between pAz and AciNIR, suggesting that Cyt c_{551} ($E_{1/2}$ = +241 mV) would be an electron donor for AxgNIR (type 1 Cu, $E_{1/2}$ = +290 mV vs. NHE).[2]

Experimental

Purification of Cyt c_{551}. The cultured *Alcaligenes xylosoxidans* GIFU 1051 cells were suspended in 20 mM phosphate buffer (pH 7.0) and sonicated. The cell-free extract was dialyzed against 4 mM phosphate buffer (pH 6.0) and loaded onto a CM-Sephadex column equilibrated with the same buffer. Azurins, NIR, and cytochromes *c*, were adsorbed on the column, and a red fraction of crude Cyt c_{551} was eluted with 20 mM phosphate buffer (pH 6.0). After the repeating of CM-Sephadex chromatography, the fraction of Cyt c_{551} was loaded onto a Superose 12HR gel filtration column (20 mM phosphate buffer (pH 6.0)) for the final purification. SDS-PAGE gave the single band of Cyt c_{551} (0.8 kDa).

Spectroscopic Measurements. Electronic absorption spectra were measured with a Shimadzu UV-2200 spectrophotometer and MCD spectra were measured with a JASCO J-500 spectrophotometer equipped with an electromagnet at the magnetic field of 1.4 T.

Electrochemical Measurements. Cyclic voltammetry was carried out by using a Bioanalytical Systems Model CV-50 W voltammetric analyzer with a three-electrode system consisting of an Ag/AgCl reference electrode, a gold-wire counter electrode, and a 6-mercaptopurine-modified gold working electrode in 0.1 M potassium phosphate buffer (pH 6.0) at 25 °C.

This work was supported by a Grant-in-Aid for Scientific Research (B) No. 11440198 from the Ministry of Education, Science, Sports and Culture.

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