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Electronic Absorption Spectra and Redox Properties of *C* Type Cytochromes in Living Microbes**

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Unlike most gram-negative bacteria, anaerobically cultivated *Shewanella* and other related microbes, such as *Geobacter*, have a significant content of *c* type cytochromes (*c* Cyts) in their outer membrane.[1] Owing to their unique location on the cellular surface, the *c* Cyts have been predicted to mediate direct electron transfer (ET) from the cell surface to solid minerals, such as iron oxide and manganese oxide, during the course of their respiratory electron-transfer process.[2] This phenomenon has been studied extensively because of its importance in microbial activities in anaerobic subsurface environments.[2] Herein we describe the development of optical absorption spectroscopy to characterize the functions of outer-membrane *c* Cyts in a living microbe.

Electrochemical and spectroscopic studies have been performed on purified outer-membrane *c* Cyts[3,4] from *Shewanella*. Their ability to mediate electron transfer to a graphite electrode has been confirmed by protein film voltammetry (PFV)[5] and scanning tunneling microscopy.[6] However, as purified proteins may behave differently to in vivo protein complexes, the molecular spectroscopic study of a whole-cell system will provide more meaningful information. In the whole-cell system, proteins embedded in a lipid membrane function together as a whole and their properties should be largely influenced by a shift in the equilibrium, which is characteristic of living systems (electron transfer theory in a thermodynamically open system developed by Tributsch et al.[7]). Very recently, Busalmen et al. reported the application of infrared spectroscopy in a whole-cell system in which the electron transfer between *Geobacter* and a gold electrode was monitored.[8] Using surface-enhanced infrared absorption (SEIRA) of the amide bands of outer-membrane *c* Cyts as spectral indicators, they showed the spectroelectrochemical data of the outer-membrane *c* Cyts in the living cell.[8] This success demonstrated the feasibility of using molecular spectroscopic techniques for the analysis of whole-cell systems.

UV/Vis absorption spectroscopy is a desirable method for investigating the in vivo functions of *c* Cyts because of the large molar absorption coefficient of heme irons. It is possible not only to investigate the electronic states of in vivo *c* Cyts, but also to combine it with various techniques, such as circular dichroism (CD) and magnetic circular dichroism (MCD), which reflect the conformation of polypeptides and the electron spin states of heme irons, respectively.[9] However, the application of UV/Vis spectroscopy to a living microbe has been limited by the amount of scattered light from microbial surfaces, which although useful for estimating bacterial numbers, hinders the application of other measurements.

Herein we report the first application of UV/Vis spectroscopy in diffuse transmission mode for measuring the electronic absorption spectra of the multiheme *c* Cyts in intact cells. Furthermore, by using the UV/Vis evanescent wave (EW) spectra, we also determined the spectroelectrochemical properties of the cell-membrane associated *c* Cyts. Our results revealed a large discrepancy in the redox properties between purified proteins and protein complexes in a living microbe.

Shewanella loihica PV-4,[10] used in this work, exhibits an intense reddish–orange color when cultured (insert, Figure 1a). The cell suspension was added into a Pyrex cell, and it was mounted in front of an integrating sphere to measure the diffuse transmission light. The diffuse transmission spectrum of strain PV-4 (Figure 1a) shows an intense absorption band at 419 nm (the Soret band) and weak absorption bands at 522 and 552 nm (the Q band).[11] These peak positions are characteristic of the reduced form of the heme groups of *c* Cyts, and are almost identical to the outer-membrane decaheme *c* Cyts purified from strain MR-1.[5] Note that the spectral interferences owing to the light scattered by cell surfaces were not observed in the spectrum, demonstrating the effectiveness of the diffuse transmission technique for measuring the electronic absorption spectra of intact cells. The concentration of the heme groups in the cell suspension was estimated to be approximately 0.5 mM, when the molar absorption coefficient of the Soret band was

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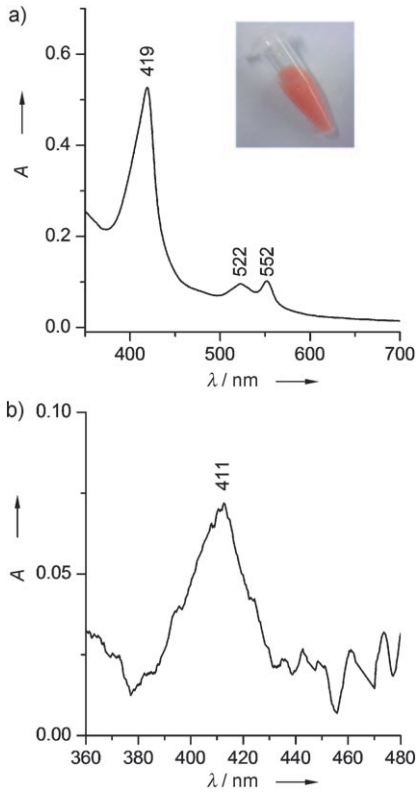

Figure 1. a) UV/Vis spectrum of a whole cell of strain PV-4 suspended in a HEPES buffer solution containing 10 mM of lactate measured in diffused transmission mode. Optical length of the cell is 0.1 mm. The spectrum was taken under aerobic condition. Insert: photograph of a suspension of strain PV-4. b) UV/Vis EW spectrum of a whole cell of PV-4 grown on an ITO electrode; electrode potential 0.32 V.

assumed to be 10^5.[9] This concentration of heme proteins is of the order of magnitude required for applying various spectroscopic methods developed in studies with purified proteins.[3–6,9] Therefore, strain PV-4 is an appropriate candidate for a whole-cell study based on the use of molecular spectroscopic techniques.

We applied UV/Vis EW spectroscopy to monitor the in vivo role of *c* Cyts in the interfacial electron transfer from the cells to the electrode surface. A quartz optical waveguide coated with a tin-doped indium oxide (ITO) film was placed on the bottom surface of the electrochemical reactor as an internal reflection element that generates an evanescent wave at the interface between the electrode and the electrolyte solution. Lactate was added as a carbon source and an electron donor to sustain the metabolic activity of strain PV-4. The UV/Vis EW spectrum of strain PV-4 with an electrode potential at 320 mV (versus standard hydrogen electrode) is shown in Figure 1b. In the course of microbial current generation, the spectrum exhibited a Soret absorption band at approximately 411 nm characteristic of the oxidized form of *c* Cyts.[5] The peak intensity increased gradually with time, and saturated approximately 2 h after inoculation, which indicates the completion of attaching the cells to the electrode surface. The penetration depth that the evanescent wave field decays to $1/e$ was estimated to be ~110 nm at a wavelength of 400 nm. Accordingly, the appearance of the Soret peak at approximately 411 nm demonstrates that the oxidized form of the *c* Cyts is adjacent to the electrode surface during the respiratory electron transfer reaction.

Figure 2a shows the change in the EW spectra during a potential step experiment between 820 and −180 mV. Upon stepping the electrode potential from 320 to −180 mV, there is an immediate red-shift (to 419 nm) of the Soret band, which is

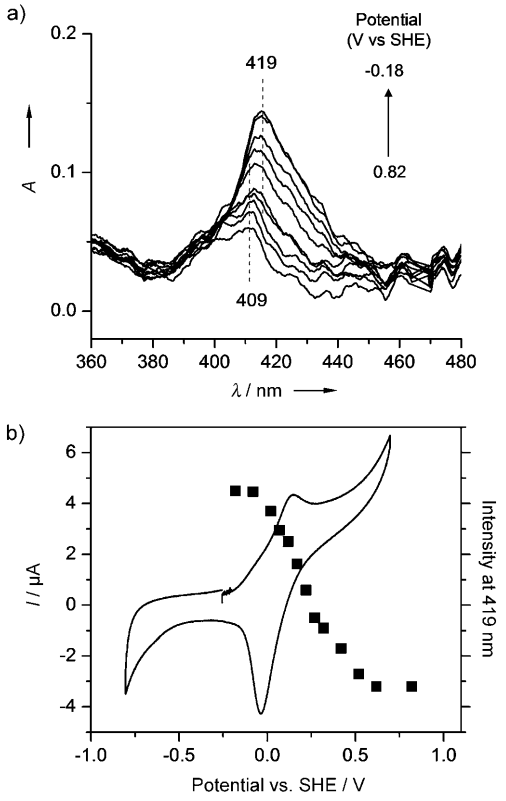

Figure 2. a) UV/Vis EW spectra of a whole cell of strain PV-4 on an ITO electrode obtained at electrode potentials of 0.82, 0.54, 0.42, 0.32, 0.27, 0.22, 0.17, 0.12, 0.02, and −0.18 V. Arrow indicates the direction of a spectral change with a potential shift from 0.82 to −0.18 V. b) A plot of absorbance at 419 nm as function of electrode potential (■) and a cyclic voltammogram, 10 mV s^{-1}, of a whole cell of strain PV-4 measured under the same conditions as in (a).

assigned to the reduced form of *c* Cyts. The spectral changes are fully reversible for both the positive and negative direction of the scan. The plot of the peak intensity (at 419 nm) against electrode potential (Figure 2b) shows that the *c* Cyts have a potential distribution ranging approximately 250 mV, from fully oxidized (270 mV) to fully reduced (20 mV). This is within the potential distribution of the whole cells of strain PV-4 obtained by cyclic voltammetry (Figure 2b). From Figure 2a and b, a midpoint potential (E_m) of the *c* Cyts under operating conditions was estimated to be 145 ± 50 mV.

Interestingly, this value is largely shifted to a positive potential region from those reported for the multiheme *c* Cyts purified from strain MR-1.[5,6] Namely, the PFV and spec-

troelectrochemical measurements of purified OmcA, MtrC, and CymA showed that these proteins have an E_m in the range of −350 to 10 mV independent of the substrates used as an electrode.[5,6] In the living system, contrary to purified proteins, electrons are continuously supplied to the heme groups of the outer-membrane *c* Cyts through the oxidative reaction of lactate. Accordingly, the positive shift of the E_m value may reflect the displacement of the equilibrium to produce the reduced form of outer-membrane *c* Cyts in the living systems as a result of this constant influx of electrons.

Herein we have demonstrated the first optical absorption spectrum of cell-membrane associated *c* Cyts during metabolism, and showed a large positive shift of the E_m value compared to the purified proteins. This effect is most likely a result of a continuous supply of electrons to the heme groups. Such a shift in the equilibrium demonstrates the importance of exploring the in vivo function of membrane proteins. In addition, the high concentration (ca. 0.5 mM) of *c* Cyts in strain PV-4 is also an intriguing finding as this enables the application of electron spin resonance, CD, and MCD. These are all powerful techniques for the identification of the electron spin states of heme groups and the surrounding configuration of the *c* Cyt polypeptide. Therefore, the present study may provide a new platform for molecular spectroscopy to examine membrane proteins in living microbes, which is of importance not only for the fundamental study of biological electron transfer[7] but also for the fabrication of bioanode materials.[12]

Experimental Section

UV/Vis EW spectra of intact cells of *S. loihica* PV-4 were measured using a SIS-5000 Surface and Interface Spectrometer System (System Instruments). A quartz optical waveguide coated with a tin-doped-In_2O_3 (ITO) film was used as a working electrode. A single-chamber electrochemical reactor was mounted onto the ITO substrate and sealed with a silicon rubber O-ring. A silver and a platinum wire were used as the counter and reference electrode, respectively. The incident light from a 150 Watt xenon lamp was inclined at an angle of 8° parallel to the surface of the ITO substrate.

Strain PV-4 was grown aerobically in Marine Broth (10 mL; 20 g L^{-1}) at 30 °C for 24 h. Subsequently, this was centrifuged, and the Marine Broth was replaced with 10 mL of defined media [DM; $NaHCO_3$ (2.5 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.08 g L^{-1}), NH_4Cl (1.0 g L^{-1}), $MgCl_2{\cdot}6H_2O$ (0.2 g L^{-1}), NaCl (10 g L^{-1}), and HEPES (7.2 g L^{-1}). The cells were further cultivated aerobically in DM at 30 °C for 2 days using lactate as a carbon source. The suspension was centrifuged for 10 min and the resultant cell suspension was washed with DM three times prior to being used for spectroelectrochemical experiments.

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