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出版情報：Analytical Sciences/Supplements, pp.i1357-i1358, 2002. 日本分析化学会
バージョン：

Diffusionless Electrochemistry of Cytochrome *c* Covalently Immobilized on 11-Carboxyundecanethiol Monolayer Electrode

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Cytochrome *c* molecules were covalently immobilized on a self-assembled monolayer (SAM) electrode from 11-carboxyalkanethiol. The electrode with the protein/SAM/gold interfacial structure gave well-defined, stable redox waves which were attributed for the direct electron transfer (ET) reaction of the proteins. The formal potential was 234±4 mV (vs SHE, *n*=8) and the electroactive surface coverage of the protein was estimated to be 9.3±2.3 pmol cm^{-2} (*n*=8) which was consistent to submonolayer coverage. Detailed analysis for the ET reaction gave a rate constant of 2.5±1.0 s^{-1} (*n*=8).

(Received on August 7, 2001; Accepted on September 13, 2001)

The study of direct electron transfer (ET) of proteins and enzyme has been attracting considerable research interest for several decades since it represents a basic feature for the application of biocatalysts in chemical sensors and other electrochemical devices.[1,2] It is well-known that electrical communication between a biocatalyst and an electrode relies, to a considerable extent, on a controlled chemical modification of electrode surfaces. Self-assembled monolayers (SAMs) offer a simple yet effective means for that purpose[3,4] and also have proven to be useful for studying an interfacial ET reaction.

We have previously reported that SAM-modified electrode of the ω-carboxyalkanethiol/gold type accommodated cytochrome *c* and provided a favorable surface for the ET reaction of the protein in an adsorbed state. A (sub) monolayer coverage is attained through electrostatic interaction between the anionic SAM and cytochrome *c* having positively charged, lysine-patches. The interaction, in the same time, led to a specific orientation of cytochrome *c* molecule so that a facilitated electron transfer occurred between the heme in the protein molecule and the underlying gold electrode.[5]

Obviously, protein immobilization through electrostatic interaction has a drawback that stable attachment of protein molecules is only attained in low ionic strength solutions of appropriate pH. With regard to this, covalent attachment of cytochrome *c* has been studied by us[6] and by other researchers.[7,8] In the present paper, we report electrochemistry of cytochrome *c* covalently immobilized on ω-carboxyalkanethiol monolayer electrodes.

Experimental

Chemicals and biochemicals

11,11'-Dithiodiundecanoic acid was synthesized according to the literature.[9,10] 1-Cyclohexyl-3-(2-morpholonoethyl)carbodi-imide metho-*p*-toluene sulfonate (CMC) was purchased from Tokyo Kasei and used as received. Horse heart cytochrome *c* (Sigma, type VI) was chromatographically purified on (carboxymethyl)cellulose (CM-52, Whatman), concentrated by using an Amicon ultrafiltration cell with a PLCC membrane (NMWL 5,000, Millipore).[11] The solution was then passed through a desalting column (DE-52, Whatman) equilibrated with 4.4 mM potassium phosphate buffer (pH 7.0, 10 mM ionic strength). The desalted solution containing purified cytochrome *c* was stored at 5 °C.

Procedure

A one-chamber cell containing a horizontally mounted electrode was used for electrochemical measurement (0.32 cm^2 exposed electrode area). Gold mirror electrodes were purchased from Kinoene Kogaku (Tokyo, Japan). They were placed in the cell in contact with an aqueous 0.1 M H_2SO_4 solution (0.01 M KCl). The electrode was first subjected to anodic potential cycling to give a fresh surface exposed. After rinsing, 0.3 cm^3 of a 10 mM alkanethiol solution (in ethanol) was placed in the cell and the SAM was allowed to form for 24 h at RT. The cell was rinsed thoroughly with ethanol and water, then background CVs were obtained in 4.4 mM potassium phosphate buffer (pH 7.0, 10 mM ionic strength).

Cytochrome *c* was covalently immobilized on the SAM electrode by a procedure similar to the previous study.[6] The cell was first treated with a 0.3 cm^3 portion of 0.1 mM CMC solution (0.1 M potassium phosphate buffer, pH 7.0) for 30 min at 5 °C and activation of the surface carboxyliate was made. After disposal of the CMC solution, cytochrome *c* solution was placed immediately into the cell and was allowed to react for 1 h at 5 °C. Typically, a 0.1 cm^3 portion of 22 μM protein solution in potassium phosphate buffer (4.4 mM, pH 7, 10 mM ionic strength) was used for this procedure. The cell finally rinsed with water, filled with the same buffer solution and stored at 5 °C before and between uses.

Results and Discussion

Studies of biological ET reaction are primarily examined by a homogeneous bimolecular format comprises a protein and a redox molecule both of which are freely dissolved in an aqueous solution. On the other hand, application of electrochemical methods, *i.e.*, a non-mediated ET reaction between a protein and an electrode is now almost commonplace and proving itself as an useful tool for characterizing protein bioenergetics. We especially have been focusing our research interest on cytochrome *c* strongly adsorbed on an electrode surface. Under this heterogeneous reaction format, mechanistic simplification is attained by eliminating diffusion and the ET reaction is essentially *intramolecular* with a unimolecular rate constant.

Figure 1 shows typical CVs for the blank and the cytochrome *c*-immobilized gold electrode measured in 4.4 mM potassium phosphate buffer solution. The blank electrode only gives capacitive currents which are the product of the sweep rate, the double-layer capacitance (C_{dl}), and the electrode surface area. The C_{dl} value calculated from CVs was 6.0±2.2 μF cm^{-2} (n=8) which was *ca.* one-forth to that of an electrochemically pretreated, fresh gold electrode (15 — 30 μF cm^{-2} at +0.2 V in aqueous 0.5 M sodium fluoride solution). The cytochrome *c*-immobilized electrode, on the other hand, gives well-defined redox waves in its CV. The voltammetric responses were stable for several hours at room temperature even if the electrode was exposed to a solution of saturated potassium nitrate.

We attributed the faradaic response to ET reaction between the adsorbed protein and the underlying Au electrode. The formal potential $E°'$, taken as (E_{pc}+E_{pa})/2, was 234±4 mV vs SHE (n=8), which is consistent to previous data.[5] For an one-electron transfer reaction for an adsorbate, the full-width-at-half-maximum (FWHM) value is 90 mV at an ideal state. The present system showed a FWMH value of 105 mV, indicating only a slight $E°'$ dispersion. By integrating the anodic peak of the CV, the electroactive surface coverage of the protein is estimated to be 9.3±2.3 pmol cm^{-2}. This was somewhat small than that of the electrostatic immobilization which typically gave 16 pmol cm^{-2}.[5]

As seen in Fig. 1, the CV response is quasi-reversible. With using Laviron's analysis,[12] an apparent ET rate constant at zero driving force was determined to be 2.5 ± 1.0 s^{-1}. For a nonadiabatic ET reaction, the following equation can be valid:

$$k_{et}° = \nu \exp[-\beta(d - d_0)] \exp(-\Delta G^*/RT) \quad (1)$$

where $k_{et}°$ is the ET rate constant, ν is the frequency factor (1.2 × 10^{12} s^{-1}), β is an electronic tunneling factor, d is ET distance, d_0 is nucleus-to-nucleus ET distance at donor-acceptor closest approach (3 Å), and ΔG^* is the intrinsic free energy of activation (8 kJ mol^{-1}).[5] Using this equation, we calculated an ET distance of d=28 Å for the present system. CPK model building suggested a tunneling distance of 19 Å along the chain direction of the SAM monomer. If we assume that cytochrome *c* is immobilized with its exposed heme edge facing the film, a distance of 5 Å should be added. The estimated tunneling distance is 24 Å which agrees fairly well to the data from CVs.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan. Financial support from The Zeneraru-Sekiyu Foundation (K.N.) is gratefully acknowledged.

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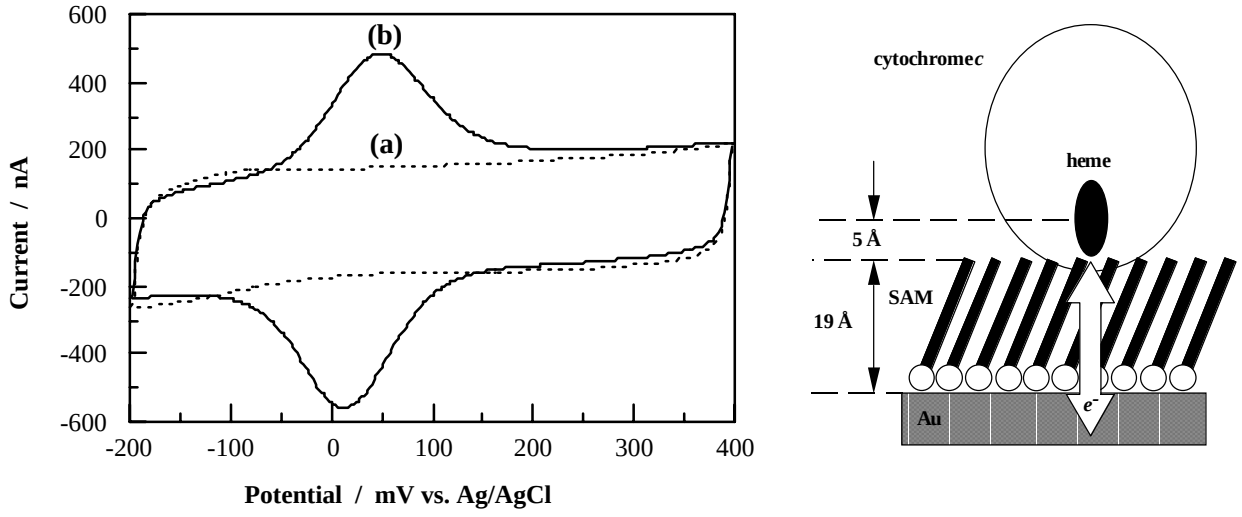

Fig. 1 Cyclic voltammograms for cytochrome *c*/SAM/Au electrode: (**a**) SAM only, (**b**) after immobilization of the protein. Electrolyte solution, 4.4 mM potassium phosphste (10 mM ionic strength, pH 7.0) ; scan rate, 100 mV s^{-1}.