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Direct measurement of cyclic current–voltage responses of integral membrane proteins at a self-assembled lipid-bilayer-modified electrode: Cytochrome *f* and cytochrome *c* oxidase

(electrochemistry)

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ABSTRACT **Direct cyclic voltage–current responses, produced in the absence of redox mediators, for two detergent-solubilized integral membrane proteins, spinach cytochrome *f* and beef heart cytochrome *c* oxidase, have been obtained at an optically transparent indium oxide electrode modified with a self-assembled lipid-bilayer membrane. The results indicate that both proteins interact with the lipid membrane so as to support quasi-reversible electron transfer redox reactions at the semiconductor electrode. The redox potentials that were obtained from analysis of the cyclic "voltammograms," 365 mV for cytochrome *f* and 250 and 380 mV for cytochrome *c* oxidase (vs. normal hydrogen electrode), compare quite well with the values reported by using conventional titration methods. The ability to obtain direct electrochemical measurements opens up another approach to the investigation of the properties of integral membrane redox proteins.**

It is well known that the use of direct electrochemical methods to obtain thermodynamically correct values for the redox potentials of electron-transfer proteins has proven to be rather difficult as a consequence of strong interactions between these proteins and the electrode surface, which often lead to adsorption and structural alteration (1–7). Attempts to circumvent this problem have resulted in the development of a variety of strategies for chemically modifying metal and semiconductor surfaces so as to provide an electron transfer pathway that maintains protein native structure and eliminates strong binding (1, 8–29). In previous studies from our laboratory (30–35), we reported on a method of coating such solid supports. This method uses a self-assembled lipid bilayer at the electrode/aqueous interface that greatly facilitates electroactivity, as has been shown with a variety of small soluble redox proteins of various surface charge and active-site composition (cytochrome *c*, plastocyanin, ferredoxin, thioredoxin) without any artifactual structural modifications accompanying interaction with the electrode.

Because many redox proteins are strongly membrane-associated and normally function within the liquid crystalline environment of a phospholipid bilayer, it is clearly desirable to develop direct electrochemical systems that can accommodate proteins of this type as well. Although a planar lipid-bilayer membrane separating two aqueous phases has been an excellent model for biological membranes and is well suited for electrical measurements (36–38), the fragility and small size (typically <1 mm^2) of the bilayer membrane do not allow corroborative spectroscopic assays of membrane-protein biochemical activities or structural properties. As a consequence, several different types of interfacial films deposited on solid supports have been developed that can incorporate integral membrane proteins (for review, see ref. 39; also see refs. 29, 40–43) and that offer the possibility of manipulating mechanical stability and geometry to permit electrical, chemical, and structural measurements to be made on the same film. As an example, such systems have successfully been used for investigating electric field effects on the structure of ATPases and membrane-channel proteins, the activities of which are modulated by membrane potentials (39, 44–48). Another aspect of this technology lies in the possibility of incorporating functional membrane enzymes and receptors into biochemical sensing devices, which use changes in film electrical (or photoelectrical) and optical properties to detect charge-transfer reactions or substrate binding (39).

A major conclusion that we arrived at on the basis of our above-mentioned experiments on the use of lipid-bilayer-modified electrodes to carry out direct electrochemical measurements on redox proteins was that specific, but relatively weak, adsorption processes involving electrostatic or hydrophobic forces were an important component of the ability of these systems to function in mediating protein electron transfer reactions. It seemed reasonable to us, then, that integral membrane proteins, which normally are embedded in phospholipid bilayers, should also find such electrode coatings a hospitable environment, allowing them to directly participate in electron transfer reactions with an underlying metal or semiconductor surface. Our experiments have, indeed, borne this out, and we report below cyclic voltage–current measurements of the redox reactions between a lipid-bilayer-modified electrode and two integral membrane proteins: cytochrome *f* and cytochrome *c* oxidase.

Cytochrome *f* is a heme c-containing component of the chloroplast b_6f complex that is involved in the photosynthetic transport of electrons from plastoquinol to photosystem I via plastocyanin (49). It has been isolated in a monomeric, water-soluble form from *Spirulina* (a cyanobacterium) and from spinach (as an octameric aggregate), rape, and turnip (higher plants) (50, 51). Structural evidence obtained with the spinach protein suggests that a 20-residue segment between residues 251 and 270 of the 285-residue protein may be involved in a single membrane-spanning α-helix, with the heme-containing domain (residues 1–250) protruding from the membrane surface (52–54).

Cytochrome *c* oxidase is an intensively studied, tightly bound integral membrane metalloprotein that carries out a crucial function in cellular respiration in both prokaryotes and eukaryotes (55–57)—i.e., reduction of dioxygen to water by using electrons derived from cytochrome *c*, a process that is coupled with energy conservation in the form of a transmembrane pH gradient and potential. The redox centers are two heme a moieties (a and a_3) and two copper ions (CuA and CuB). The heme a and CuA centers are clearly involved in the

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entry of electrons into the protein, whereas the heme a_3 and CuB moieties form a binuclear cluster that binds and reduces dioxygen during the catalytic cycle. The redox potentials at which these sites operate are somewhat uncertain (56); the most frequently cited values from reductant titrations and potentiometric data are ≈0.28 V for CuA and 0.34 V for heme a for the resting state of the enzyme. However, the thermodynamic situation with respect to redox potential is complicated by extensive interactions among the centers (56).

As we will show below, we have been able to use cyclic voltage-current measurements at an optically transparent lipid-bilayer-modified semiconductor electrode to obtain redox potentials for these two metalloproteins. The present work constitutes a report of direct (i.e., unmediated) electrochemical measurements involving integral membrane electron transfer proteins and opens up another approach to the study of these important materials.

MATERIALS AND METHODS

Spinach cytochrome *f* was purchased from Sigma and used without further purification. Purified beef heart cytochrome *c* oxidase was provided by L. P. Pan and S. I. Chan of the California Institute of Technology.

Lipid-bilayer membranes were deposited on solid substrates, using a described technique (58), from a membrane-forming solution containing egg lecithin at 10 mg/ml (Sigma) in squalene (Fluka)/butanol, 1:3. An optically transparent tin-doped indium oxide semiconductor electrode of 15 Ω/cm² (donated by Donnelly Corporation, Tucson, AZ) was used as a solid substrate, playing the role of a working electrode in our electrochemical device. The electrode was cleaned immediately before use by successive 15-min sonications in Alconox solution (Alconox, New York) in 95% ethanol and in purified water. A thin-layer electrochemical cell was used, as described (35); this cell had a sample volume of 0.4 ml and a variable solution thickness (0.08–0.25 cm). It is important to note that this geometry permits optical measurements to be made on the protein sample, although we do not report such results here. All cyclic voltammetric experiments were done at room temperature (22 ± 1°C) by using a home-made functional generator with a current interface (31). Potential and current outputs were digitized by a Heath–Zenith SD 4850 digital oscilloscope, and the digital waveforms were stored in a computer. All potentials were measured relative to a saturated Ag/AgCl reference electrode. Solutions were degassed with high-purity humidified argon before use.

RESULTS AND DISCUSSION

The functional reconstitution of integral membrane proteins into phospholipids is generally based on detergent removal from the protein solution in the presence of a lipid bilayer. A number of different methodologies, such as dilution, dialysis and gel filtration, active detergent absorption, and other less conventional techniques, have been used for this purpose (for review, see refs. 39 and 59). Of these various reconstitution procedures, detergent dilution is the simplest and most rapid technique and seemed the most appropriate in our experiments where we have a preformed solid surface-bound lipid bilayer in an electrochemical cell, as described (35). Thus, concentrated solutions of the purified detergent-solubilized proteins were injected into the electrolyte-containing compartment of the cell, allowing the reconstitution of protein into the lipid bilayer.

Interaction between protein molecules and the lipid-bilayer-modified electrode resulted in voltammetric peak currents that were superimposed on sloping backgrounds. Therefore, a digital background subtraction procedure was used to improve the precision and accuracy of peak potential determinations. This procedure involved the use of a computer program that identified the beginning and end of a single potential sweep cycle and subtracted point-by-point the appropriate background current from the total current measured in the presence of redox proteins. The background current was obtained separately by using the same experimental conditions but without protein. Peak currents and potentials were determined by using such background-subtracted "voltammograms," and the half-wave potential was calculated as a midpoint between oxidation and reduction peaks.

Fig. 1 shows a cyclic voltammogram (Fig. 1*A*) and the electrode response after background subtraction (Fig. 1*B*) obtained after injection of a concentrated octyl glucoside detergent solution of cytochrome *f* into a supporting electrolyte consisting of 5 mM phosphate buffer, pH 7.0/20 mM $NaClO_4$ and dilution of proteins and detergent to their final concentrations of 50 μM and 0.1%, respectively, at a self-assembled egg phosphatidylcholine lipid-bilayer-modified indium oxide electrode. Fig. 2 shows similar results obtained with 45 μM cytochrome *c* oxidase from beef heart mitochondria in an electrolyte of 10 mM Tris, pH 7.4/0.5 mM EDTA/40 mM $NaClO_4$/0.05% lauryl maltoside at the same electrode system. In both cases, measurements taken under anaerobic conditions (see *Materials and Methods*) produced well-defined voltammograms, with one redox wave for cytochrome *f* (Fig. 1) and two separate waves for cytochrome *c* oxidase (Fig. 2). In the latter case, when dioxygen was present, additional signals were also observed; however, these require further study to evaluate their significance. There was no measurable response of a bare indium oxide electrode without the lipid-bilayer modification to either cytochrome *f* or to cytochrome *c* oxidase.

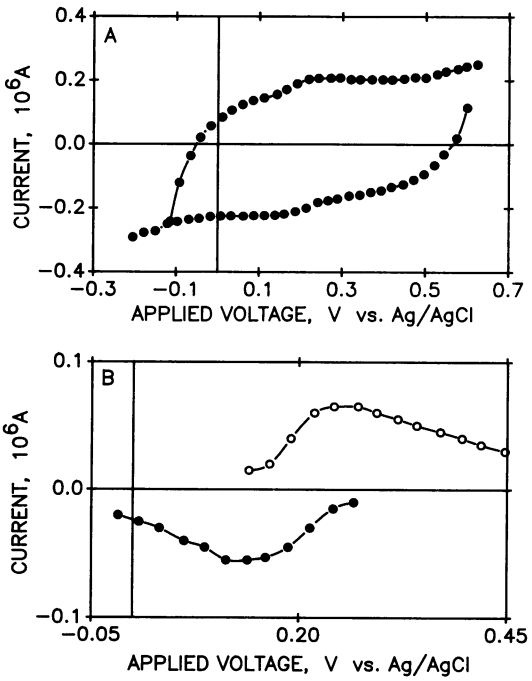

FIG. 1. Cyclic voltammogram (*A*), and shapes of current–voltage oxidation (○) and reduction (●) curves obtained after background subtraction (*B*) for an anaerobic solution of spinach cytochrome *f* at an indium oxide electrode modified with a phosphatidylcholine bilayer. The cytochrome *f* concentration was 50 μM in a supporting electrolyte consisting of 5 mM phosphate buffer, pH 7.0/20 mM $NaClO_4$/0.1% octyl glucoside (original octyl glucoside concentration in added protein solution was 0.5%). A potential scan rate of 16 mV/s and a Ag/AgCl reference electrode were used.

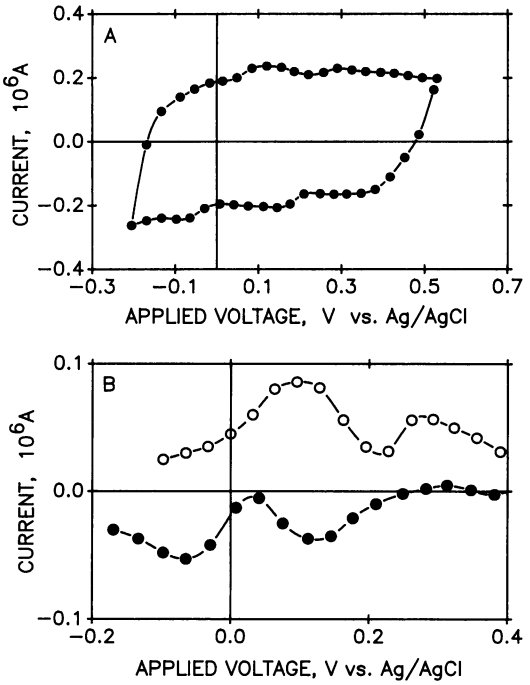

FIG. 2. Cyclic voltammogram (*A*), and shapes of current–voltage oxidation (○) and reduction (●) curves obtained after background subtraction (*B*) for beef heart cytochrome *c* oxidase at an indium oxide electrode modified with a phosphatidylcholine bilayer. Samples were made anaerobic, as described. The enzyme concentration was 45 μM in electrolyte containing 10 mM Tris, pH 7.4/0.5 mM EDTA/40 mM $NaClO_4$/0.05% lauryl maltoside (original detergent concentration in added protein solution was 0.1%). A potential scan rate of 32 mV/s and a Ag/AgCl reference electrode were used.

Typical electrochemical parameters [oxidation and reduction peak separation (ΔE) and half-wave potential ($E_{1/2}$)] for both proteins obtained under the experimental conditions described in Figs. 1 and 2 are listed in Table 1. Although the lack of correction of peak potentials for uncompensated resistance of both the electrode and the lipid bilayer should not have a measurable impact on the half-wave potentials measured here, it might affect the value of the peak separation (ΔE). Thus, the resistivity of the lipid bilayer might be large enough to shift the oxidation and reduction peaks in opposite directions (58, 60–62), and protein molecules could penetrate the bilayer, thereby altering its resistivity. It is important to point out that the half-wave potentials obtained here (365 mV for cytochrome *f* and 250 mV and 380 mV for cytochrome oxidase, calculated vs. the normal hydrogen electrode) compare quite well with the values reported for the heme of cytochrome *f* (63), and the CuA and heme a redox centers of cytochrome *c* oxidase, respectively, obtained by conventional titration methods (56).

Although these preliminary results require further experimentation to fully understand the mechanism of interaction between the lipid-bilayer-modified electrode and these two membrane-bound protein molecules, as well as to better characterize both the kinetic and thermodynamic parameters of the measured redox reactions, the above results unequivocally demonstrate the occurrence of a direct electrochemical reaction between the working electrode and the two redox proteins. Thus, unmediated electrochemistry with these two integral membrane proteins has now been accomplished. We believe that this accomplishment opens up another approach to the investigation of the properties of integral membrane redox proteins, by allowing simultaneous measurements of thermodynamically accurate potentials and spectroscopic parameters (optical as well as other types of spectroscopy) in a biocompatible phospholipid environment for which composition can be readily varied.

Table 1. Peak separations (ΔE) and half-wave potentials ($E_{1/2}$) calculated vs. normal hydrogen electrode for cytochrome *f* and cytochrome *c* oxidase

	Peak separation, ΔE/mV	Half-wave potential, $E_{1/2}$/mV
Cytochrome *f*	105	365 ± 15
Cytochrome *c* oxidase		
I wave	160	250 ± 20
II wave	160	380 ± 20

All conditions were as in Figs. 1 and 2, respectively.

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