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Direct measurement of the electrogenic activity of *o*-type cytochrome oxidase from *Escherichia coli* reconstituted into planar lipid bilayers

(patch pipets/open-circuit membrane potential/applied voltage/respiratory control)

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ABSTRACT **Turnover of *o*-type cytochrome oxidase purified from *Escherichia coli* and reconstituted into proteoliposomes leads to the generation of a transmembrane electrical potential (interior negative) by means of vectorial electron flow. In the experiments reported here, purified oxidase is reconstituted in planar lipid bilayers formed at the tip of patch pipets, and open-circuit membrane potentials generated by electron transfer are measured directly. Potentials of up to 4 mV (substrate side positive) are generated in the presence of reduced phenazine methosulfate or ubiquinol-1, and with both substrates, electrogenic activity is inhibited by cyanide. Furthermore, the membrane potential generated during oxidase turnover is inhibited progressively with applied voltages (substrate side positive), decreasing almost to zero at an applied voltage of 150 mV.**

Cytochrome *o*-type oxidase purified from the membrane of *Escherichia coli* contains four polypeptides (M_rs 66,000, 35,000, 22,000, and 17,000), two mol of cytochrome *b*-type heme (b_{558} and b_{563}), and two mol of copper per mol of enzyme (1–4). The oxidase utilizes ubiquinol-1 (Q_1H_2), reduced phenazine methosulfate (PMS), and certain other electron donors as substrates but does not oxidize reduced cytochrome *c*, and its activity is highly dependent upon exogenous phospholipids and/or nonionic detergent. Proteoliposomes formed by detergent dilution or dialysis in the presence of oxidase and phospholipids, followed by freeze-thaw/sonication, generate a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) of −115 to −140 mV (interior negative and alkaline) during turnover of the oxidase. Furthermore, proteoliposomes containing both the oxidase and purified *lac* permease transport lactose against a concentration gradient in a fashion that mimics intact cells and right-side-out membrane vesicles (3).

By measuring external and internal pH changes in proteoliposomes reconstituted with the *o*-type oxidase, it has been shown (4) that during Q_1H_2 oxidation, protons are released on the external surface of the membrane and consumed on the internal surface. In contrast, with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, an electron donor that carries few protons at neutral pH, little change in external pH is observed until a protonophore is added, at which point the medium becomes alkaline. These and other results (4) are consistent with the interpretation that oxidase turnover generates an electrical potential ($\Delta\Psi$, interior negative) due to vectorial electron flow from the outer to the inner surface of the membrane. The pH gradient (ΔpH, interior alkaline), on the other hand, results from scalar (i.e., nonvectorial) reactions that consume and release protons at the inner and/or outer surfaces of the membrane, respectively. Thus, as opposed to mitochondrial cytochrome oxidase (5) and certain bacterial terminal oxidases (6–8), the *o*-type oxidase from *E. coli* does not appear to catalyze vectorial proton translocation.

In this paper, we report direct measurements of the electrogenicity of the *o*-type cytochrome oxidase reconstituted into planar lipid bilayers formed at the tip of patch pipets. Although the potentials obtained are small relative to those reported for reconstituted proteoliposomes, both systems exhibit similar properties qualitatively.

A preliminary account of this research has been presented elsewhere (9).

EXPERIMENTAL PROCEDURES

Materials. Sodium ascorbate, PMS, dithiothreitol, and *N*-tris[hydroxymethyl]methylglycine (tricine) were purchased from Sigma, 4-morpholinepropanesulfonic acid (Mops) was from Boehringer Mannheim, and 3,3′-diisopropylthiocarbocyanine was from Molecular Probes (Junction City, OR). Q_1 was generously provided by Hoffmann-La Roche.

Methods. *Preparation and reconstitution of* o*-type cytochrome oxidase.* The *o*-type cytochrome oxidase was solubilized from the membrane of *E. coli* GR19N (*Cytd*⁻), purified and reconstituted into proteoliposomes with *E. coli* phospholipids as described (3, 4). The functional integrity of the preparations was assessed by measurements of $\Delta\Psi$ (interior negative) and ΔpH (interior alkaline) with appropriate probes (3, 4). Proteoliposomes to be used for planar bilayer formation were washed once in 10 mM sodium tricine (pH 7.5), frozen in liquid nitrogen, and stored at −70°C until use.

Patch pipets. Pipets were produced as described (10) with hematocrit capillaries made of flint glass (BLUE-TIP, plain; i.d., 1.1–1.2 mm; Lancer, St. Louis, MO). The capillaries were cleaned in 1.0 M nitric acid and rinsed overnight under running deionized water. Before being pulled, the capillary tubes were rinsed with methanol and dried by flushing with a nitrogen stream. A commercially available pipet puller (David Kopf Instruments, model 700C, Tujunga, CA) was used.

Electrical recordings. Membrane potentials under current clamp were recorded with an extracellular patch-clamp system (10) (List L/M EPC-5, List Electronics, Darmstadt, FRG). Constant voltage was applied from a dc source, and the compartment under continuous perfusion was defined as zero voltage. The signal from the clamp was recorded on FM

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Abbreviations: PMS, phenazine methosulfate; Mops, 4-morpholinepropanesulfonic acid; Q_1H_2, ubiquinol-1; $\Delta\bar{\mu}_{H^+}$, transmembrane proton electrochemical gradient; ΔV, open-circuit membrane potential; $\Delta\Psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient.

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(frequency modulation) tape (RACAL 4DS, Hythe, Southampton, England) and displayed on a strip chart recorder (Gould 2200S) after being filtered at 700 Hz on an eight-pole Bessel low-pass filter (Frequency Devices, Haverhill, MA).

Formation of planar bilayers at patch pipet tips. Proteoliposomes prepared and stored as described above were thawed immediately before the experiment, sonicated in a water-bath sonicator (Branson) for 30 s, and stirred for 15 s. A 2-μl aliquot of proteoliposomes (3 mg of protein per ml at a protein-to-lipid ratio of 1:5, wt/wt) was added to a Teflon chamber containing 200 μl of 50 mM KCl/1 mM $CaCl_2$/10 mM Mops (potassium salt), pH 7.1. Under these conditions, the proteoliposomes generate monolayers at the air/water interface (11–14). After a few minutes, a patch pipet was introduced into the solution, and the resistance of the open pipet was measured. The pipet was then removed from the solution and immediately reimmersed into the same solution, thereby leading to the formation of a lipid bilayer by apposition of the hydrocarbon tails of the two monolayers (15) at the tip of the patch pipet (14, 16–18). Occasionally, positive or negative pressure was applied in order to increase the bilayer resistance (10). The aqueous compartments separated by the bilayer were connected to the patch-clamp circuit via Ag/AgCl electrodes (In Vivo Metric, Healdsburg, CA). The electrode outside the patch pipet was connected to the Teflon chamber through a salt bridge (1.0 M KCl in 2% agar) in order to avoid artifactual signals arising from the interaction between the Ag/AgCl electrode and the redox reagents under study. Substrates and inhibitors were introduced into the bilayer chamber by continuous perfusion with medium (1–2 ml/min) containing the appropriate reagent. All bilayer experiments were conducted at room temperature (22 ± 2°C).

RESULTS

Generation of $\Delta\Psi$ in Proteoliposomes. As indicated by fluorescence quenching studies with 3,3′-diisopropylthiocarbocyanine (3) and subsequently confirmed with other probes (4), proteoliposomes containing purified cytochrome *o* generate both a $\Delta\Psi$ (interior negative) and a ΔpH (interior alkaline) during turnover in the presence of Q_1H_2. Similarly, addition of ascorbate and PMS to proteoliposomes containing the oxidase also quenched the fluorescence of 3,3′-diisopropylthiocarbocyanine; moreover, quenching was enhanced in the presence of nigericin (Fig. 1). Nigericin is an ionophore that catalyzes electrically neutral exchange of protons for K^+ or Na^+, thereby collapsing ΔpH with a compensatory increase in $\Delta\Psi$ (19–21; for review, see ref. 22). In contrast, immediately after addition of valinomycin, the signal returned rapidly to the baseline, an observation consistent with the known ability of this ionophore to collapse $\Delta\Psi$ in the presence of K^+ (19–21; for review, see ref. 22). Inhibition of oxidase activity by cyanide also reversed the signal, although the effect was somewhat slower than that observed with valinomycin.

The magnitude of the $\Delta\Psi$ generated during oxidase turnover can be estimated by comparison to standard curves constructed from experiments in which fluorescence quenching is induced by imposition of K^+ diffusion gradients of known magnitude in the presence of valinomycin (4). Values for $\Delta\Psi$ obtained by this means ranged from −100 to −120 mV (after addition of reduced PMS and nigericin), and similar values have been estimated from steady-state distribution measurements with [^{3}H]tetraphenylphosphonium ion (cf. ref. 4).

Generation of Open-Circuit Membrane Potential (ΔV) in Planar Bilayers. The data presented in Fig. 2 illustrate the time course of change in ΔV upon perfusion of a planar lipid bilayer containing *o*-type cytochrome oxidase with reduced PMS (substrate) and/or cyanide (inhibitor) according to the pulse sequence shown. Perfusion with buffer containing substrate led to generation of a ΔV of approximately 2 mV, positive on the substrate side (i.e., outside of the pipet). When substrate was washed out of the chamber (substrate pulse off), ΔV returned to the initial baseline. This response could be repeated virtually indefinitely by repeating the substrate-pulse sequence shown. Introduction of cyanide, an inhibitor of the oxidase (3, 4), collapsed the ΔV generated in the presence of reduced PMS and markedly inhibited generation of ΔV on subsequent perfusion with the electron donor (Fig. 2). The magnitude of the ΔV generated by oxidase turnover was linearly dependent on bilayer resistance (R_m) from 5 to 20 GΩ. Although not shown, similar results are obtained when Q_1H_2 was used in place of reduced PMS.

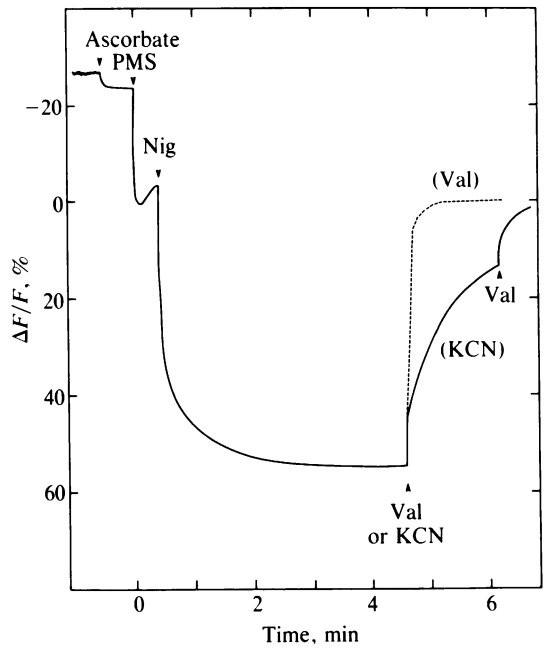

FIG. 1. 3,3′-Diisopropylthiocarbocyanine fluorescence quenching induced by reduced PMS in proteoliposomes containing *o*-type cytochrome oxidase. Reaction mixtures contained 50 mM potassium phosphate (pH 7.5), proteoliposomes (4.0 μg of protein) with a cytochrome *o*-to-phospholipid ratio of 1:5 (wt/wt), and 1 μM 3,3′-diisopropylthiocarbocyanine. Reactions were initiated by addition of 2.5 mM ascorbate and 10 μM PMS, followed by addition of 0.025 μM nigericin (Nig), 1.0 μM valinomycin (Val), and/or 2.5 mM potassium cyanide (KCN) as indicated. Excitation was at 622 nm, and fluorescence emission was recorded at 670 nm. Data are plotted as $\Delta F/F$ (%) versus time. Since PMS causes quenching, the baseline fluorescence (i.e., $\Delta F/F = 0$) is taken as that observed after addition of valinomycin.

Results similar to those shown were obtained with 23 independently formed bilayers. Values of ΔV ranging from 0.6 to 3.8 mV were obtained with an average of 1.2 mV (±0.7 SD) with either reduced PMS or Q_1H_2 as electron donors.

The effect of applied electric field on the electrogenic activity of the *o*-type oxidase is shown in Fig. 3. The amplitude of the ΔV generated with reduced PMS was essentially independent of applied voltage from about +25 mV to −25 mV. At values below −25 mV (substrate side positive), however, the electrogenic activity of the oxidase was inhibited progressively and approximated zero at an applied voltage of −150 mV.

DISCUSSION

These results provide a direct demonstration of the transformation of redox energy into a transmembrane electrical potential by the *o*-type terminal oxidase from *E. coli*. Thus, turnover of oxidase molecules reconstituted into a planar bi-

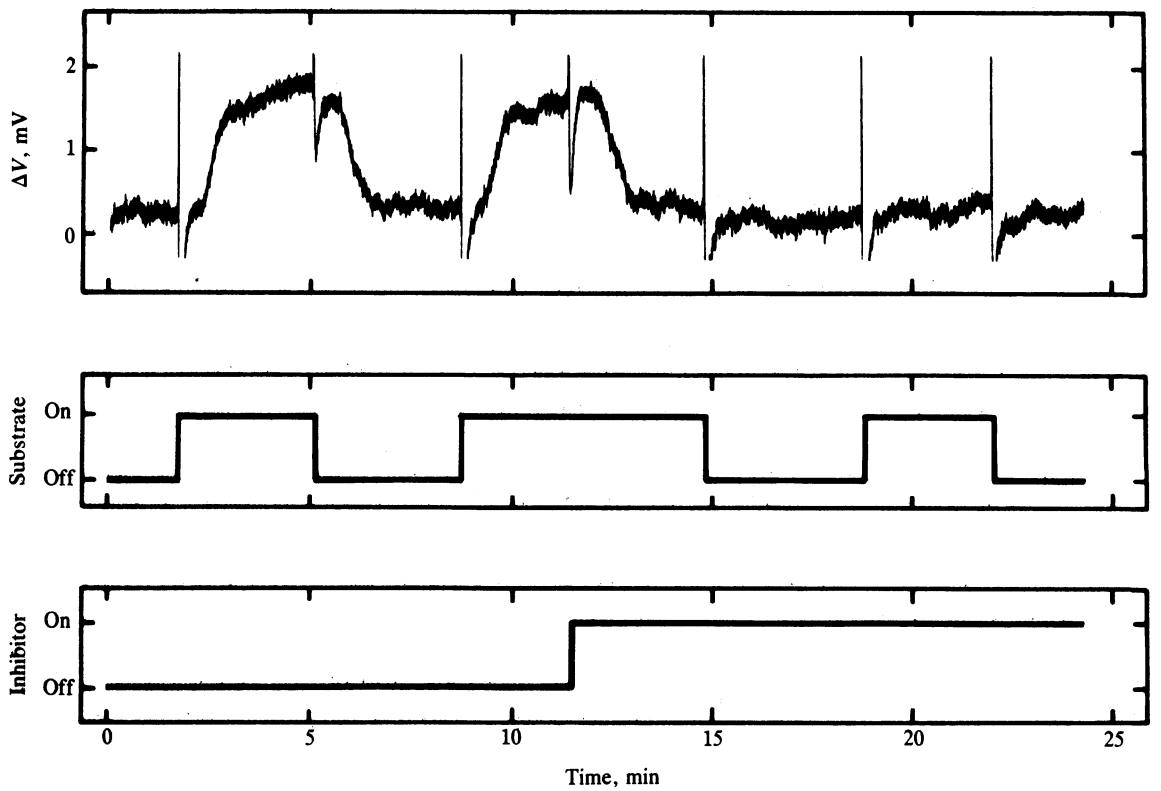

FIG. 2. Membrane potential generation by cytochrome *o* oxidase. To form the vesicle-derived monolayers at the air/water interface, 2 μl of reconstituted vesicles were added to 200 μl of buffer. After a few minutes, the bilayer was formed by the sequential removal and reimmersion of the pipet through the interface. The bilayer was perfused with medium (10 mM potassium Mops/50 mM KCl/1 mM $CaCl_2$/5 mM sodium ascorbate, pH 7.1) with or without substrate (25 μM PMS) and/or inhibitor (1 mM KCN) as indicated. The medium inside the pipet was 10 mM potassium Mops/50 mM KCl/1 mM $CaCl_2$, pH 7.1. Membrane resistance was 14 GΩ. The record illustrates the time course of change in membrane potential upon perfusion of the bilayer with substrate (25 μM PMS) and inhibitor (1 mM KCN). The transient spikes are electrical artifacts introduced by changing one medium for another.

layer at the tip of a patch pipet generates an electrical potential that averages about 1.2 mV, substrate side positive, and the activity is abolished by cyanide. Although the findings reported with the planar bilayer are highly reproducible, it is apparent that the magnitude of the electrical potential generated in this system is significantly lower than that obtained in proteoliposomes reconstituted with the same oxidase, where $\Delta\Psi$ values of −100 to −120 mV are observed with reduced

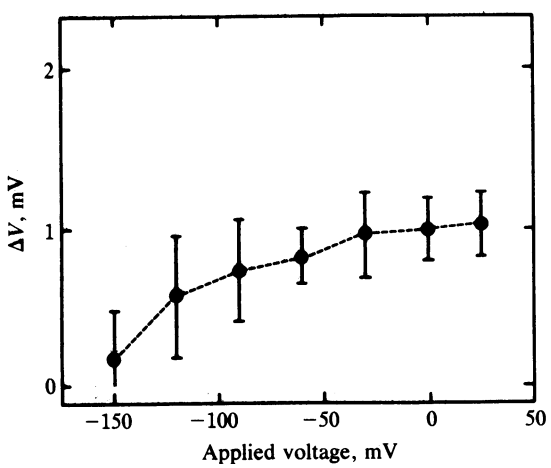

FIG. 3. Effect of applied voltage on the electrogenic activity of cytochrome *o* oxidase. Voltage was applied across the bilayer under current-clamp conditions as described. The change in membrane potential (±SD, n = 11) caused by perfusion of the substrate (25 μM PMS) is plotted against applied voltage. The compartment under perfusion (i.e., outside the pipet) was defined as zero voltage. All other conditions are the same as described in Fig. 2.

PMS or Q_1H_2. By considering some of the parameters of the planar bilayer system, however, the discrepancy can be rationalized.

An estimate of the theoretical ΔV generated by the oxidase in a planar bilayer can be made with the following considerations: (*i*) the area of the bilayer in the tip of the pipet is 2 μm^2; (*ii*) the protein-to-lipid ratio in the reconstituted bilayer is the same as that in proteoliposomes (1:900, mol/mol); (*iii*) the turnover number, T, of the oxidase in the bilayer is equivalent to that measured in proteoliposomes (190 electrons s^{-1}) (3, 4); and (*iv*) the area occupied by one phospholipid molecule is similar to that in a condensed monolayer (60 $Å^2$) (23). The current, i, generated by the oxidase reaction is therefore:

$$i = q/t = (\text{the number of oxidase molecules})\cdot(N)\cdot(T), \quad [1]$$

where q is the charge transferred across the membrane per unit time, t, N is the stoichiometry of charge translocation, and T is turnover number. By using this relationship, the estimated current is $7 \times 10^5 \times N$ charges s^{-1} or 0.1 N pA transferred across the bilayer. With a typical bilayer resistance of 10 GΩ, it follows from Ohm's Law that a ΔV of 1 mV should be generated by the oxidase reaction under the conditions described. In comparison, if the resistance of the bilayer was 1000 GΩ, the same current would produce a potential difference of 100 mV, a value more in concert with that observed in reconstituted proteoliposomes. Although these considerations represent a rough approximation, they provide an estimate at least of the expected ΔV in the reconstituted bilayer. More specifically, they focus on differences in resistance as a possible causative factor for the discrepancy between reconstituted planar bilayers and proteoliposomes.

Another possible complication that deserves comment concerns the orientation of individual oxidase molecules in the reconstituted bilayer. Although it is clear from measurements of $\Delta\bar{\mu}_{H^+}$ and H^+/O stoichiometry (4) that oxidase molecules in reconstituted proteoliposomes have a preferred orientation (i.e., substrate side out), it is conceivable that the procedure used to assemble lipid bilayers in patch-pipet tips leads to the formation of symmetric membranes in which oxidase molecules are randomly oriented. Since the electron donors utilized as substrates by the oxidase are permeant [i.e., reduced cytochrome *c* is not a substrate (3, 4)], turnover of molecules oriented in both directions across the bilayer would be expected to cancel each other electrogenically.

Although oxidase turnover in reconstituted proteoliposomes leads to generation of a relatively large $\Delta\bar{\mu}_{H^+}$, stimulation of oxidase activity by dissipation of $\Delta\bar{\mu}_{H^+}$ is minimal, and respiratory control ratios of only about 1.5 are observed (4). To an extent, this behavior is consistent with the response of the reconstituted bilayers to applied electric fields (Fig. 3). At an applied voltage of about −120 mV (substrate side positive), the electrogenic activity of the oxidase is inhibited by about 50%, a figure commensurate with the respiratory control ratio of 1.5 observed in proteoliposomes. However, imposition of an electric field of −150 mV across the planar bilayer almost completely abolishes the electrogenicity of the oxidase, while analogous experiments with reconstituted proteoliposomes do not inhibit oxidase activity nearly as effectively. Thus, when potassium diffusion potentials ($[K^+]_{in} \rightarrow [K^+]_{out}$ in the presence of valinomycin) approximating −150 mV are imposed on reconstituted proteoliposomes, respiratory control ratios of only about 1.5 are still observed.

The electrogenic nature of the oxidase reaction was experimentally tested in planar membranes by Drachev *et al.* (24). Bovine cytochrome oxidase was incorporated into proteoliposomes which, when adsorbed or fused onto black lipid films or thick films, generated cyanide-sensitive transmembrane potentials upon oxidation of external cytochrome *c*. This investigation did not deal with a genuine reconstitution of the oxidase into a planar lipid bilayer and suffers from the drawbacks that the structure of fused vesicles with thick films is not well-understood and the nature of the reactions is not clear. In contrast, our study involves the reassembly of the purified enzyme into a well-defined planar lipid bilayer membrane that exhibits functional activity. The reconstituted planar bilayer offers the unique advantage of being accessible for the direct measurements of membrane potential generated by the oxidase reaction (25) and for direct application of an electric field across the oxidase. Consequently, the effect of electric fields on the electron-transfer reaction proceeding in the reconstituted enzyme can be assessed. In addition, the pH on both sides of the membrane can be readily manipulated, allowing one to examine the effects of transmembrane pH gradients on the electron-transfer reaction. This provides an opportunity to test specific predictions of reaction mechanisms that consider the electron or the proton as the charged species carrying the current across the membrane (5, 26).

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