Reconstitution of Biological Molecular Generators of Electric Current

CYTOCHROME OXIDASE*

(Received for publication, January 30, 1975, and in revised form, December 17, 1975)

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1. Direct measurement of the electric current generation by cytochrome oxidase has been carried out. To this end, two procedures were used. The simpler one consists in formation of planar artificial membrane from the mixture of decane solution of soya bean phospholipids and beef heart cytochrome oxidase. Addition of cytochrome c and ascorbate to one of the two compartments separated by the cytochrome oxidase-containing planar membrane was found to result in a transmembrane electric potential difference being formed (plus on cytochrome c side of the membrane). Maximal values of potential differences obtained by this method were about 40 mV. Much higher potentials were observed when another ("proteoliposome-planar membrane") method was applied. In this case cytochrome oxidase was reconstituted with phospholipid to form proteoliposomes which adhered to planar phospholipid membrane in the presence of Ca²⁺ ions. Addition of cytochrome c and ascorbate to the proteoliposome-containing compartment gives rise to generation of an electric potential difference across the planar membrane, which reached 100 mV at a current of about 1 × 10⁻¹¹ A (minus in the proteoliposome-free compartment). The electromotive force of this generator was estimated as being about 0.2 V. If ascorbate and proteoliposomes were added into different compartments, a penetrating hydrogen atom carrier (phenazine methosulfate, (PMS) or tetramethyl-p-phenylenediamine (TMPD)) was required for a membrane potential to be formed. Generation of an electric potential difference of the opposite direction (plus in the proteoliposome-free compartment) was revealed in experiments with cytochrome oxidase proteoliposomes containing cytochrome c in their interior. In this case, addition of PMS or TMPD was necessary.

2. In the suspension of cytochrome oxidase proteoliposomes the uptake of a cationic penetrant (tetraphenyl phosphonium cation) was found to be coupled with electron transfer via external cytochrome c. Electron transfer via intraprotoeliposomal cytochrome c induced the uptake of anionic penetrants (tetraphenyl borate and phenyldicarba-undecaborane anions).

3. All the above effects were sensitive to cyanide and protonophorous uncouplers.

4. In proteoliposomes containing both cytochrome oxidase and bacteriorhodopsin, the light- and oxidation-dependent generations of membrane potential have been revealed.

5. The data obtained are in agreement with Mitchell’s idea of transmembrane electron flow in the cytochrome oxidase segment of the respiratory chain.

In the two previous papers we reported that bacteriorhodopsin (1) and the bacteriochlorophyll reaction center complex (2) function as molecular generators of electric current. This and the next article (3) summarize the results of the study, along the same line, of two mitochondrial enzyme complexes: cytochrome oxidase and ATPase.

According to Mitchell’s chemiosmotic theory of energy coupling (4), cytochrome oxidase catalyzes transmembranous electron transfer which results in the mitochondrial membrane being charged. Racker and associates (5) demonstrated that cytochrome oxidase proteins in mitochondria are organized across the membrane. Several pieces of indirect evidence suggesting the cytochrome oxidase reaction to be electrogenic
were obtained (6-10). Further progress in the investigation of this problem became possible after two kinds of methods were developed: (a) reconstitution of phospholipid-protein vesicles (proteoliposomes) (9-14) and (b) direct voltameter measurement of electric generation by proteoliposomes (1, 14). As was found earlier (1, 2), fusion of the planar phospholipid membrane and the proteoliposomes reconstituted from bacteriorhodopsin (or bacteriophylophyrins complexes) and phospholipids results in a system competent in electric generation which can be monitored with a voltmeter connected with two electrodes immersed into solutions on both sides of the planar membrane. In the experiments described below an attempt to apply the same procedure to cytochrome oxidase proteoliposomes has been undertaken. The results showed that cytochrome oxidase can generate a transmembrane electric potential difference whose direction depends on whether cytochrome c is localized inside or outside proteoliposomes. A simpler procedure consisting in planar membrane being formed from a cytochrome oxidase + phospholipid + decane mixture was also used to obtain the cytochrome oxidase-mediated electric responses. However, the magnitudes of these responses were always lower in the proteoliposome-planar membrane system.

The results obtained by an indirect method of membrane potential detection, namely by means of a synthetic penetrating ion probe, were found to be in agreement with the data of voltameter measurements.

Preliminary communications concerning some of these results were published elsewhere (13, 15).

EXPERIMENTAL PROCEDURES

Materials—Beef heart cytochrome oxidase was purified according to Yonelani (16). Phospholipids were obtained from the same beef heart mitochondria as cytochrome oxidase. To this end, the procedure of Folch et al. (17) was used. To form cytochrome oxidase-containing planar membrane, 15 mg of cytochrome oxidase was mixed with 0.2 ml of a solution of 0.25 M sucrose and 0.01 M Tris-HCl (pH 7.5). Then 100 mg of azolectin was added. After stirring, the mixture was supplemented with 0.2 ml of azolectin solution in decane (100 mg of azolectin/ml). One drop of the final mixture was applied onto the 0.8-mm aperture in the Teflon partition of the experimental cell.

Cytochrome oxidase proteoliposomes were reconstituted after Racker et al. (9, 11). Cholate solutions of mitochondrial phospholipids (or, in some experiments, azolectin) and cytochrome oxidase were mixed and dialyzed for 18 h at 2°C. The mixture for reconstitution contained 3 mg of cytochrome oxidase protein and 50 mg of phospholipid per ml, 2% sodium cholate, 0.125 M sucrose, 0.01 M Tris-HCl (pH 8.0), 0.1 M (NH₄)₂SO₄, and 0.25 M NaCl. The mixture for dialysis in the case of reconstitution of cytochrome oxidase proteoliposomes without cytochrome c contained 0.05 M potassium phosphate (pH 7.4), or, in experiments on K⁺ transport, 0.125 M choline chloride and 0.01 M Tris-HCl (pH 8.0). In the case of cytochrome oxidase proteoliposomes with cytochrome c inside, the dialysis mixture contained 10% methanol, 0.01 M Tris-HCl (pH 8.0), 0.2 mM EDTA, and 0.1 mM dithiothreitol. The resulting proteoliposomes were centrifuged at 15,000 × g for 60 min, suspended in a solution containing 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.5), and 1 mM EDTA and stored at 2°C. The reconstitution mixture for cytochrome oxidase proteoliposomes with cytochrome c inside was supplemented with cytochrome c (20 mg/ml). Cytochrome c bound to the outer surface of the proteoliposomes was removed by washing with 0.25 M sucrose, 0.25 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 1 mM EDTA.

Cytochrome oxidase oxidase was obtained from rabbits immunized by beef heart cytochrome oxidase.

Measurements—Electric generation by the cytochrome oxidase proteoliposomes was measured using a procedure involving Ca⁺⁺ induced fusion of the proteoliposomes and planar artificial phospholipid membrane by the method developed for bacteriorhodopsin study (for details, see the first paper of this series, Ref. 1).

Measurements of synthetic penetrating ions (PCB⁻⁻, TPB⁻⁻, and TPP⁺⁺) were carried out as described earlier (1, 7) using an artificial phospholipid membrane as an ion-sensitive electrode. The membrane was made of a decane solution of mitochondrial phospholipids supplemented, in the case of TPP⁺⁺, with phenylboran to increase TPP⁺⁺ permeability. Respiration was measured polargraphically (7). Differential spectra were taken by means of a Chance type split beam spectrophotometer.

RESULTS AND DISCUSSION

Planar Membrane Made of Phospholipid and Cytochrome Oxidase Mixture—In the first series of experiments, a very simple procedure for the cytochrome oxidase incorporation into planar membrane was tried. Cytochrome oxidase was added to a decane solution of azolectin, and the mixture was applied onto an aperture in the Teflon partition (previously this procedure was used to incorporate bacteriorhodopsin into planar membrane, see Ref. 1). Then one of the two ascorbate solutions, separated by cytochrome oxidase-containing planar membrane, was supplemented with cytochrome c to initiate electron transfer from ascorbate to oxygen. One can see (Fig. 1) that cytochrome c addition results in an electric potential difference across the planar membrane being generated (plus in the cytochrome c-containing (say, left-hand) compartment. Addition of cytochrome c into the right-hand compartment leads to a response of opposite direction. Cyanide addition to the right-hand compartment inhibits immediately the electric potential generation.
response induced by cytochrome c in the same compartment. In the opposite (left-hand) compartment, inhibition develops much more slowly. If cyanide is present in both compartments, cytochrome c addition does not induce formation of any electric potential difference.

Maximal values of electric potential obtained by this procedure were no higher than 40 mV. Higher potentials were obtained when a proteoliposome-planar membrane system was studied.

Cytochrome Oxidase Proteoliposomes with Cytochrome c Outside—To induce fusion of the proteoliposomal and planar membranes, Ca\textsuperscript{2+} ions were added. As the experiments showed, ascorbate added 20 to 30 min after proteoliposomes to the same compartment induces an electric generation across the planar membrane (minus in the proteoliposome-free compartment). A subsequent cyanide treatment abolished the electric potential difference (Fig. 2A). No ascorbate effect was found if the latter was added after cyanide. If the external resistance (R\textsubscript{ext}), shunting the planar membrane, was lower than the planar membrane resistance (R\textsubscript{memb}), differentiation of the ascorbate response took place.

Ascorbate addition to the proteoliposome-free compartment did not induce any potential difference generation unless a penetrating hydrogen atom carrier, PMS or TMPD, was added (in these experiments, cytochrome c was added into both compartments of the experimental chamber). This observation is illustrated by Fig. 3.

Maximal values of the cytochrome oxidase-generated electric potential differences observed by this method were about 100 mV at a current of above 1 \times 10\textsuperscript{-11} A.

The electric field generated across planar membrane by an external battery was found to affect the ascorbate-induced response (minus in the proteoliposome-free compartment decreased, and plus increased, the response). Estimation of the electromotive force of the cytochrome oxidase proteoliposome-planar membrane system, which was carried out counterbalancing the ascorbate-induced electric generation by external battery, gave a value of about 0.2 V (see also Fig. 8).

Cytochrome Oxidase Proteoliposomes with Cytochrome c Inside—To prepare proteoliposomes with cytochrome c inside, we supplemented the reconstitution mixture with cytochrome c, and then, after proteoliposome reconstitution, removed cytochrome c associated with the outer proteoliposome surface by NaCl washing. As spectrophotometric measurement showed, the NaCl washing gave rise to a decrease in the cytochrome c to cytochrome oxidase ratio. Experiments with TMPD activation and cytochrome oxidase antisera inhibition of the proteoliposome respiration revealed that cytochrome c in the NaCl-treated proteoliposomes is localized inside vesicles and only a half of the proteoliposomal cytochrome oxidase pool is activated (not shown in figures). Direction of electric field across the membrane of these proteoliposomes was found to be regulated by addition of external cytochrome c or PMS. Fig. 4 demonstrates that initiation of electron transfer via external cytochrome c makes

![Fig. 2. Electric potential generation by cytochrome oxidase proteoliposomes associated with the planar azolectin membrane. Incubation mixture: 0.3 M sucrose, 5 mM Tris-citrate (pH 7.2), 30 mM CaCl\textsubscript{2}, 5 mM MgSO\textsubscript{4}, 1 \times 10\textsuperscript{-4} M cytochrome c, and, in one of the compartments, cytochrome oxidase proteoliposomes (0.3 mg of protein/ml). Additions: 10 mM ascorbate and 1 mM NaCN.](http://www.jbc.org/)

![Fig. 3. PMS-induced electric response of the cytochrome oxidase proteoliposome-planar membrane system. Ascorbate (5 mM) and the proteoliposomes (0.25 mg of protein/ml) were added into different compartments. Incubation mixture: 0.2 M sucrose, 0.05 M Tris-HCl (pH 7.5), 30 mM CaCl\textsubscript{2}, and 7 \times 10\textsuperscript{-4} M cytochrome c. Concentration of PMS was 1.5 \times 10\textsuperscript{-4} M, that of NaCN, 1.5 \times 10\textsuperscript{-4} M.](http://www.jbc.org/)
Fig. 5. PCB- responses of the cytochrome oxidase proteoliposomes. Incubation mixture: 0.2 M sucrose, 0.05 M Tris-HCl (pH 7.5), and cytochrome oxidase proteoliposomes with cytochrome c inside (curve A, 0.5 mg of protein/ml, curve B, 0.6 mg of protein/ml). In Experiment A the mixture was supplemented with 2 x 10^-1 M cytochrome c. Additions: 1 x 10^-6 M PMS, 5 mM ascorbate, and 4 mM NaCN.

penetrating cations and anions of similar structure move in the opposite directions: tetraphenyl phosphonium (TPP+) is taken up, and tetraphenyl borate (TPB-) is extruded from proteoliposomes. On the other hand, the PMS-induced electron transfer via internal cytochrome c results in an uptake of a penetrating anion (PCB-) as it is seen in Fig. 5. Under the same conditions, electron transfer via external cytochrome c induced the ion movement in the opposite direction.

In Fig. 6, electric response of the cytochrome c-containing proteoliposomes associated with the planar membrane is demonstrated. It is shown that ascorbate induces generation of electric potential with minus in the proteoliposome-free compartment. Subsequent addition of PMS gives rise to the electric generation of the opposite polarity (plus in the proteoliposome-free compartment). As a result, the membrane potential returns to the zero level.

Proteoliposomes Containing Cytochrome Oxidase and Bacteriorhodopsin—Fig. 7 demonstrates interaction of two types of electric generators (cytochrome oxidase and bacteriorhodopsin) incorporated into the same proteoliposome membrane. Such proteoliposomes associated with the planar membrane were shown to be competent in the generation of electric currents of opposite directions, charging the proteoliposome-free compartment positively in response to illumination, and negatively in response to the ascorbate addition. In Fig. 8, an effect of the external electric field on the light- and ascorbate-induced responses of the proteoliposomes is shown. Linear relationships are revealed in both cases. The electromotive force estimated according to the method described above was found, under the conditions used, to be equal to 210 mV for cytochrome oxidase and to 230 mV for bacteriorhodopsin.

In conclusion, the data presented above clearly show cytochrome oxidase to be competent in electric generation. Direction of the electric field formed depends on the position of cytochrome c (plus is always formed on the cytochrome c side of the membrane).

Acknowledgments—We are greatly indebted to Professor E. A. Lihman, Professor P. Mitchell, and Professor S. E.
Severin for useful advice and discussion; to Dr. A. E. Dontsov, Dr. I. B. Nemeček, Dr. T. I. Polosina, and Dr. S. M. Smirnova for participation in some experiments; Mr. S. A. Bogoslovsky and Ms. N. M. Goreyshina for help during preparation of the manuscript, and Ms. T. I. Kheifets for correcting the English version of the paper.

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