Reconstitution of Biological Molecular Generators of Electric Current

H⁺-ATPase*

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

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In three previous papers of this series (1-3), the data on direct measurement of electric generation by bacteriorhodopsin, chlorophyll reaction center complexes, and cytochrome oxidase were reported. These results seem to be sufficient to conclude that the energy-supplying systems mentioned are competent in the utilization of the corresponding energy sources to form transmembrane electric potential.

According to Mitchell's chemiosmotic theory (4), the energy-consuming ATP synthetase reaction, localized in the same membrane as energy-supplying mechanisms, represents reversal of the ATPase reaction coupled with electrogenic uphill H⁺ transport (H⁺-ATPase).

In 1971 Kagawa and Racker (5) described reconstitution of ATPase proteoliposomes catalyzing "Pi-ATP exchange which proved to be sensitive to such a specific agent discharging membrane potential as combination "valinomycin + nigericin + K⁺." The ATP-dependent H⁺ uptake by the proteoliposomes was also observed (6).

In this work, membrane potential generation by H⁺-ATPase from beef heart mitochondria was studied by means of a penetrating ion probe and direct voltmeteter measurements in a system "proteoliposomes-planar phospholipid membrane." Preliminary notes of some of these results were published elsewhere (7, 8).

EXPERIMENTAL PROCEDURES

Materials—Hydrophobic proteins of oligomycin-sensitive H⁺-ATPase were prepared from beef heart mitochondria according to

* The part of this study in which bacteriorhodopsin was used was performed within the framework of the Research Project "Rhodopsin," organized by the U.S.S.R. Academy of Sciences and Moscow State University, and supervised by Vice-President of the U.S.S.R. Academy of Sciences Professor Yu. A. Ovchinnikov.
Kagawa and Racker (5), coupling factor F₁ according to Horstman and Racker (9), F₆ according to Balus and Racker (10). For other preparative methods, see Refs. 1 and 3. Purified phospholipids were from Koch-Light.

Reconstitution of ATPase proteoliposomes was carried out essentially after the procedure by Kagawa and Racker (5). The reconstitution mixture contained hydrophobic ATPase proteins (10 mg/ml), mitochondrial phospholipids or zcolecine (50 mg/ml), 2% sodium cholate, 0.125 mM succrose, 0.1 M (NH₄)₂SO₄, 0.01 M Tris·HCl (pH 8.0), 0.5 mM dithiothreitol, and 5 x 10⁻⁴ M EDTA. The mixture was dialyzed at 0-2°C for 18 h against the solution containing 10% methanol, 0.01 M EDTA. Dialysis and centrifugation of the reconstituted particles were performed as described above for ATPase proteoliposomes. The particles obtained were washed by a solution of 0.25 M NaCl, 0.25 mM succrose, 0.01 M Tris·HCl (pH 7.4), and 2 mM EDTA to remove cytochrome c adsorbed on the outer surface of the proteoliposomes. Then the particles were suspended in 0.25 M sucrose, 0.05 M Tris·HCl (pH 7.5), and 5 mM MgSO₄, and were reconstituted with F₆ (see above).

Measurements—ATPase activity was measured by a recording pH meter, and the PCB⁻ level by the phospholipid membrane (12). For other measurements, see previous papers of this series (1, 3).

RESULTS

ATPase Proteoliposomes—Electron microscope examination on the ATPase proteoliposomes revealed closed vesicular structures similar to proteoliposomes of other types. The ATPase activity measurements showed that ATP hydrolysis by the proteoliposomes is stimulated by protonophorous uncouplers and is strongly inhibited by oligomycin. Direct measurement of the ATPase-produced electric potential has been carried out with the method developed earlier for the bacteriorhodopsin, chlorophyll reaction center complexes, and cytochrome oxidase proteoliposomes (1–3). ATPase proteoliposomes were incorporated into planar phospholipid membrane by means of Ca²⁺ treatment. Potential difference across the planar membrane was measured by Ag/AgCl electrodes and a voltmeter. The ATP addition to such a system was found to give rise to electric generation which disappeared after oligomycin treatment (Fig. 1A). The form of the voltmeter-measured electric response was very similar to that of the ATP-induced PCB⁻ response of the proteoliposome suspension (Fig. 1B). The PCB⁻ uptake indicates the positive charging of the proteoliposome interior (7, 12).

Other measurements showed that the ATP-induced electric response can be decreased by addition of a protonophorous uncoupler or shunting the planar membrane by external resistance (not shown in figures).

Effect of Phospholipid Composition—Table I reports the values of the membrane potential, measured by means of the PCB⁻ method and directly by a voltmeter, at variations of lipid and protein composition of proteoliposomes. One can see that proteoliposomes containing phosphatidylcholine as a lipid component were quite ineffective whatever protein component was used. As to the phosphatidylserine, it was effective in the case of cytochrome oxidase and bacteriorhodopsin rather than

![FIG. 1. Electric generation by ATPase proteoliposomes. A, direct voltameter measurement in the system "proteoliposomes-planar membrane." Incubation mixture: 0.2 M sucrose, 0.05 M Tris·HCl (pH 7.3), 5 mM MgSO₄, 0.05 M CaCl₂, and proteoliposomes (0.5 mg of protein/ml). B, PCB⁻ probe. Incubation mixture: 0.25 M sucrose, 0.05 M Tris·HCl (pH 7.5), 5 mM MgSO₄, and proteoliposomes (0.5 mg protein/ml). Additions: 1.5 mM ATP and oligomycin (25 μg/ml).]

| Phospholipids | ATPase | Cytochrome oxidase | Bacteriorhodopsin |
|---------------|--------|-------------------|------------------|
| Mitochondrial| 2.5    | 15                | 1.8              |
| Phosphatidyl ethanolamine | 1.0    | 10                | 2.2              |
| Phosphatidyl serine | 0      | 4.4               | 16               |
| Phosphatidyl choline | 0      | 0                | 0.1              |

1 The abbreviations used are: F₁ and F₆, Racker's coupling factors; CCCP, trifluoromethoxybenzylcarbonyl inethylbenzylalanine; PMS, phenazine methosulfate; ΔHᵢ, membrane gradient of electrochemical potential of H⁺ ions.
ATPase. Mitochondrial phospholipids and phosphatidylethanolamine were effective in all cases.

Measurements of electric resistance of the planar membranes made of different phospholipids showed that it is several times lower in the case of phosphatidylicholine than in the case of other phospholipids. Electron microscopic study revealed only small amounts of membrane vesicles when phosphatidylicholine was used for the reconstitution. Apparently, these facts may explain why membrane potential-generating proteoliposomes cannot be obtained with this phospholipid. Other effects of lipid composition should be due to the specific demands of proteins on their phospholipid partners during the reconstituting or the functioning of proteoliposomes.

Proteoliposomes with ATPase and Bacteriorhodopsin or Cytochrome Oxidase—In the last series of experiments, proteoliposomes were reconstituted from the mixture containing ATPase together with bacteriorhodopsin or cytochrome oxidase. As one can see in Fig. 2, ATPase + bacteriorhodopsin proteoliposomes generate electric potential at the expense of ATP, or alternatively of light energy, the process being revealed by both PCP- and voltmeter measurements. Usually, the ATP-supported potential was lower than the light-supported one. In both cases, the electric fields were found to be of the same direction (plus inside proteoliposomes).

The study on the interaction of two types of generators was continued in the experiments with ATPase + cytochrome oxidase proteoliposomes. In this case, direction of the field formed by the cytochrome oxidase generator was regulated by actuation of the extra- or, alternatively, intravesicular cytochrome c. As the experiments showed, the electron transport via inner cytochrome c generates the field of the same direction as ATPase does (plus inside the proteoliposomes), whereas that via external cytochrome c generates the field of the opposite direction (minus inside). These relationships are demonstrated in Fig. 3, where PCP- responses are shown. It is noteworthy that ATP-supported membrane potential collapses when external cytochrome c is actuated. Subsequent addition of cyanide abolishes the ascorbate effect, so that ATP-supported membrane potential can be observed again.

In Fig. 4 one can see PCP- response of the proteoliposomes containing cytochrome oxidase (+ cytochrome c inside) and hydrophobic proteolipids of ATPase. Reconstitution of the proteoliposomes with F₁ was not carried out. According to the data obtained, the membrane potential, generated by cytochrome oxidase, can be increased by oligomycin. This effect corresponds to the “coupling” action of oligomycin on the respiratory chain-supported membrane potential formation in the F₁-deficient submitochondrial particles (12).

DISCUSSION

Direct Measurement of Electric Generation by Membranous Proteins and Verification of Chemiosmotic Principle of Energy Coupling—The data reported in this and the three previous papers of the series demonstrate the fact that energy-transducing systems of coupling membranes can function as molecular generators of electric current which may be measured directly by conventional electrometer techniques. To carry out such measurements, a special method of association of membranous proteins with artificial planar phospholipid membrane has

![Fig. 2. Electric generation by ATPase + bacteriorhodopsin proteoliposomes. A, direct voltmeter measurement. Incubation mixture: 0.3 M sucrose, 5 mM Tris-citrate (pH 7.2), 0.05 M CaCl₂, and 5 mM MgSO₄. One of compartments contained proteoliposomes (0.3 mg of protein/ml). Additions: 2.5 mM ATP, oligomycin (90 μg/ml). B, PCP- probe. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM MgSO₄, and proteoliposomes (1.3 mg of protein/ml). Additions: 0.4 mM ATP and oligomycin (6.5 μg/ml).](http://www.jbc.org/content/177/3/7078/F2)

![Fig. 3. PCP- responses of the ATPase + cytochrome oxidase proteoliposomes. Upper curve, incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM MgSO₄, 1 mM ascorbate, and ATPase + cytochrome oxidase proteoliposomes with cytochrome c inside (0.9 mg of protein/ml) obtained as described previously (3). Additions: 1 mM ATP, oligomycin (3.0 μg/ml), 4 x 10⁻⁷ M PMS, and 1 mM NaCN. Lower curves, incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM MgSO₄, 2 x 10⁻⁷ M cytochrome c, and proteoliposomes (2.8 mg of protein/ml). Additions: 1 mM ATP, 5 mM ascorbate, 1 mM NaCN, and oligomycin (1.6 μg/ml).](http://www.jbc.org/content/177/3/7078/F3)
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references, see the first paper of this series (1)). Now it seems to be worthy that both energy-providing reactions of respiratory chain and H\(^+\)-ATPase systems prove to be a part of the planar membrane. Experiments confirmed the first scheme. It was found (1) that electric responses of the bacteriorhodopsin proteoliposome, associated with planar membrane, are sensitive to gramicidin A, added in concentrations which do not reduce the planar membrane resistance in the light. This can be accounted for by the fact that gramicidin, a compound forming K\(^+\), Na\(^+\), and H\(^+\)-permeable channels through thin (black), rather than thick, phospholipid membranes, shunts the proteoliposomal membrane whose thickness is of about 70 A according to the electron microscope data (1). As to the planar membrane, it remains unaffected, being too thick for a gramicidin channel to be organized. It is noteworthy that, under the same conditions, the decrease in the electric response of proteoliposomes caused by proton carriers such as CCCP correlates with the drop in the electric resistance of the planar membrane (1). These facts fail to be accounted for in terms of the second and the third schemes mentioned above.

The observation incompatible with the second scheme (the opening of proteoliposomes attached to the black planar membrane) was described when cytochrome oxidase proteoliposomes were studied. It was shown (3) that cytochrome c and ascorbate, added to the proteoliposome-free compartment, did not induce any electric response until the mixture was supplemented with a penetrating H atom carrier. This means that cytochrome oxidase originally incorporated into the proteoliposomal membrane cannot interact with cytochrome c if proteoliposomes and cytochrome c are separated by the planar membrane. So, the proteoliposomal membrane, if associated with the planar one, is still inaccessible to the solution on the opposite side of the planar membrane.

Another fact testifying against the second scheme is that not only black (bilayer) but also thick planar membranes can be

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**Mode of Proteoliposome Association with Planar Membrane**

As was mentioned in the first paper of the series (1), variations in the direction of the bacteriorhodopsin-generated photo electromotive force could be observed when bacteriorhodopsin was added directly into the planar membrane-forming solution. On the other hand, this parameter was invariant when bacteriorhodopsin proteoliposomes were used. In the cases of proteoliposomes of other types, the direction of electric field was also regular. Results of measurements of the electric field direction in proteoliposomes as detected by PCB\(^-\) probe and by voltmeter are summarized in Table II. It is seen that the signs of the electric potential difference on the proteoliposome-free side of the planar membrane always coincide with those in the proteoliposomal interior, as can be judged by the PCB\(^-\) flux direction.

Three possible versions of the proteoliposome association with planar membrane may be considered.

1. Proteoliposomes are attached to the surface of the planar membrane. The charges, e.g. H\(^+\) ions accumulated inside these proteoliposomes, move electrophoretically from the proteoliposomal interior to the extraproteoliposomal compartments: (a) across the proteoliposomal membrane-to the same chamber which proteoliposomes were added to; or (b) across the planar membrane-to the opposite chamber. In the latter case, an electric potential difference between the two chambers separated by the planar membrane should arise (Fig. 5).

2. Attachment of proteoliposomes to the black planar membrane entails proteoliposomes opening in such a way that their membranes prove to be a part of the planar membrane.

3. The opening of the proteoliposome, attached to the thick planar membrane, results in some areas of the planar membrane surface being covered by the proteoliposomal material.

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**Fig. 4. “Coupling” effect of oligomycin on the respiration-supported PCB\(^-\) uptake by ATPase + cytochrome oxidase proteoliposomes.** Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM ascorbate, and ATPase + cytochrome oxidase proteoliposomes with cytochrome c inside (1 mg of protein/ml). Additions: 4 × 10\(^{-6}\) M PMS, oligomycin (3.3 \(\mu\)g/ml), and 5 mM NaCN.

The procedure includes (a) reconstitution of the protein with phospholipid to form vesicles (proteoliposomes) according to the method of Kagawa and Racker (5), (b) association of proteoliposomes with planar membrane induced by Ca\(^2+\) ions, and (c) measurement of electric potential difference across planar phospholipid membrane by electrodes immersed into electrolyte solutions on both sides of this membrane.

Using this method, we found the electric current to be produced by light-dependent systems (bacteriochlorophyll reaction centers of *Rhodospirillum rubrum* chromatophores and bacteriorhodopsin from *Halobacterium halobium*), by the complex forming an energy coupling site of the mitochondrial respiratory chain (cytochrome c-cytochrome oxidase), and by the mitochondrial oligomycin-sensitive H\(^+\)-ATPase. The good correlation of the data obtained by this method and by the synthetic penetrating ion probe is noteworthy.

These results directly confirm Mitchell’s postulate (4) on electric potential generation as a process intrinsic of respiratory and photosynthetic energy coupling systems. The validity of the postulate in question was supported by several independent pieces of evidence provided by indirect methods. Nevertheless there were some grounds for skepticism left, mainly because of the failure of an attempt to measure the membrane potential in mitochondria with a voltameter (for references, see the first paper of this series (1)). Now it seems to be high time to throw away the remaining doubts and accept the point that molecular electric generators exist in coupling membranes.

When considering energy coupling mechanisms, it is noteworthy that both energy-providing reactions of respiratory (photosynthetic) chain and H\(^+\)-ATPase systems prove to be competent in electric generation. It means that H\(^+\)-ATPase, if reversible, can utilize electric membrane potential energy to form ATP. Reversibility of ion-transporting ATPases, including H\(^+\)-ATPase of coupling membranes, is well established (for review, see Ref. 13). So, energy-providing reactions can be coupled with phosphorylation via membrane potential as was originally postulated by Mitchell (4).

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**Incubation mixture:** 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM ascorbate, and ATPase + cytochrome oxidase proteoliposomes with cytochrome c inside (1 mg of protein/ml). Additions: 4 × 10\(^{-6}\) M PMS, oligomycin (3.3 \(\mu\)g/ml), and 5 mM NaCN.
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used in electric generation experiments (1). As a matter of fact, most of the experiments described in these four papers were carried out in thick planar membranes since black ones treated with proteoliposomes proved to be unstable.

Mechanism of Electric Generation by Proteoliposome-Planar Membrane System—The schemes illustrating mechanism of electric current generation by different types of proteoliposomes associated with planar membrane are given in Fig. 5. It is shown that electrogenic transfer of electrons or protons across proteoliposomal membrane is carried out by a specific protein system utilizing the corresponding energy source: light (bacteriorhodopsin and chlorophyll), hydrogen donor and oxygen (cytochrome oxidase), or ATP (H⁺-ATPase). In all the cases studied, formation of electrochemical H⁺ potential gradient (ΔφH) between extra- and intraproteoliposomal compart-
ments proves to take place. Movement of H⁺ ions down ΔφH when it occurs through planar membrane results in an electric potential difference between two planar membrane-separated solutions. In fact, this is the value that is measured by a voltmeter.

According to the schemes, proteins localized in the region of spherical and planar membrane fusion, might, in principle, generate an electric field of the direction opposite to that produced by other membrane regions. Apparently, this does not take place since native arrangements of biological electric generators requires opposite parts of them to be on the water-lipid interphases. This requirement is not fulfilled if the protein complex is immersed into thick phospholipid membrane, as it should take place in the fusion area.

Asymmetry of Proteoliposome Reconstitution—In the case of both bacteriorhodopsin and chlorophyll, there should be reasons for the fact that the majority of bacteriorhodopsin and chlorophylls, incorporated into proteoliposomes, transfer proteins or, respectively, electrons across the proteoliposomal membrane from outside to inside. The simplest explanation might consist in that chromophores, localized on the outer surface of the proteoliposomal membrane, are better saturated with light than the chromophores on the inner surface. However, this can hardly account for the phenomenon observed because the sign of the photoeffect does not depend on whether the planar membrane was illuminated from the proteoliposome or proteoliposome-free side (1, 2). Apparently, the effect discussed is due to asymmetric reconstitution of the proteoliposome membrane. This may be a consequence of different factors affecting the reconstitution process. One of them may be the difference in the extra- and intraproteoliposo-

![Fig. 5. Schemes illustrating electric current generation in the proteoliposome-planar membrane system. A, bacteriorhodopsin proteoliposomes; B, bacteriochlorophyll proteoliposomes; C, cytochrome oxidase proteoliposomes with cytochrome c outside; D, same as C but cytochrome c inside; E, H⁺-ATPase proteoliposomes. Rh, bacteriorhodopsin; A, primary electron acceptor of the bacteriochlorophyll reaction center complex; Chl, bacteriochlorophyll; c, a, a', cytochromes; AH₂, hydrogen donor, reducing cytochrome c; HP, hydrophobic proteins of the oligomycin-sensitive H⁺-ATPase.](http://www.jbc.org/)
nal volumes. If formation of the phospholipid vesicles is assumed to be the first step of proteoliposome reconstitution, and protein incorporation is the second step, the outer surface of the vesicles should bind more protein than the inner one. This should be due to the source of the protein which can be inlaid on the inner membrane surface being limited by the protein amount present in the intravesicular compartment.

Another reason for proteoliposome asymmetry may be the difference in the areas of the inner and outer surfaces of the reconstituted vesicles. It was found (14) that in liposomes formed from the mixture of negatively charged and neutral phospholipids, the former were localized mainly on the outer, and the latter on the inner surface of the membrane.

In the case of cytochrome oxidase proteoliposomes, no measurable asymmetry of reconstitution was found (see, however, Ref. 15). Both oxygen consumption and electric generation measurements showed that about 50% of cytochrome oxidases are arranged in such a way that cytochrome a is localized near the outer surface of the membrane, the others being oriented in the opposite fashion. No respiratory control or electric generation were found when both external and internal cytochromes a were actuated (3). Impermeability of the proteoliposomal membrane to cytochrome c and ascorbate is the factor allowing asymmetric electron flow and membrane charging to be revealed.

As to hydrophobic proteins of H⁺-ATPase, we do not know whether it is symmetric or asymmetric reconstitution that takes place. In any case, the proteoliposome membrane impermeability to ATP seems to be sufficient to induce asymmetry of the field generation.

Acknowledgments—We thank Dr. A. D. Kaulen and Dr. A. A. Kondrashin for participation in some experiments, Professor S. E. Severin and Professor E. A. Liberman for discussion, Professor L. P. Kayushin and Dr. L. N. Chekulaeva for providing Halobacterium halobium cells, Mr. S. A. Bogoslovsky and Ms. N. M. Goreyshina for the help during preparation of the manuscripts, and Ms. T. I. Kheifets for correcting the English version of the paper.

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Reconstitution of biological molecular generators of electric current. H+-ATPase.
L A Drachev, A A Jasaitis, H Mikelsaar, I B Nemecek, A Y Semenov, E G Semenova, I I Severina and V P Skulachev

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