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Membrane potential generation by two reconstituted mitochondrial systems: Liposomes inlayed with cytochrome oxidase or with ATPase

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SUMMARY

Formation of a membrane potential in two types of liposomes, one inlayed with cytochrome *c* + cytochrome oxidase, and another, with oligomycin-sensitive ATPase, has been demonstrated. To detect a membrane potential, phenyl dicarbaundecaborane (PCB^-), a penetrating anion probe, was used.

The first type of liposome was reconstituted from a solution of purified cytochrome oxidase, mitochondrial phospholipids and cytochrome *c*, the latter being enclosed inside liposomes. Cytochrome *c* bound to the outer surface of the liposome membrane was removed by washing with NaCl. Such liposomes catalyzed oxidation of ascorbate by oxygen in the presence of phenazine methosulfate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The oxidation was found to support the PCB^- uptake by liposomes. The PCB^- response was prevented and reversed by cyanide, protonophorous uncouplers and external cytochrome *c*.

Liposomes of the second type were prepared from a solution of mitochondrial phospholipids, coupling factors F_1 and F_c, and the hydrophobic proteins of the oligomycin-sensitive ATPase. These liposomes catalyzed ATP hydrolysis coupled with the PCB^- uptake. The latter effect was prevented and reversed by oligomycin and uncouplers.

The conclusion is made that membrane potential can be independently formed by enzymic reactions of two different kinds: (1) redox (*e.g.* cytochrome *c* oxidase) and (2) hydrolytic (ATPase).

Abbreviations: ANS^-, anilinonaphthalene sulfonate anion; PCB^-, phenyl dicarbaundecaborane anion; PMS, phenazine methosulfate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*- tetramethyl-*p*-phenylenediamine.

Mitchell's[1] chemiosmotic theory of oxidative phosphorylation postulates that the coupling membrane possesses two types of electric potential generators: one utilizing the energy of electron transfer reactions; the other actuated by ATP hydrolysis. In this laboratory, evidence has been obtained suggesting that membrane potential in mitochondria can be supported (1) by redox chain electron transfer without the involvement of high-energy intermediates and (2) by ATPase without electron transfer[2-4] The present paper summarizes the data obtained from experiments on separation and reconstitution of two systems for generation of membrane potential.

The present investigation is based on the results of Racker and co-workers[5-7] who have recently succeeded in reconstituting the oxidative phosphorylation system from proteins of the inner mitochondrial membrane and phospholipids. These authors reported that vesicles made of soya bean phospholipids, coupling factors F_1 and OSCP and some colourless hydrophobic mitochondrial proteins catalyzed the oligomycin-sensitive $^{32}P_i$–ATP exchange and ATPase reactions. If the reconstitution mixture was supplemented with cytochrome oxidase and cytochrome *c*, the resulting particles carried out phosphorylating oxidation of ascorbate by oxygen. If cytochrome oxidase was the only protein component of the reconstitution mixture, the vesicles catalyzed ferrocytochrome *c* oxidation which was greatly stimulated by uncouplers.

Some observations suggest that Racker's reconstituted particles were able to form a membrane potential: (1) Cytochrome oxidase vesicles catalyzed K^+ influx during oxidation (2) ATPase-containing particles demonstrated an ATP-dependent, oligomycin-sensitive enhancement of anilinonaphthalene sulfonate anion (ANS^-) fluorescence, the fluorescent penetrating anion probe signalling formation of the membrane potential[8-10]. (3) Both respiration and the ATPase activity of the reconstituted particles were activated by valinomycin + nigericin. This treatment specifically discharges the membrane potential[1,10]. Valinomycin + nigericin inhibited both $^{32}P_i$–ATP exchange and oxidative phosphorylation in the reconstituted particles.

We have studied similar systems using phenyl dicarbaundecaborane anion (PCB^-), which is a membrane potential probe[11,12]. This compound is a negatively charged penetrant for artificial[13,14] and mitochondrial[9] membranes. As was found earlier, formation of the membrane (potential positive charge inside the closed space) results in PCB^- uptake by particles. This process can be followed using an artificial phospholipid membrane as a PCB^--sensitive electrode[11,14]. The procedure for particle reconstitution was essentially the same as that recommended by Racker and co-workers. Cholate solutions of mitochondrial phospholipids and the corresponding proteins were mixed and then dialyzed for 18 h at 0–2 °C to remove cholate. The particles reconstituted during dialysis were sedimented at 165 000 × *g* for 60 min, suspended in a solution containing 0.25 M sucrose, 10 mM Tris buffer (pH 7.8) and 1 mM EDTA, and stored at 2 °C.

The mixture for reconstituting the cytochrome oxidase particles was composed of mitochondrial phospholipids (50 mg/ml), cytochrome oxidase (5 mg/ml) purified according to the method of Yonetani[15], cytochrome *c* (18.75 mg/ml), 2% sodium cholate, 0.125 M sucrose, 10 mM Tris buffer (pH 8.0), 0.1 M $(NH_4)_2SO_4$ and 0.25 M NaCl. The

particles obtained after dialysis and centrifugation were washed once with 0.25 M NaCl, 10 mM Tris buffer (pH 7.8) and 1 mM EDTA.

The ATPase particles were reconstituted in a solution including phospholipids (50 mg/ml), coupling factor F_1 (0.1 mg/ml) obtained by the method of Horstman and Racker[16], coupling factor F_c (1.0 mg/ml) prepared according to the method of Bulos and Racker[17], mitochondrial hydrophobic proteins required for the oligomycin-sensitive ATPase (10 mg/ml) obtained by the method of Kagawa and Racker[5], 2% sodium cholate, 5 mM dithiothreitol, 0.125 M sucrose, 10 mM Tris buffer (pH 8.0), 0.05 mM EDTA and 0.1 M $(NH_4)_2SO_4$.

Phospholipids were found to compose the greater part of the reconstituted particles. In the cytochrome oxidase particles, the phospholipid:protein ratio was about 2. In the ATPase particles, phospholipids amount to about 90% of the total weight. Both the electron microscope analysis and the functional characteristics suggest that the reconstituted particles represent closed vesicles, a fact which, together with data on the chemical composition, allows these systems to be defined as liposomes inlayed with enzyme proteins.

The PCB^- responses of the cytochrome oxidase-inlayed liposomes are given in Fig.1. It is seen that addition of phenazine methosulfate (PMS) to the liposomes preequilibrated with PCB^- in the presence of ascorbate results in a PCB^- uptake (Curves a, c and e) which is reversed (Curve a) and prevented (Curve b) by cyanide. These PCB^- responses were in good correlation with changes in the respiration rate measured polarographically: PMS initiated and cyanide arrested the oxygen consumption of the particles. The PMS-induced PCB^- response was also inhibited by protonophorous uncoupler[5], carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (see Fig.1, Curves c and d) and tetrachlorotrifluoromethyl benzimidazole, added at low concentrations that did not affect the diffusion potential of PCB^- across the artificial membrane. The PMS effect on the PCB^- level could be reversed (Curve e) and prevented (Curve f) when the reaction mixture was supplemented with cytochrome *c*. Polarographic measurements showed that cytochrome *c* induced an increase in the respiration rate.

The effect similar to that of PMS could be produced by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). Liposomes reconstituted from phospholipids, cytochrome *c* and inactivated cytochrome oxidase or active cytochrome oxidase but without cytochrome *c* were found to display no responses of the kind shown in Fig.1. None of the above effects was observed when ascorbate was excluded from the reaction mixture. Succinate, NADH, NADPH+ NAD^+ and ATP did not induce any PCB^- responses.

Fig. 2 demonstrates the PCB^- responses of the liposomes inlayed with proteins of the oligomycin-sensitive ATPase complex. One can see that, in this case, PCB^- uptake is induced by addition of ATP (Curves a and c). This effect could be reversed (Curve a) and prevented (Curve b) by FCCP. Oligomycin showed a similar inhibitory action in the PCB^- experiments (Curves c and d). Measurement of ATPase activity revealed that FCCP stimulated, whereas oligomycin inhibited, the rate of ATP hydrolysis by the liposomes. In the same experiments, it was shown that ascorbate+PMS and other respiratory substrates were ineffective in

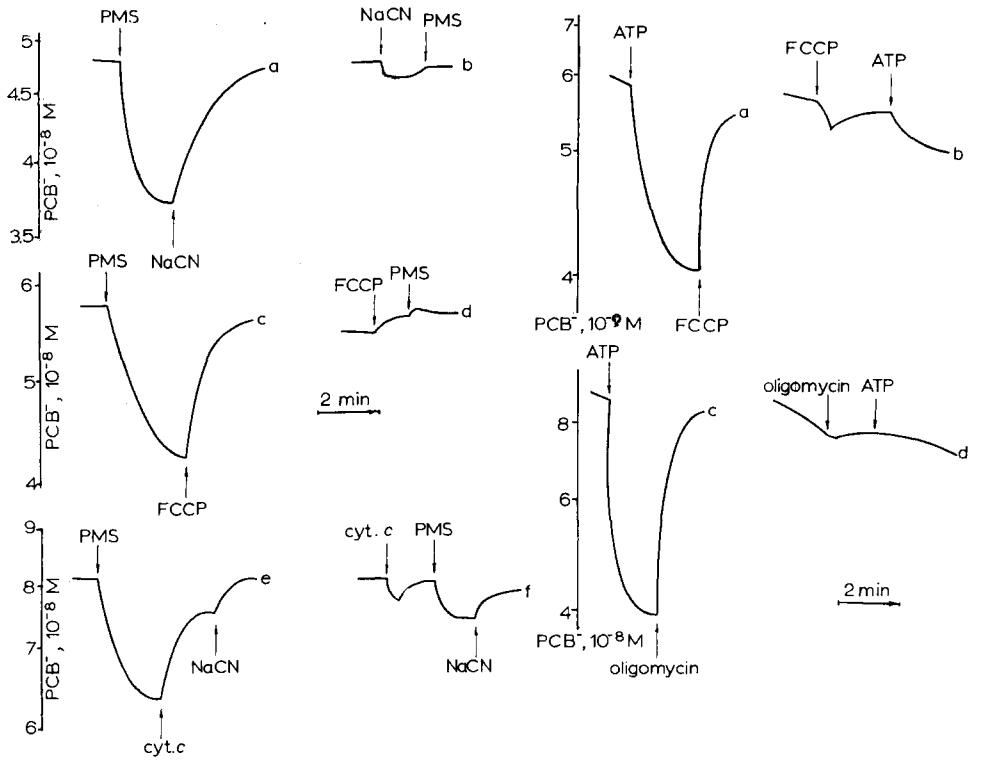

Fig.1. The PCB^- responses in liposomes inlayed with cytochrome oxidase and cytochrome *c*. Reaction mixture contained 0.25 M sucrose, 50 mM Tris–HCl (pH 7.5), and NaCl-washed liposomes inlayed with cytochrome oxidase and cytochrome *c* oxidase and cytochrome *c* (protein concentration, 0.4 mg/ml). The initial PCB^- concentration (before addition of liposomes) was $3 \cdot 10^{-6}$ M. Additions: $1 \cdot 10^{-6}$ M PMS, 4 mM NaCN, $3 \cdot 10^{-5}$ M cytochrome *c*, $1 \cdot 10^{-7}$ M FCCP.

Fig.2. The PCB^- responses in liposomes inlayed with the proteins of the oligomycin-sensitive ATPase complexes. Reaction mixture contained 0.25 M sucrose, 50 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$, and ATPase liposomes (protein concentration, 1.3 mg/ml). The initial PCB^- concentration was $3 \cdot 10^{-6}$ M. Additions: 1 mM ATP, $6 \cdot 10^{-7}$ M FCCP, 8 M oligomycin.

the ATPase liposomes. Neither the rate nor the amplitude of the ATP-dependent PCB^- response of these liposomes was affected by ferricyanide, dithionite or respiratory chain inhibitors. No cytochrome absorption maxima were observed in the spectrum of the ATPase liposomes.

The above results indicate that liposomes reconstituted from phospholipids and the corresponding enzyme proteins by the method of Racker and co-workers display PCB^- responses similar to those of "inside–out" submitochondrial particles, *i.e.* anion uptake under energization (*cf.* Grinius *et al.*[14]). This fact suggests the formation of an electric potential difference on the liposome membrane, the positive charge being inside the vesicle. The membrane potential can be discharged by arresting the energy-supplying reaction in lipo-

somes by a specific enzyme inhibitor (cyanide for the "respiring" liposomes, oligomycin for the ATPase liposomes) or increasing the electric conductance of the liposomal membrane by an uncoupler.

In liposomes inlayed with cytochrome oxidase and cytochrome *c*, formation of a membrane potential can be supported by oxidation of ascorbate *via* a lipid-soluble hydrogen carrier (PMS or TMPD), cytochrome *c* localized inside particles, and membraneous cytochrome oxidase. It is most probable that, in this case, the membrane potential is formed by a cytochrome oxidase-mediated transmembrane electron flow from cytochrome *c*, bound at the inner side of liposome membrane, to the outer oxygen. This suggestion is supported by the fact that (1) washing of the liposomes with NaCl, a procedure resulting in removal of the externally-bound cytochrome *c*, was necessary for the respiration-dependent PCB^- uptake to be observed, and (2) addition of external cytochrome *c* to washed liposomes strongly inhibited the respiratory PCB^- response. Apparently, cytochrome *c*, when present on both sides of the liposome membrane, can initiate the transmembrane electron flow in both directions: from outside to inside and *vice versa*. In such a case no membrane potential can be formed.

It is noteworthy that external cytochrome *c* suppressed oxidative phosphorylation in the reconstituted system of Racker and Kandrash[6], catalyzing the ATP formation coupled with electron transfer *via* the inner cytochrome *c*.

It is not possible to explain the above effects of external cytochrome *c* by liposome damage, *etc.* Our experiments showed that cytochrome *c* added to the ATPase liposome did not inhibit the ATP-supported PCB^- uptake, Hinkle *et al.*[7] reported that oxidation of external cytochrome *c* can be stimulated by valinomycin in liposomes reconstituted from phospholipids and cytochrome oxidase without cytochrome *c*. These observations can be nicely explained if one assumes that oxidation of external cytochrome *c* forms a membrane potential in the direction opposite to that required for PCB^- influx or ATP formation. Indeed, electron transfer from outer cytochrome *c* to inner cytochrome oxidase should charge the liposome interior negatively, whereas Racker's phosphorylating particles and the PCB^--consuming respiring particles described in this paper have a positively charged interior. All effects of the cytochrome *c* arrangement, if considered in terms of the chemical energy coupling schemes, including high-energy intermediates between electron transfer and membrane potential, seem to be quite obscure.

Generally, it is highly improbable that any high-energy compounds are involved in the respiration-dependent formation of membrane potential in such a simple system as liposomes inlayed with purified cytochrome oxidase and cytochrome *c*. Moreover, membrane potential formation in liposomes inlayed with the ATPase proteins seems to be independent of electron transfer reactions.

In conclusion, the reconstitution of the two types of enzymic mechanism for the generation of membrane potential has been demonstrated. One of these mechanisms utilizes electron transfer energy; the other ATP hydrolysis energy. This fact together with observations on the reversibility of the energy transduction between membrane potential and ATP ("ion transfer phosphorylation", for review see Skulachev[4,10,18]) add up to the assumption that

oxidation produces a membrane potential which can be consumed by ATP synthetase. It means that oxidation can be coupled with phosphorylation *via* membrane potential as postulated by Mitchell[1].

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REFERENCES

1 Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin.
2 Dontsov, E.A., Grinius, L.L., Jasaitis, A.A., Severina, I.I. and Skulachev, V.P. (1972) *J. Bioenerg.* 3
3 Jasaitis, A.A., Severina, I.I., Skulachev, V.P. and Smirnova, S.M. (1972) *J. Bioenerg.* 3
4 Skulachev, V.P. (1972) *J. Bioenerg.* 3, 25
5 Kagawa, J. and Racker, E. (1971) *J. Biol. Chem.* 246, 5447
6 Racker, E. and Kandrash, A. (1971) *J. Biol. Chem.* 246, 7069
7 Hinkle, L.L., Jung Ja Kin and Racker, E. (1972) *J. Biol. Chem.* 247, 1338
8 Jasaitis, A.A., Kuliene, V.V. and Skulachev, V.P. (1971) *Biochim. Biophys. Acta* 234, 177
9 Jasaitis, A.A., La Van Chu and Skulachev, V.P. (1972) in preparation
10 Skulachev, V.P. (1972) *Energy Transformation in Biomembranes*, Nauka Press, Moscow
11 Liberman, E.A., Topali, V.P., Tsofina, L.M., Jasaitis, A.A. and Skulachev, V.P. (1969) *Nature* 222, 1076
12 Liberman, E.A. and Skulachev, V.P. (1970) *Biochim. Biophys. Acta* 216, 30
13 Liberman, E.A. and Topali, V.P. (1968) *Biochim. Biophys. Acta* 163, 125
14 Grinius, L.L., Jasaitis, A.A., Kadziauskas, J.P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vladimirova, M.A. (1970) *Biochim. Biophys. Acta* 216, 1
15 Yonetani, T. (1967) in *Methods in Enzymology* (Estabrook, R.W. and Pullman, M.E., eds), Vol. 10, p. 332, Academic Press, New York
16 Horstman, L.L. and Racker, E. (1970) *J. Biol. Chem.* 245, 1336
17 Bulos, B. and Racker, E. (1968) *J. Biol. Chem.* 243, 3891
18 Skulachev, V.P. (1971) *Curr. Top. Bioenerg.* 4, 127