A Study on the Mechanism of Energy Coupling in the Redox Chain

2. ATP-Supported Generation of Membrane Potential in the Respiratory Chain-Deficient Submitochondrial Particles

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Abstract

Generation of a membrane potential in the respiratory chain-deficient particles of beef heart mitochondria has been studied. For detection of membrane potential, phenyl dicarbaundecaborane (PCB^-) and anilino-naphthalene sulphonate (ANS^-) probes were used. The respiratory chain-deficient submitochondrial particles were prepared after Arion and Racker (E-SMP), the procedure including complete disappearance of membrane structures and subsequent reconstitution of membrane vesicles as judged by the electron microscopy study. E-SMP were found to be deficient in cytochromes a, a_3 and transhydrogenase, the cytochrome b, c_1 and c content being lowered. Addition of NADH, succinate and tetramethyl-p-phenylenediamine + ascorbate did not induce either any oxygen consumption or membrane potential formation. Treatment of E-SMP with $NADPH + NAD^+$ or with $NADH + CoQ_0$ did not entail generation of membrane potential, in contrast to that of parent, "pyrophosphate" submitochondrial particles (PP-SMP).

E-SMP displayed an oligomycin-sensitive ATPase activity which could be increased by reconstitution of E-SMP with coupling factor F_1. Addition of ATP resulted in an uptake of PCB^- and enhancement of ANS^- fluorescence, the facts testifying to the formation of the membrane potential with "plus" inside E-SMP. Membrane potential formation was arrested by oligomycin, rutamycin, and uncouplers. Addition of respiratory chain inhibitors (antimycin + rotenone + cyanide), complete reduction of respiratory carriers by dithionite and oxidation by ferricyanide were without effect on ATP-supported formation of membrane potential in E-SMP. It was concluded that utilization of ATP energy for the membrane potential generation does not depend on the state of the respiratory carriers and can be demonstrated under the conditions when none of redox chain coupling sites were functioning.

Abbreviations: phenyl dicarbaundecaborane, PCB^-; anilinonaphthalene sulfonate, ANS^-; the respiratory chain-deficient submitochondrial particles, E-SMP; "pyrophosphate" submitochondrial particles, PP-SMP.

removal of cholate by dialysis, was employed without major modifications. As was seen on electron microscopy sections of the glutaraldehyde-fixed preparations (Fig. 1), cholate treatment resulted in
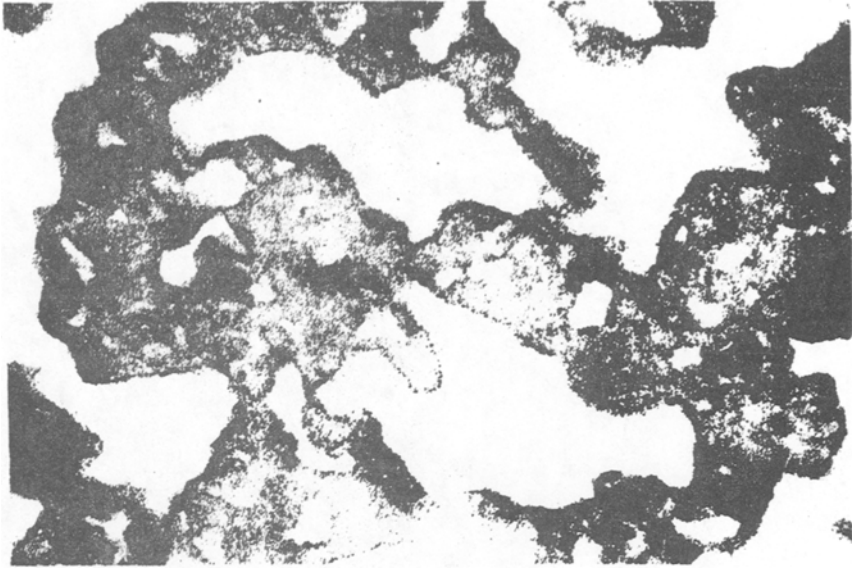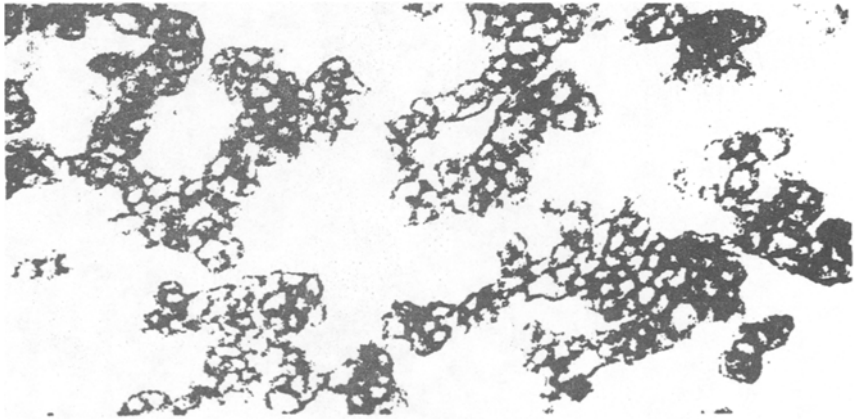

Figure 1(b)

Figure 1(c)

Figure 1. Electron micrographs of ' pyrophosphate" submitochondrial particles (PP-SMP, Fig. 1(a)), material obtained after disruption of PP-SMP by cholate (Fig. 1(b)) and E-SMP reconstituted after removing cholate (Fig. 1(c)). Samples were fixed, with 5% glutaraldehyde and treated with OsO_4, alcohols, uranyl acetate and epoxide resin Epon 812. Thin sections were prepared with an LKB-4800 ultramicrotome. Electron microscopic studies were carried out using a Hitachi HU-11B microscope. Magnification x 120,000.

complete disappearance of membranes and formation of some amorphous material. Removal of cholate induced reconstitution of membrane vesicles (E-SMP) whose size was rather standard and much smaller than the average size of the original PP-SMP.

Polarographic and fluorimetric measurements showed that E-SMP did not respire (NADH, succinate and TMPD + ascorbate were used as substrates of respiration) and did not catalyse transhydrogenase reaction (for measurement technics see the previous paper [5]). Low-temperature spectrophotometric analysis of E-SMP demonstrated that amounts of cytochromes b, c and c_1 per mg of protein were decreased and concentrations of cytochromes a and a_3 proved to be negligible (Fig. 2). ATPase activity per mg protein was higher in E-SMP than in PP-SMP. Oligomycin inhibited this activity almost completely.

For measuring membrane potential in PP-SMP and E-SMP, phenyl dicarbaundecaborane (PCB$^-$) and anilinonaphthalene sulphonate (ANS$^-$) probes were used [5]; applicability of these probes to this end was demonstrated by the previous experiments [8, 9].
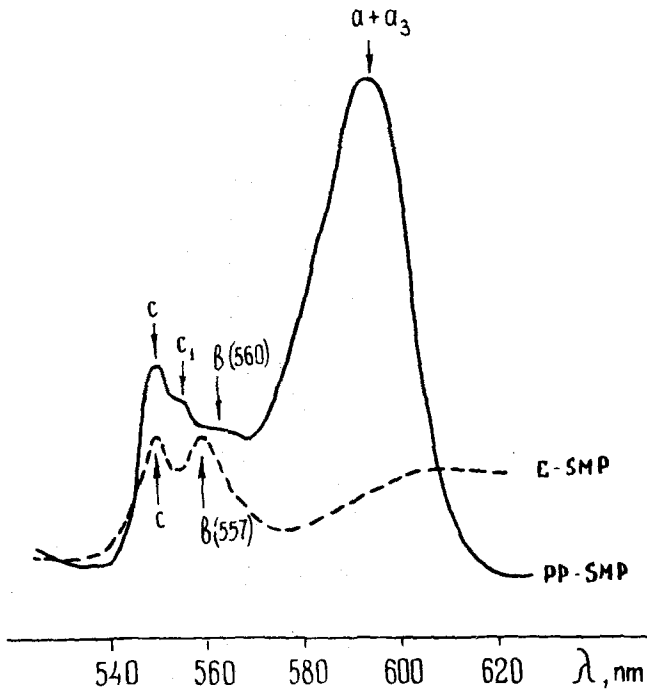

Figure 2. Cytochrome spectra of PP-SMP and E-SMP treated by dithionite. Samples contained 2.4 and 4.6 mg protein of PP-SMP and E-SMP per ml, respectively. Spectra were recorded at liquid nitrogen temperature.

Results

PCB⁻ responses of PP-SMP and E-SMP are compared in Fig. 3. It is seen that PP-SMP are competent in both ATP- and redox chain-dependent PCB⁻ transport, the fact, confirming the earlier observation for Mg-SMP [8]. In PP-SMP, the PCB⁻ responses supported by electron transfer via individual coupling sites could be demonstrated (1st site—on treatment with NADH + CoQ, 3rd site—with ascorbate + TMPD, 4th site—with NADPH + NAD⁺). E-SMP demonstrated only one type of the energy-dependent PCB⁻ response, namely, the PCB⁻ uptake induced by the ATP treatment. Addition of electron donors and acceptors actuating electron transfer-dependent PCB⁻ responses in PP-SMP, proved to be without any effect in E-SMP. If one takes into account that the PCB⁻ uptake is a very sensitive probe for membrane potential generation in submitochondrial particles, a conclusion may be drawn from the data of Fig. 3 that E-SMP lost the capacity of charging the membrane by mechanisms of the 1st, 3rd and 4th redox chain coupling sites. The membrane potential-generating capacity of the 2nd coupling site cannot be estimated in SMP because of the lack of a penetrating electron acceptor for reduced cytochrome c localized on the inner surface of SMP membrane [10].

In the further experiments the ATP effect in E-SMP was studied in some detail. It was shown that it is sensitive to oligomycin, rutamycin, and FCCP. As Fig. 4 demonstrates, factors affecting the redox chain, i.e. addition of some redox chain inhibitors (rotenone + antimycin + cyanide), reductant (dithionite) and oxidant (ferricyanide) were almost without influence on either size or kinetics of ATP-induced PCB⁻ influx into, and rutamycin (or FCCP)-induced PCB⁻ efflux from E-SMP.

Figures 5 and 6 show ANS⁻ responses of PP-SMP and E-SMP. The obtained data confirmed those of the PCB⁻ experiments. PP-SMP demonstrated both the ATP- and respiration-supported ANS⁻ responses (fluorescence increase) while E-SMP showed the ATP effect only. Again, ATP-induced response proved to be resistant to the respiratory chain inhibitors.

Discussion

The data presented above confirm the original results of Arion and Racker [7] that respiratory chain-deficient submitochondrial particles (E-SMP) have no energy coupling at the 1st and 3rd sites. Judging from PCB⁻ and ANS⁻ experiments, treatment of E-SMP with electron donors and acceptors of the 1st and 3rd coupling sites does not induce formation of a membrane potential. Transhydrogenase-linked mechanism of membrane potential generation proves to be ineffective either. Under
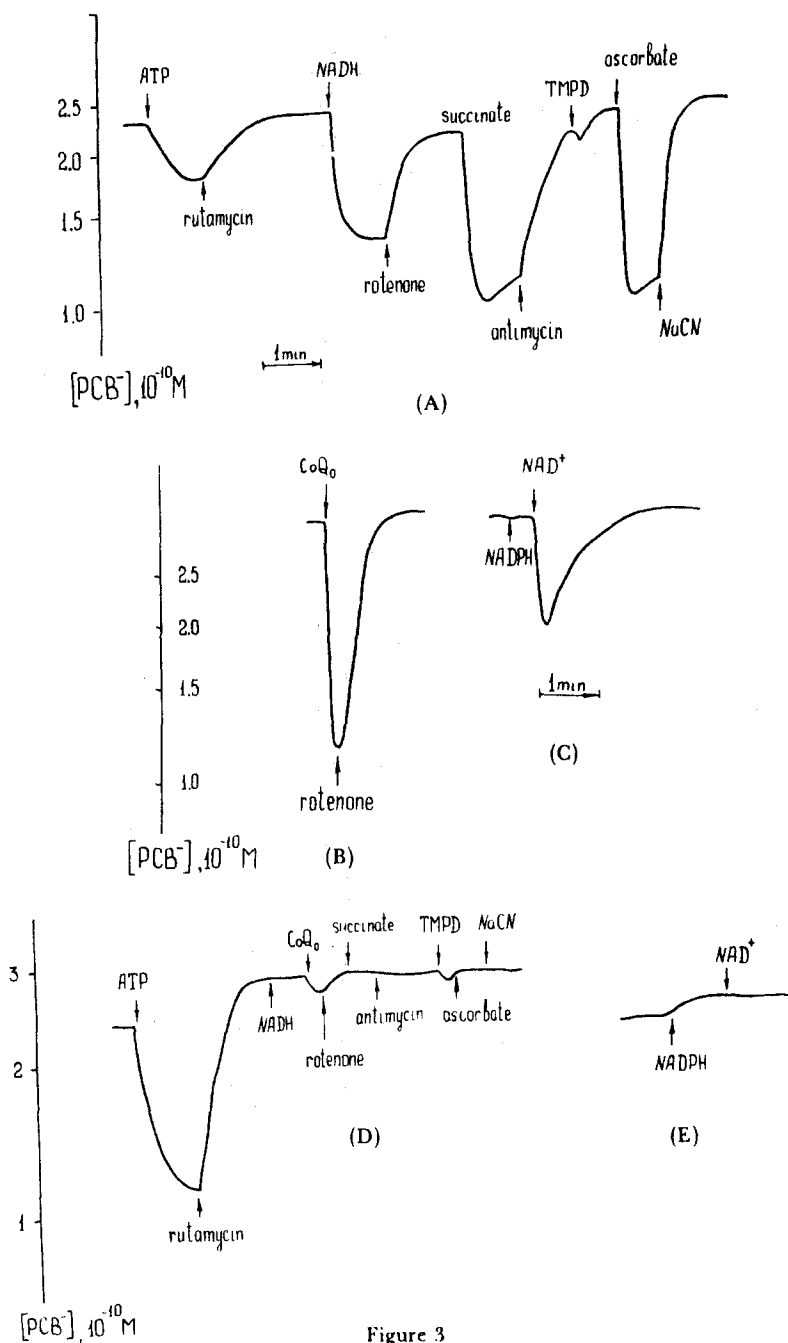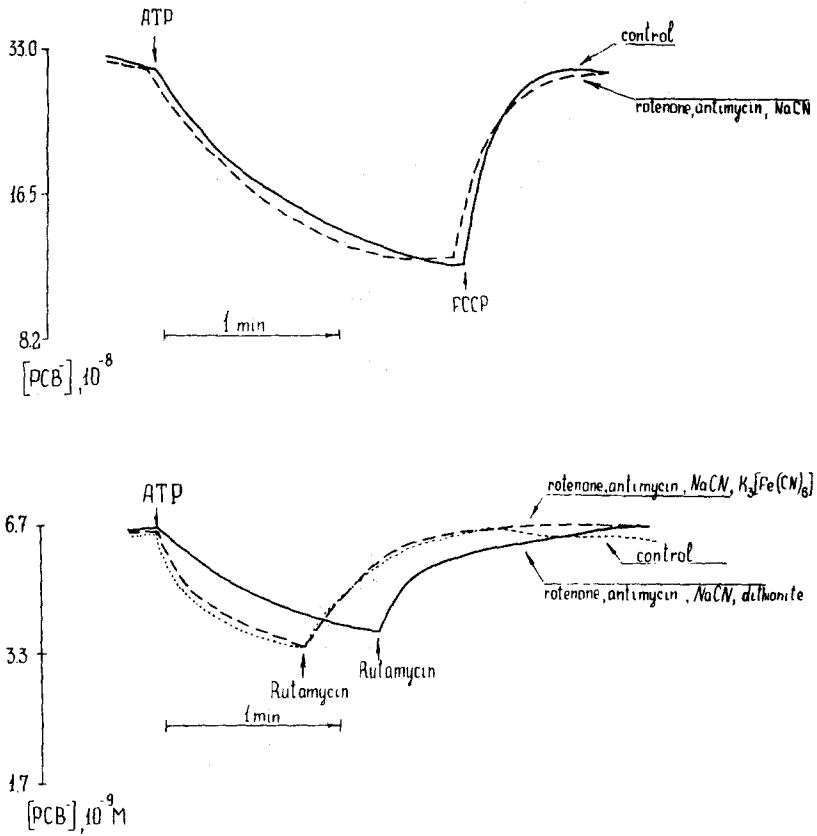
Figure 3

Figure 4. ATP-induced PCB⁻ responses of E-SMP after various treatments affecting the redox chain. Incubation mixture: 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM MgSO₄, 0.45 mg of E-SMP protein per ml, and where indicated 1 µM rotenone, 2 µM antimycin, 3 mM NaCN, 5 mM K₃Fe(CN)₆. Additions: 3 mM ATP, 2.5 µg/ml rutamycin, 1 µM FCCP.

Figure 3. PCB⁻ responses of PP-SMP and E-SMP. Incubation mixture: 0.25 M sucrose, 50 mM Tris-HCl, 5 mM MgSO₄, pH 7.5. Samples contained 0.93 and 0.45 mg protein of PP-SMP (A, B, C) and E-SMP (D, E) per ml, respectively, 2% ethanol, 0.05 mg/ml alcohol dehydrogenase (A, B, D), 2 µM antimycin (B) and 1 µM rotenone (C, E). Additions: 2 mM ATP, 2.5 µg/l rutamycin, 0.05 mM NADH, 1 µM rotenone, 5 mM succinate, 2.4 µM antimycin, 0.1 mM TMPD, 4 mM ascorbate, 3 mM NaCN, 1 mM CoQ₀, 1.2 mM NADPH, 1.2 mM NAD⁺.
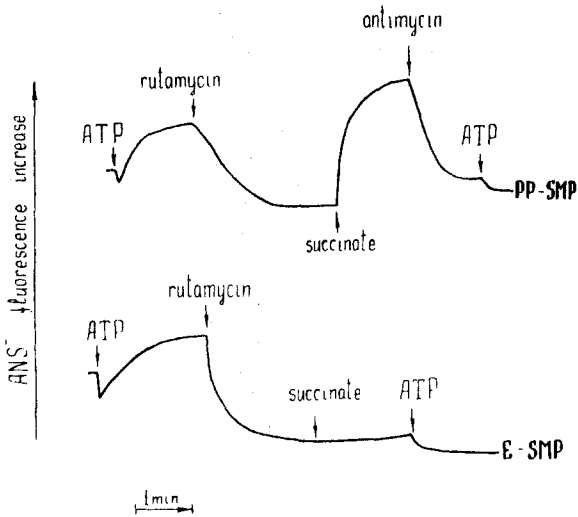
Figure 5. ANS⁻ responses of PP-SMP and E-SMP. Incubation mixture: 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM MgSO₄, 3 μM ANS⁻. Samples contained 3.0 mg protein of PP-SMP and 1.35 mg protein of E-SMP per ml. Additions: 0.4 mM ATP, 2.5 μg/ml rutamycin, 5 mM succinate, and 4 μg antimycin per ml.
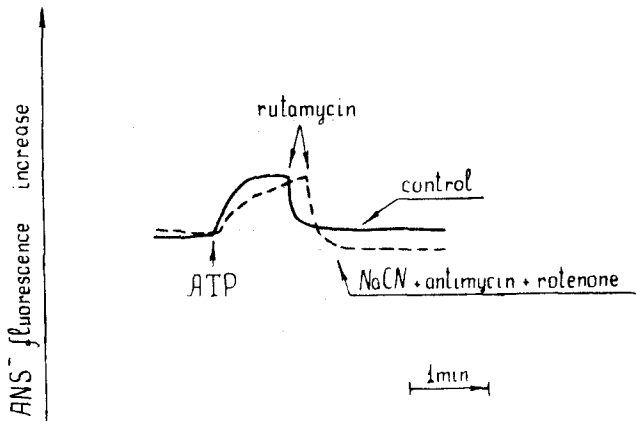

Figure 6. ATP-induced ANS⁻ responses in the absence and in the presence of respiratory chain inhibitors. Incubation mixture as in Fig. 5. Samples contained 1.9 mg of E-SMP protein per ml, and where indicated 1 μM rotenone, 2 μM antimycin and 1 mM NaCN. Additions: 0.4 mM ATP and 2.5 μg rutamycin per ml.

the same conditions, E-SMP are competent in ATP-supported formation of membrane potential which is characterized by the same properties as in the original PP-SMP: (1) "plus" is localized inside the particles since PCB$^-$ anions are taken up and ANS$^-$ fluorescence increases under energization; (2) energy transfer inhibitors and (3) uncouplers arrest membrane potential generation.

Some indications as to the formation of membrane potential in E-SMP were presented by Arion and Racker [7]. These authors noticed that phosphorylation coupled with electron transfer via the 2nd coupling site of E-SMP could be inhibited by combined addition of valinomycin and nigericin, a treatment collapsing a membrane potential. The 2nd coupling site was the only step of electron transfer chain capable of actuating the phosphorylation system of E-SMP.

These properties of E-SMP suggest that such type of submitochondrial particles should be a handy object for studying the relationships between electron transfer, membrane potential and phosphorylation. As a matter of fact, E-SMP can be studied under conditions when none of electron transfer coupling sites are operative. To this end, it is enough to arrest by an inhibitor the electron transfer via the 2nd coupling site since the other three coupling site mechanisms are lost during preparation of E-SMP. Fortunately, such inhibitor exists—it is antimycin. Nobody has ever reported any energy-linked responses of electron transfer via the 2nd coupling site if antimycin was present.* Any effects which can be demonstrated in the antimycin-treated E-SMP should be defined as independent of electron transfer via coupling sites. Respectively, ATP-induced responses of PCB$^-$ (Fig. 4) and ANS$^-$ (Fig. 6) may be considered as such redox chain independent effects. It means that a mechanism utilizing ATP energy for membrane potential generation does not involve electron transfer via coupling sites of the mitochondrial redox chain. The conclusion made is in agreement with the data of the paper by Groot, Kovač and Schatz [13] which appeared when this article was in preparation. The authors showed some ATP-dependent responses, connected with formation of a membrane potential, in mitochondria from anaerobic yeasts ("promitochondria") containing no cytochromes b, c_1, c, a, a_3, CoQ, and non-heme iron proteins with $g = 1.94$. Treatment of promitochondria with ATP resulted in an ANS$^-$ fluorescence decrease which was sensitive to an uncoupler. P_i-ATP exchange reaction of promitochondria was inhibited by rutamycin, uncouplers, valinomycin + K$^+$. The authors concluded that energy transfer in promitochondria requires no electron transfer chain.

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* It may be noted that there are no such specific inhibitors for electron transfer via other coupling sites. Energy-dependent reverse electron transfer was demonstrated in the 1st coupling site in the presence of rotenone [11] and in the 3rd coupling site in the presence of cytochrome oxidase inhibitors [12]. For transhydrogenase coupling site, there are no effective inhibitors at all.

Conclusion

Data reported in the first paper of this series demonstrate formation of a membrane potential by redox chain with no high-energy intermediate involved. On the other hand, results summarized in this paper, as well as those of Groot et al. [13] show the ATP-supported formation of the membrane potential with no electron transfer involved. It means that the following chain of events has been demonstrated:

electron transfer → membrane potential ← ATP hydrolysis (1).

Taking into account that conversion of ATP energy into membrane potential is a reversible process ("ion transfer phosphorylation", see reference [14]) one can transform Eq. (1) as follows:

electron transfer → membrane potential → ATP synthesis (2).

Assuming this point we may conclude that oxidation and phosphorylation can be coupled via membrane potential as it was postulated by Mitchell [1] (for discussion, see reference [15]).

Acknowledgements

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