Reconstitution of Ion Transport and Respiratory Control in Vesicles Formed from Reduced Coenzyme Q-Cytochrome c Reductase and Phospholipids*

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Reduced coenzyme Q-cytochrome c reductase from bovine heart mitochondria (complex III) was incorporated into phospholipid vesicles by the cholate dialysis procedure. Soybean phospholipids or mixtures of purified phosphatidylcholine, phosphatidylethanolamine, and cardiolipin could be used. Oxidation of reduced coenzyme Q₂ by the reconstituted vesicles with cytochrome c as oxidant showed the following energy-coupling phenomena.

1. Protons were translocated outward with a coupling ratio, H⁺/2e⁻, of 1.9 ± 0.2. Measurements with mitochondria under similar conditions showed an H⁺/2e⁻ ratio of 1.8. Proton translocation was not seen in the presence of uncoupling agents and was in addition to the net acidification of the medium from the overall oxidation reaction.

2. Potassium ions were taken up by the reconstituted vesicles in the presence of valinomycin in a reaction coupled to electron transfer. The coupling ratio for K⁺ uptake, K⁺/2e⁻, was 2.0 in the vesicles and approximately 1.5 in mitochondria.

3. The rate of oxidation of reduced coenzyme Q₂ by the reconstituted vesicles was stimulated up to 10-fold by uncouplers or by valinomycin plus nigericin and K⁺ ions. Addition of valinomycin alone in a K⁺ medium caused a transient stimulation of electron transfer. The results indicate that energy coupling can be observed with isolated reduced coenzyme Q-cytochrome c reductase if the enzyme complex is properly incorporated into a phospholipid vesicle.

The approach of resolution and reconstitution has been successfully applied to the study of oxidative phosphorylation in the case of the mitochondrial ATPase complex (1), cytochrome c oxidase (2, 3), and NADH-coenzyme Q reductase (4, 5). In each case an enzyme complex of oxidative phosphorylation was isolated by fractionation after solubilization with cholate or other detergents. Reconstitution to a vesicular membrane was achieved by addition of phospholipids plus cholate and removal of the cholate by dialysis (1) or by simple sonication with phospholipids (6). The remaining segment of the respiratory chain, reduced coenzyme Q-cytochrome c reductase, has been purified by Haufl et al. (7) and Rieske et al. (8, 9) using cholate solubilization and fractionation. This paper describes the reconstitution of cytochrome h-c₃ segment into phospholipid vesicles which showed uncoupler-stimulated rates of electron transfer and coupled proton translocation similar to mitochondria. A preliminary report of this work has been published (10).

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EXPERIMENTAL PROCEDURE

Materials

Deoxycholate and cholate were purchased from Sigma and recrystallized from 70% ethanol after treatment with charcoal. Soybean phospholipids (Sigma) were washed with acetone as described (1), but without antioxidant. Cardiolipin was from Calbiochem. Phosphatidylethanolamine and phosphatidylcholine were purified from soybean phospholipids as described (4). Coenzyme Q₂ was a generous gift of Dr. H. Morimoto, Takeda Chemical Industries, Osaka, Japan. Cytochrome c type III was from Sigma and dialyzed at 4°C against 10 mM potassium phosphate buffer, pH 7.5, for 22 hours followed by a final dialysis against distilled water for 2 hours before use. Antimycin A, rotenone, valinomycin, and CCCP were from Sigma. The uncoupler 1799 was a gift of Dr. P. Heytler, Du Pont. Nigericin was a gift of Dr. David Wong, Eli Lilly.

Preparations

Mitochondria—Heavy layer bovine heart mitochondria prepared on a large scale (11) were provided by Dr. E. Racker, this department. They were used fresh for proton translocation and potassium ion uptake experiments.

Complex III—Reduced coenzyme Q-cytochrome c reductase was purified as described (9) and stored at -70°C in small aliquots. The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; CoQ₂, coenzyme Q₂; 1799, bis(hexafluoroacetyl)acetone.
specific activity was 100 μmol of cytochrome c reduced/min/mg, assayed at 22°C as described under "Analytical Procedures" but extrapolated to saturating quinol concentration.

Reduced Coenzyme Q₁₀-CoQ₂ (2 to 10 mg) were dissolved in ethanol and reduced with dithionite according to Rieske (9). The final product was dissolved in nitrogen-saturated ethanol and stored under nitrogen in the dark. The concentration of reduced CoQ₂ was estimated in a Clark oxygen electrode by mitochondrial oxidation. Solutions of reduced CoQ₂ were rechecked from day to day and not used if the concentration was less than 80% of original quinol. The quinol was stable for at least 1 week by this criterion.

Preparation of Complex III Vesicles—Acetone-washed soybean phospholipids (30 mg) were added to 1 ml of 0.1 M potassium phosphate buffer, pH 7.8, containing 20 mg of potassium cholate. The mixture was sonicated until clear in a bath type sonicator. Purified complex III (2 mg) was added and the mixture was dialyzed for 4 hours versus 125 ml of 0.1 M potassium phosphate, pH 7.8, and then overnight against 250 ml of the same buffer. In proton translocation and potassium ion uptake experiments, a final dialysis of 2 hours was performed against 0.15 M potassium chloride or 0.15 M choline chloride, respectively, to largely remove the buffer or potassium ions from the preparation.

Analytical Procedures

Assay of Reduced Coenzyme Q₁₀-Cytochrome c Reductase Activity—The reduction of cytochrome c was measured with an Aminco DW-2 spectrophotometer at 550 - 540 nm. A reduced minus oxidized extinction coefficient of 19 μM⁻¹ was used for cytochrome c (12). The assay medium was 25 mM potassium phosphate, 25 μM EDTA, final pH 7.5, 8 nmol of cytochrome c, 0.3 mg of soybean phospholipids previously sonicated in the same potassium phosphate/EDTA medium, and 0.5 to 1 μg of enzyme in a final volume of 1 ml at 22°C. The reaction was initiated by the addition of 15 nmol of reduced CoQ₂ in 1 μl of ethanol. The rate of cytochrome c reduction was estimated after reduction of 1 nmol of cytochrome c. The rates were corrected for a blank rate of cytochrome c reduction in the absence of the enzyme which was less than 20% of the enzymatic rates (approximately 1.5 nmol/min). Specific activity is defined as micromoles of cytochrome c reduced per min per mg of protein. When the reconstituted enzyme was assayed, additional phospholipids gave no stimulation and were not added, and ionophores were added before reduced CoQ₂. The oxidation control ratio is defined as the ratio of specific activity in the presence and in the absence of ionophores. Cytochrome c reduction was inhibited 95% by antimycin A at 0.1 μg/ml of assay medium with no preincubation.

Proton Translocation and Potassium Ion Uptake—Measurements of proton translocation were made with a recording pH meter at a sensitivity of 0.1 pH full scale as described previously (13). The buffering power of the medium was measured in each experiment by additions of standardized 5 mM HCl, and the noise level was less than 0.001 pH units. A cation-sensitive glass electrode (Beckman 38047) was used for K⁺ measurements. Other details are shown in the figure legends.

Protein Determination—Protein was determined by the biuret method in the presence of 0.5% deoxycholate with bovine serum albumin as standard (14).

RESULTS

Stimulation of Electron Transfer by Ionophores

Table I shows the effect of ionophores on the rate of cytochrome c reduction by complex III vesicles. Complex III alone was not affected by the various ionophores tested. Furthermore, when the enzyme was subjected to the dialysis procedure described under "Preparations," but without added phospholipids, no stimulation by ionophores was observed, showing that the endogenous phospholipids in complex III (9, 10) were not sufficient to give reconstitution by this method. The reconstituted system with added phospholipids showed a stimulation of the rate of cytochrome c reduction by as much as 10-fold by the uncouplers CCCP or 1799 (Table I). Valinomycin, a potassium ionophore (cf. Ref. 15), stimulated the initial rate of cytochrome c reduction by 2-fold. However, this stimulation was transient, decreasing to the control rate by the time 50% of the cytochrome c was reduced. Nigericin, which catalyzes an electrically neutral proton for potassium exchange (11, 16), had no effect on the rate of cytochrome c reduction in the reconstituted vesicles. However, nigericin showed a synergistic effect with valinomycin, since addition of both caused maximal uncoupling. Examination of this preparation with an electron microscope after thin sectioning showed single bilayer liposomes with no multilamellar structures.

The effect of varying the protein to phospholipid ratio during the reconstitution of complex III vesicles is shown in Fig. 1. In this experiment, different amounts of purified complex III were dialyzed with 30 mg of phospholipids/ml in the presence of 2% cholate as described under "Preparations." The vesicles formed were assayed for cytochrome c reduction in the presence and absence of 10 μM CCCP. Little variation in the oxidation control ratio was observed above 2 mg of complex III/30 mg of phospholipid although a slight maximum was observed at this ratio. Accordingly, this ratio of protein to phospholipid was used routinely for reconstitution. The cytochrome c reductase activity of the vesicles increased slightly as the proportion of phospholipid was increased.

Fig. 2 shows the oxidation control ratio observed when varying amounts of cholate were used during the reconstitution. The oxidation control ratio was close to 1 when no cholate

![Fig. 1](https://via.placeholder.com/150)

**FIG. 1.** Effect of protein to lipid ratio on oxidation control ratio. Complex III vesicles were prepared as described under "Experimental Procedure" except that the amount of complex III was varied as shown. The amount of cholate (20 mg) and phospholipids (30 mg) used was constant in a final volume of 1 ml. The specific activity reported was in the presence of 30 μM CCCP.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Complex III</th>
<th>Complex III vesicles</th>
<th>Oxidation control ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30.9</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>CCCP, 10 μM</td>
<td>31.9</td>
<td>34.1</td>
<td>11.0</td>
</tr>
<tr>
<td>1799, 40 μM</td>
<td>31.7</td>
<td>22.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Nigericin, 50 ng</td>
<td>30.1</td>
<td>4.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Valinomycin, 50 ng</td>
<td>31.9</td>
<td>6.3-4.1</td>
<td>9.0-1.3</td>
</tr>
<tr>
<td>Valinomycin + nigericin</td>
<td>30.8</td>
<td>31.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>
was present to solubilize the phospholipid and protein. A maximum ratio occurred when 20 mg/ml of cholate were present, as was observed in other reconstituted systems (2-4). In addition, this concentration of cholate resulted in a nearly maximum specific activity of the enzyme.

Complex III could also be reconstituted with purified phospholipids (Table II). Purified phospholipids were used at a final concentration of 30 mg/ml in the molar ratios indicated. The highest specific activity and oxidation control ratio were obtained with soybean phospholipids. Cytochrome c reductase activity in complex III vesicles reconstituted with phosphatidylycholine and phosphatidylethanolamine alone was low. This activity was enhanced when cardiolipin was included in the phospholipid mixture. This may have been caused by increased binding of cytochrome c to the negative surface of the vesicles. However, the oxidation control ratio was not significantly improved by the presence of cardiolipin.

**Proton Translocation**—An oxidation pulse technique similar to the oxygen pulse method of Mitchell and Moyle (17) was used to assay for proton translocation in the reconstituted complex III vesicles (Fig. 3). Vesicles were suspended in a potassium chloride medium and valinomycin was added to provide a permeant counter-ion for proton movements. The reaction was initiated by a pulse of ferricyanide after an equilibration period of approximately 5 min. A typical trace is shown in Fig. 3A (trace a). A rapid acidification of the medium was observed on addition of the oxidant. Part of this rapid acidification reversed with half-time of 43 s and could be distinguished as the uncoupler-sensitive component. Fig. 3A (trace b) shows that in the presence of the uncoupler CCCP, formation of protons stoichiometric to the amounts of oxidant added was observed. This rapid acidification is the net proton production caused by quinol oxidation. Only slow acidification was observed in the presence of antimycin A, a specific inhibitor to coenzyme Q-cytochrome c reductase (Fig. 3A, trace c). The amount of proton translocation was calculated as the initial acid formation above the uncoupler-insensitive net reaction. The H+/2e⁻ ratio of proton translocation shown in Fig. 3A was 1.8.

Proton translocation by the CoQ-cytochrome c reductase segment of whole mitochondria is shown in Fig. 3B. Heavy layer bovine heart mitochondria were suspended in a potas-

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**Table II**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Oxidation Control Ratio</th>
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<tbody>
<tr>
<td></td>
<td>Alone</td>
</tr>
<tr>
<td>PC:PE = 1:1</td>
<td>2.1</td>
</tr>
<tr>
<td>PC:PE = 1:2</td>
<td>7.1</td>
</tr>
<tr>
<td>PC:PE = 2:1</td>
<td>2.8</td>
</tr>
<tr>
<td>PC:PE:C = 1:1:1</td>
<td>7.8</td>
</tr>
<tr>
<td>Soy phospholipids</td>
<td>5.6</td>
</tr>
</tbody>
</table>

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.png

**FIG. 2.** Effect of cholate concentration on reconstitution. Complex III vesicles were reconstituted as described under "Experimental Procedure" using 2 mg of complex III and 30 mg of phospholipids/ml, except that the amount of cholate was varied as shown. The specific activity reported was in the presence of 10 μM CCCP.

**FIG. 3 (left and center).** A, proton translocation by complex III vesicles. Complex III vesicles (0.2 mg of protein) were suspended in 1 ml of nitrogen-saturated medium containing 150 mM KCl, 5 mM MgCl₂, 1 μg of valinomycin, 70 μM reduced CoQ₀, and 12 μM cytochrome c. Additions of 2.0 nmol of potassium ferricyanide are shown. CCCP (10 μM) and antimycin A (1 μg) were present in trace b and trace c, respectively. B, proton translocation in the CoQ-cytochrome c reductase segment of mitochondria. Heavy layer bovine heart mitochondria (3.8 mg of protein) were suspended in 1 ml of nitrogen saturated medium containing 150 mM KCl, 5 mM MgCl₂, 2 mM glycylglycine, 2 μg of oligomycin, 2 μM rotenone, 1 mM KCN, 1 μg of valinomycin, 140 μM reduced CoQ₀, and 12 μM cytochrome c. Additions of 5 nmol of nitrogen-saturated potassium ferricyanide are shown. CCCP (10 μM) and antimycin A (1 μg) were present in trace b and trace c, respectively.

**FIG. 4 (right).** Effect of different amounts of oxidant on the H⁺/2e⁻ ratio in complex III vesicles. Complex III vesicles (0.4 mg of protein) were suspended between pH 7.4 and 7.5. Different amounts of reduced CoQ₀ were added as required. Other details as in Fig. 3A.
sium chloride medium containing cyanide and rotenone to eliminate contributions from other segments of the respiratory chain. The $H^+/2e^-$ ratio of proton translocation shown in Fig. 3B (trace a) was 1.8 without correction for decay (17). Fig. 3B also shows the uncoupler and antimycin A sensitivity of the mitochondrial reaction.

A series of experiments performed under conditions of Fig. 3A are summarized in Fig. 4. The $H^+/2e^-$ ratio was calculated for a wide range of amounts of ferricyanide added. This ratio was close to 2 for small amounts of ferricyanide added and decreased slightly up to 1.5 nmol of oxidant.

Potassium Ion Uptake—The uptake of potassium ions by the vesicles driven by the electron transfer reactions was monitored by a potassium ion-sensitive electrode (Fig. 5A). Complex III vesicles were suspended in choline chloride medium containing reduced CoQ$_2$, cytochrome c, and valinomycin. Addition of a small pulse of sodium ferricyanide caused a rapid decrease in potassium ion concentration in the medium, indicating K$^+$ uptake by the vesicles. A $K^+/2e^-$ ratio of 2.0 was calculated for the trace shown. Fig. 5A (trace b) shows that this K$^+$ uptake was uncoupler-sensitive.

The corresponding reaction in cyanide and rotenone-treated bovine mitochondria is shown in Fig. 5B. The $K^+/2e^-$ ratio calculated for the trace shown was 1.5 (trace a). The rapid potassium ion uptake was also abolished by CCCP (trace b).

Comparisons of Figs. 3, A and B, and 5, A and B, show that the half times of relaxation of the ion gradients following the oxidation pulse were longer in the reconstituted system than that in the heavy layer bovine heart mitochondria. This may account for the slightly higher coupling ratios calculated for the reconstituted system since the relatively slow response of the ion-specific electrodes tend to underestimate the amount of transport when the decay of the gradient is rapid.

**DISCUSSION**

The phenomena of respiratory control and coupled ion transport were demonstrated in a reconstituted coenzyme Q-cytochrome c reductase vesicle preparation similar to previous studies with cytochrome oxidase (2). Protons were transported outward by the vesicles and potassium ions were taken up when valinomycin was present during the oxidation of reduced CoQ$_2$ by cytochrome c. This polarity was expected because cytochrome c was added externally after formation of the vesicles. We did not attempt to reconstitute the system with internal cytochrome c as was done with cytochrome oxidase vesicles, because we do not have a permeant oxidation-reduction mediator which will oxidize the internal cytochrome c without reacting directly with the reduced CoQ$_2$. It appears that the complex is oriented predominately in the mitochondrial configuration since the maximum rates of electron transfer in the reconstituted system were close to the rate with the unreconstituted complex. However, the large effect of lipid composition on the activity of complex III may cause an error in calculation of sidedness based on activity with external cytochrome c. For example, cardiolipin greatly stimulated the reaction under the conditions we have used for assay (Table II) and we do not know how much reconstitution affects the turnover number of the complex.

The effect of valinomycin on the rate of electron transfer in the reconstituted vesicles is complex. A large stimulation by valinomycin was reported previously (10) because the high nonenzymatic reduction of cytochrome c by durohydroquinone limited observations to the initial phase (less than 10 s after initiation of the reaction by quinol). Studies reported here with reduced CoQ$_2$ as substrate showed that valinomycin stimulated electron transfer in complex III vesicles transiently with the rate of cytochrome c reduction decreasing to control levels. This transient uncoupling can be interpreted in terms of the chemiosmotic hypothesis (18, 19). Electron transfer reactions generate an electrochemical proton gradient ($\Delta\mu_{H^+}$) which inhibits the oxidation-reduction reactions. The initial $\Delta\mu_{H^+}$ generated by the oxidation-reduction reaction is comprised mainly of the membrane potential component before the pH gradient can develop. As a result, valinomycin induces transient uncoupling by dissipation of the membrane potential. Transient uncoupling by valinomycin has also been observed in studies of the initial rate of ATP synthesis by oxidative phosphorylation (20).

The coupling ratios for proton translocation and potassium ion uptake driven by electron transfer in the complex III vesicles were found to be close to 2 protons translocated per pair of electrons. The corresponding ratios measured for electron transfer in the cytochrome b-c$_1$ region of mitochondria gave slightly lower values. This result agrees with coupling ratios determined in cytochrome oxidase vesicles (2) and with studies by Mitchell and Moyle (17, 21) of all three coupling regions in rat liver mitochondria. However, different methods have been used by others who found higher coupling ratios (22).

A scheme (23) for the topology of the respiratory chains in the b-c$_1$ region which is consistent with our observations on ion transport is shown in Fig. 6. Cytochrome c and ferricyanide are

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**Fig. 5. A,** potassium ion uptake in complex III vesicles. Complex III vesicles (0.2 mg of protein) were suspended in 1 ml of nitrogen-saturated medium containing 100 mM choline chloride, 50 mM morpholinopropanesulfonic acid-Tris, 5 mM MgCl$_2$, 1.5 mM KCl, 1 pg of valinomycin, 140 mM reduced CoQ$_2$, and 12 mM cytochrome c, pH 7.5. Additions of 30 nmol of sodium ferricyanide are shown. CCCP (10 mM) was present in trace b. **B,** potassium ion uptake in the CoQ-cytochrome c segment of mitochondria. Heavy layer bovine heart mitochondria (4.5 mg of protein) were suspended in 1 ml of 100 mM choline chloride, 50 mM morpholinopropanesulfonic acid-Tris, 5 mM MgCl$_2$, 2 mM rotenone, 1.5 mM KCN, 1 pg of valinomycin, 80 mM reduced CoQ$_2$, and 12 mM cytochrome c, pH 7.5. Additions of 21.8 nmol of nitrogen saturated sodium ferricyanide are shown. CCCP (10 mM) was present in trace b.
impermeant and are shown reacting on the outside surface of the membrane. Coenzyme $Q_2$ is permeant and could react on either side. It is shown to release protons into the external medium on oxidation because this is consistent with our observations of pH changes in this phase. The major question concerning this scheme is the identity of the hypothetical component $Z$. This is apparently not the iron-sulfur protein in this region since its midpoint potential is not pH dependent (24), although there is a chemical basis for suggesting that iron-sulfur proteins may be hydrogen carriers (25). It is interesting to note that if the reactions occur as depicted, the reconstituted system consists of the electron transfer half of the second loop and the hydrogen carrier half of the third loop of the respiratory chain as defined by Mitchell (18).

REFERENCES


![Diagram](image-url)
Reconstitution of ion transport and respiratory control in vesicles formed from reduced coenzyme Q-cytochrome c reductase and phospholipids.

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