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# Studies on the reconstitution of the structure and function of mitochondrial inner membrane. I. Structure and function of the membrane formed by the purified complex 3 and complex IV of the mitochondrial electron transfer chain

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# Studies on the reconstitution of the structure and function of mitochondrial inner membrane. I. Structure and function of the membrane formed by the purified complex 3 and complex IV of the mitochondrial electron transfer chain*

Hideo Hayashi

**Abstract**

In order to elucidate the molecular organization of mitochondrial inner membrane, biochemical and electron microscope observations were made on the formation of membrane structure and function by the purified complexes of the electron transfer chain of beef heart mitochondria. Purified complex III (CoQ-cytochrome c reductase) and complex IV (cytochrome oxidase) were soluble in the presence of bile salts. They were, however, aggregated to form membrane by washing out the bile salts. When the membranous complexes III and IV were mixed, both membranes were separate by density gradient centrifugation and the vesicle which contained both complexes could not be formed and CoQH2-oxidase activity was hardly re;tored. When the mixture of the solubilized complexes III and IV were diluted to remove the bile salts, a membranous vesicle in which both complexes were assembled was formed. CoQH2-oxidase activity was restored in accordance with the formation of the membrane. The membrane which contained any desired propotion of each complexes could be obtained. These facts indicate that the complexes of the electron transfer chain conjugate two-dimentionally each other and form the membrane to carry electrons from substrate to oxygen most efficiently.

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# STUDIES ON THE RECONSTITUTION OF THE STRUCTURE AND FUNCTION OF MITOCHONDRIAL INNER MEMBRANE

## I. STRUCTURE AND FUNCTION OF THE MEMBRANE FORMED BY THE PURIFIED COMPLEX III AND COMPLEX IV OF THE MITOCHONDRIAL ELECTRON TRANSFER CHAIN

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The components of the electron transfer and oxidative phosphorylation systems have been believed to be organized in the mitochondrial inner membrane. Electron transfer chain consists of four complexes; these are complex I (NADH-coenzyme Q oxidoreductase), complex II (succinic-coenzyme Q oxidoreductase), complex III (reduced coenzyme Q-cytochrome *c* oxidoreductase), and complex IV (reduced cytochrome *c* oxidase) as illustrated in Fig. 10 (1). Validity of these complexes has been shown by many workers and their biological and ultrastructural characterizations have already been confirmed. (2—6). However, it is uncertain how these complexes are organized in the mitochondrial inner membrane (7).

In order to resolve this problem, systematic analysis of the membrane is one of the most effective ways and reconstitution is another important way. Studies in our laboratory have shown that the components of mitochondrial inner membrane can be solubilized from the membrane step by step in proportion to the concentration of bile salts used for the treatment (8, 9). From the results of these studies we suggested that there should be a close correlation between the isolated complexes and the particulate arrangements on the surface of the membrane which were obtained by the treatment with detergents.

From the point of reconstitution, formation of the membrane by purified complex IV and phospholipid was reported by McCONNELL *et al.* (10). HATEFI *et al.* (11) reported previously the functional reconstitution of NADH- and succinic-oxidase activities by purified complexes. Recently TZAGOLOFF *et al.* (12) reported the structural and functional reconstitution of NADH- and succinic-oxidase membranes by complex (I+III) and comp-

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lex IV or complex (II+III) and complex IV. Both complex (II+III) and complex (I+III) contain many other components of the membrane and their morphological characterizations have not been made clear, therefore the membrane reconstituted from these complexes is not appropriate for study on the molecular structure of the membrane.

In this study we used the purified complex III and complex IV for the reconstitution of the membrane, both of which have been made clear on the biochemical and ultrastructural properties. Evidence will be presented to prove that the purified complex III and complex IV can form the membraneous structure and restore over-all enzymic activity of $QH_2$-oxidase. The relationship of the molecular organization between the reconstituted membrane and the original inner mitochondrial membrane will be discussed.

## MATERIALS AND METHODS

*Beef heart mitochondria* (BHM) were isolated in the medium of 0.25M sucrose solution containing 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA by the method described by CRANE *et al.* (13) with slight modifications.

*Mitochondrial inner membrane* (Electron Transfer Particle; ETP) (14) was prepared from BHM as follows: The mitochondrial suspension (20 mg of protein per ml of 0.25 M sucrose solution) was subjected to sonic oscillation with an ultrasonic oscillator for 1 minute per 5 ml of suspension (Kaijo Dempa Co. Ltd., 7 ϕ tip, 20 Kc), then centrifuged at 20,000 x g for 10 minutes to remove unbroken mitochoneria, and by further centrifugation at 140,000×g for 30 minutes ETP fraction was obtained. ETP was suspended in 0.66 M sucrose solution containing 50 mM Tris-HCl, 1 mM histidine, pH 8.0.

*Isolation of complex III and complex IV* Isolation of complex III was conducted from ETP by the method of HATEYI *et al.* (5) with modifications described by SEKI *et al.* (15). Complex IV was isolated from ETP by the method of FOWLER *et al.* (6) with modification described by SEKI *et al.* (16).

*Reconstitution of membrane.* The solution of the purified complex or the mixture of purified complexes at high concentration of protein over 15 mg/ml was diluted by the addition of 0.25 M sucrose solution containing 10 mM Tris-HCl (pH 7.6) (sucrose-Tris) until the protein concentration reached to 0.5 mg/ml. After being kept in the ice bath (0-4°C) for 60 minutes, the diluted solution of the complexes was centrifuged at 105,000 × g for 60 minutes by Spinco Model L2 rotor 30. The pellet obtained after centrifugation was suspended in sucrose Tris solution at protein concentration of 5-10 mg per ml. In order to examine about the effect of cytochrome *c* on the reconstitution, 54 mμ moles of cytochrome *c* were added to 0.5 ml of solution or the mixture, and then dilution, incubation, centrifugation were carried out as described above.

*Reduced coenzyme Q-cytochrme c reductase and cytochrome oxidase activities.* $QH_2$-cytochrome *c* reductase activities were assayed by the method described by RIESKE *et*

*al.* (17). The absorbancy at 550 m$\mu$ was recorded by an autospectrophotometer ETS-3T. (Hitachi). Cytochrome oxidatse activity was assayed by the method of SMITH (18) or SEKUZU (19) with the modification described by SEKI (16).

*Coenzyme $QH_2$-oxidase activity* was assayed by the method of HATEFI *et al.* (20) with modifications as follows: Reduced coenzyme $Q_6$ was prepared by the method of RIESKE *et at.* (17) and $Q_6H_2$ was suspended in final concentration ot 15 $\mu$ moles per ml of ethanol. Reaction medium contained 40 $\mu$ moles of phosphate buffer, pH 7.4 and 0.2 $\mu$ moles of EDTA. Ten to fifty $\mu$g of enzyme protein were mixed with the medium and the final volume was adjusted to 2 ml with deionized water. After preincubation for 5 minutes at 37°C, the reaction was started by the addition of 0.01 ml of $Q_6H_2$ into solution. Reaction was stopped by the addition of 1 ml of 16 % cold perchrolic acid after 30 seconds, 1 minute, and 2 minutes respectively. Then 1 ml of 95 % ethanol and 3 ml of cyclohexane were added and the mixtnre was vigorously stirred for 30 seconds. The portion of cyclohexane was applied to spectrophotometry directly. From the rctio of absorbancy at 273 m$\mu$ and 292 m$\mu$ the oxidation ratio of $Q_6H_2$ was calculated. Extinction coefficiency of $Q_6$ and $Q_6H_2$ in cyclohexane was used: $\varepsilon_{2\cdot 3m\mu}=17.1\ mM^{-1}cm^{-1}$ and $\varepsilon_{292m\mu}=5.0\ mM^{-1}cm^{-1}$ respectively (21). $Q_6H_2$-oxidase activity was assayed without addition of cytochrome *c* and with the addition of 0.1 m$\mu$ moles of cytochrome *c* (as the solution of 0.2 mM in deionized water) to the reaction medium.

*Determination of cytochromes.* The concentrations of cytochromes were estimated from the recording of the difference-spectra between reduced and oxidized forms with autospectrophtometer ETS-3T (Hitachi) and calculated with respective absorption band described by CHANCE (22) and GREEN *et al.* (23).

The extinction coefficients in this computation are as follows;

$\varepsilon=16\ mM^{-1}cm^{-1}$ ($\Delta 605-\Delta 630$ m$\mu$) for cytochrome *a* (heme *a*)
$\varepsilon=22\ mM^{-1}cm^{-1}$ ($\Delta 562-\Delta 575$ m$\mu$) for cytochrme *b*
$\varepsilon=19.1\ mM^{-1}cm^{-1}$ ($\Delta 553-\Delta 540$ m$\mu$) for cytochromme $c_1+c$
$\varepsilon=18.5\ mM^{-1}cm^{-1}$ ($\Delta 550-\Delta 530$ m$\mu$) for cytochrome *c*

*Protein estimation.* Protein was estimated by the biuret method of GORNALL *et al.* (24).

*Discontinuous sucrose density gradient centrifugation.* Sucrose solutions at density of 1.08, 1.10, 1.12, 1.14, 1.16, 1.18, 1.20, 1.23, and 1.25 (g/ml at 20°C) were prepared and 0.5 ml of each density solution was layered in centrifugation tube (Spinco Model L2 SW 39). Half ml of the sample solution was loaded on the top of the gradient layers and centrifuged at 39,000 rpm for 60 minutes.

*Electron microscopic observation.* Samples were prepared for examination in the electron microscope, either by negative staining with 1 % phosphotungstate (pH 7.0) or by positive staining in conjunction with appropriate procedures for fixation, embedding, and sectioning. Samples were immersed in 1.2 % $KMnO_4$ (in 0.02 M phosphate buffer) for 15 minutes, and then washed, dehydrated through a series of ethanol solution of graded concentration, embedded in Epon 812 and sectioned with a glass knife. Specimens were examined in a Hitachi HU-11-D electron microscope.

## RESULTS

*Membrane formation by complex III or complex IV.* The purified complex III was soluble in sucrose-Tris buffer at high concentration of protein in consequence of solubilization of the complex by high concentration of bile salts used in the preparation. Electron microscopic observation of the solubilized Complex III is shown in Fig. 1. When it was frozen and thawed, however, these particles tended to aggregate as shown in Fig. 2. The suspension of frozen-thawed complex III showed optically clear and these aggregations had no clear feature of membrane formation, but had some transitional stages to vesicular formation. They could be fairly redispersed by ultra-sonic irradiation. When the suspension of complex III at the dispersed state was applied to sucrose density gradient centrifugation, the sample remained at the top layer and no band was formed at any other sucrose density. The solution at high concentration of complex III was applied to reconstitution of membrane described in the methods above. A reddish pellet was obtained, and it was suspended in sucrose-Tris at the concentration of 5—10 mg protein per ml. The suspension showed visible cloudiness and the turbidity was slightly decreased by sonic oscillation. The suspension was applied to sucrose density gradient centrifugation. The result is shown in Fig. 11. A single band was observed at the bottom of density 1.18 and there was no other band. Electron microscopic observation revealed formation of vesicular membranes measuring 0.1 to 0.5 $\mu$ in diameter. Dispersed particles were not observed. The characteristic feature of the surface of the membrane showed regular arrangement of uniform particles measuring about 95 A in center-to-center distance (Fig. 5). This membrane was named membranous complex III (Mb. III) hereafter. The protein recovery to the membrane was 56 % of the solubilized complex, and the total amount of cytochromes recovered to the membrane was about 50 % (Table 1). The reduced-coenzyme $Q_6$-cytochrome *c* reductase activity of the membranous and solubilized complex III were 3.48 and 4.15 $\mu$ moles $Q_6H_2$-oxidised per minutes per mg of protein respectively, showing a little inhibition by membrane formation.

Fig. 1 Purified complex III. Negatively stained with 1 % of phosphotungstate immediately after purification. In this and subsequent plates the bar represents 1,000 A.

Fig. 2 Purified complex III after freezing-thawing. There develop small aggregations or sheet-like structure of the particles.

Fig. 3 Purefied complex IV. Negatively stained with PTA. The particulate structure has uniform size about 90 A in diameter and homogeneously dispersed.

Fig. 4 Sheet-like structure of membranous complex IV. It looks likely on the way of reconstitution of membrane. Negatively stained with PTA.

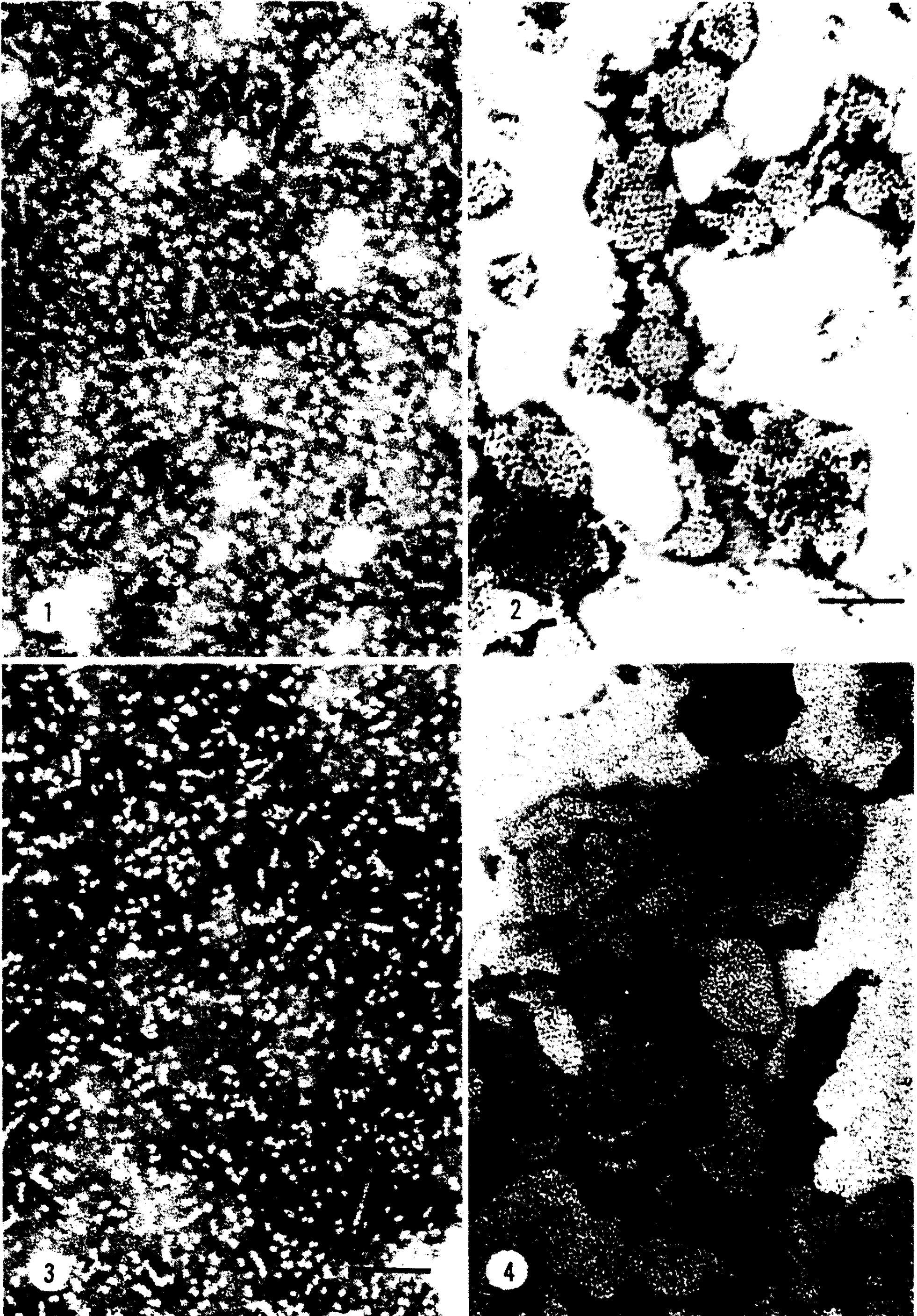
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Table 1. Recovery of protein and cytochromes in the membranes reconstituted from purified complex III, IV or the mixture of them

| Reconsituted membranes | | Solubilized complexes or the mixture of them | | Reconstituted membranes | | % recovery into membranes | |
|---|---|---|---|---|---|---|---|
| | | total cytochromes ($m\mu$ moles) | ratio/$a$* | total cytochromes ($m\mu$ moles) | ratio/$a$* | cytochromes | protein |
| Mb. III | b | 75.5 | | 34.8 | | 46 | 56 |
| | $c_1$ | 24.4 | | 12.5 | | 51 | |
| Mb. IV | a | 97.3 | | 60.6 | | 62 | 75 |
| Mb. (IV+c) | a | 97.3 | 1 | 57.5 | 1 | 59 | 78 |
| | c | 54.0 | 0.56 | 24.0 | 0.42 | 45 | |
| Mb. (III+IV) | a | 48.6 | 1 | 20.7 | 1 | 43 | |
| | b | 38.0 | 0.78 | 20.2 | 0.98 | 53 | 54 |
| | $c_1$ | 12.2 | 0.25 | 7.1 | 0.34 | 58 | |
| Mb. (III+IV+c) | a | 48.6 | 1 | 23.4 | 1 | 48 | |
| | b | 38.0 | 0.79 | 19.3 | 0.86 | 51 | 88 |
| | $c_1$ | 12.2 | 0.26 | 6.8 | 0.30 | 54 | |
| | c | 54.0 | 1.17 | 12.3 | 0.55 | 23 | |
| Mb. ($III_2+IV_1+c$) | a | 24.3 | 1 | 11.6 | 1 | 47 | |
| | b | 38.0 | 1.60 | 20.4 | 1.75 | 54 | 77 |
| | $c_1$ | 12.2 | 0.51 | 8.3 | 0.31 | 68 | |
| | c | 54.0 | 2.20 | 12.0 | 1.04 | 22 | |
| Mb. ($III_1+IV_2+c$) | a | 48.6 | 1 | 22.5 | 1 | 46 | |
| | b | 18.8 | 0.39 | 7.6 | 0.34 | 40 | 57 |
| | $c_1$ | 6.1 | 0.13 | 3.1 | 0.14 | 51 | |
| | c | 54.0 | 1.10 | 10.9 | 0.49 | 20 | |

* Ratio/$a$ represents ratio at concentration of cytochrome $b$, $c_1$, or $c$ with concentration of cytochrome $a$.
Abbreviation on the table are described in the text.

(Table 2). The complex IV was soluble in water at high concentration of protein as shown in Fig. 3. The complex could constitute sheet-like or vesicular membrane in the same way as complex III. On the surface of membranous complex IV (Mb. IV), there were obseved regular arrays of small particles, measuring about 50—60 Å in diameter about 80 Å (Fig. 4, 6) in center-to-center distance. Studies on the fine structure of membranous complex IV were reported by SEKI *et al.* (16) in details. By sucrose density gradient centrifugation, membranous complex IV formed a single band at the bottom of sucrose density 1.20. The protein recovery of solubilized complex IV to the membrane was 75 %. Sixty-eight % of the total cytochromes were recovered to the membrane, indicating that purified complex IV aggregated into the membrane homogeneously. The cyto-

Table 2. Reduced coenzyme $Q_6$-cytochrome *c* reductase and cytochrome oxidase activities of the membrane reconstituted from complex III, complex IV or the mixture of them

| Reconstituted membranes | $Q_6H_2$-cytochrome *c* reductase activities | | Cytochrome oxidase activities | |
|---|---|---|---|---|
| | Unit* per mg protein of | | Unit** per mg protein of | |
| | total complexes | complex III | total complexes | complex IV |
| Mb. III | 3.84 | 3.84 | | |
| Mb. IV | | | 20.60 | 20.60 |
| Mb. (IV+c) | | | 15.30 | 15.30 |
| Mb. (III+IV) | 2.06 | 4.25 | 3.15 | 6.30 |
| Mb. (III+IV+c) | 1.20 | 2.48 | 2.85 | 5.70 |
| Mb. ($III_2+IV_1$+c) | 2.56 | 3.94 | 2.08 | 6.24 |
| Mb. ($III_1+IV_2$+c) | 1.28 | 4.04 | 4.65 | 7.00 |

* Unit of $Q_6H_2$-cytochrome *c* reductase activities are expressed as $\mu$ moles of cytochrome *c* reduced per minute per mg of protein.

** Unit of cytochrome oxidase activities are expressed as $sec.^{-1}$ per mg of protein as indicated.

Abbreviation on the table and assay conditions are described in the text.

chrome oxidase activity of membranous and solubilized conplex IV was 20.6 $sec.^{-1}$ per mg of protein and 21.8 $sec.^{-1}$ per mg of protein respectively. The membrane formation did not inhibit the cytochrome oxidase activity. The conjugation of cytochrome *c* to the complex IV was examined. 97.3 m$\mu$ moles of solubilized complex IV and 54 m$\mu$ moles cytochrome *c* were mixed and then reconstitution was carried out by the method described above. They also reconstituted membranous structure, and there was no remarkable difference from the membranous complex IV. Cytochrome oxidase activity was slightly inhibited by membrane formation with addition of exogeneous cytochrome *c* (Table 2).

*Mixture of the membranous complex III and complex IV.* If the membranous complex III and membranous complex IV are mixed, what effect does occur on its over-all enzymatic activity and structure of the membrane? When the mixture of the two membranous complexes was applied to sucrose density gradient centrifugation, two bands were formed at the bottom of the layers of sucrose density 1.18 and 1.20 separately, which correspond to Mb. III and Mb. IV respectively. The result indicates that in the mixture of Mb. III and Mb. IV each membrane does not fuse or interact. $Q_6H_2$-oxidase activity of the mixture of Mb. III and Mb. IV could not be detected in the absence of cytochrome *c* and 0.35 $\mu$ moles of $Q_6H_2$ was oxidized per minute per mg of protein in the presence of 0.1 m$\mu$

moles of cytochrome *c*. By electron microscopic observation we could distinguish membranous complex III from complex IV. These results indicate that in the mixture of Mb. III and Mb. IV, the complex of one vesicle does not shift to the other vesicle in the absence of detergents and that the membranous complexes do not fuse each other.

*Membrane formation by solubilized mixture of complex III and complex IV.* If the solubilized complex III and solubilized complex IV are mixed at high concentration of protein and if the mixture is used for membrane formation, what kind of vasicular membrane can be constituted? To solve this problem an experiment was carried out as follows: The solubilized complex III and complex IV, each at a protein concentration of 20 mg per ml, were mixed in an equal amount of protein, and then the mixture was applied to sucrose density gradient centrifugation. After centrifugation at 39, 000 rpm for 60 minutes, the sample remained at the top layer of sucrose gradient and no bands were observed at any other portion of density (Fig. 11). Electron microscopic observation revealed that the mixture of the complexes presented dispersed particles distinguishing the particles of complex III from complex IV. None of the vesicular membrane was observed under the condition used for electron microscopic observation by negative staining (Fig. 7). The mixture was applied to reconstitution of membrane as described in the methods, then the homogeneous reddish-brown pellet was obtained. The pellet was suspended in sucrose-Tris at the protein concentration of 5 to 10 mg per ml. The suspension was applied to sucrose density gradient centrifugation. One single band appeared at the middle of density 1.20 (Fig. 11). By electron microscopic observation, it was found that there developed vesicular membranes into which particulate complexes had organized, and isolated particles of complexes could not be seen. The membrane 50—300 m$\mu$ in diameter showed electron thick margin of 95 Å in width in negative staining and revealed granular surface structure 70—100 Å in diameter, and 70—100 Å, average 95 Å, in center-to-center distance (Fig. 9). By the thin section specimen fixed with $KMnO_4$, there showed vesicular membranes with tri-lamellar picture, with a width of about 80 Å (Fig. 8). Protein

Fig. 5 Membranous complex III (Mb. III) obtained from purified complex by the method as described in the text. There develop vesicular membranes of 0.1—0.5 $\mu$ in diameter. The particulate structure shows uniform pattern about 95 Å in center-to-center distance. Negatively stained with PTA.

Fig. 6 Membranous complex IV (Mb. IV). Reconstituted from solubilized complex IV by the method as described in the text. There shows regular arrangement of uniform particles about 60 Å in diameter and about 70 Å in center-to-center-distance.

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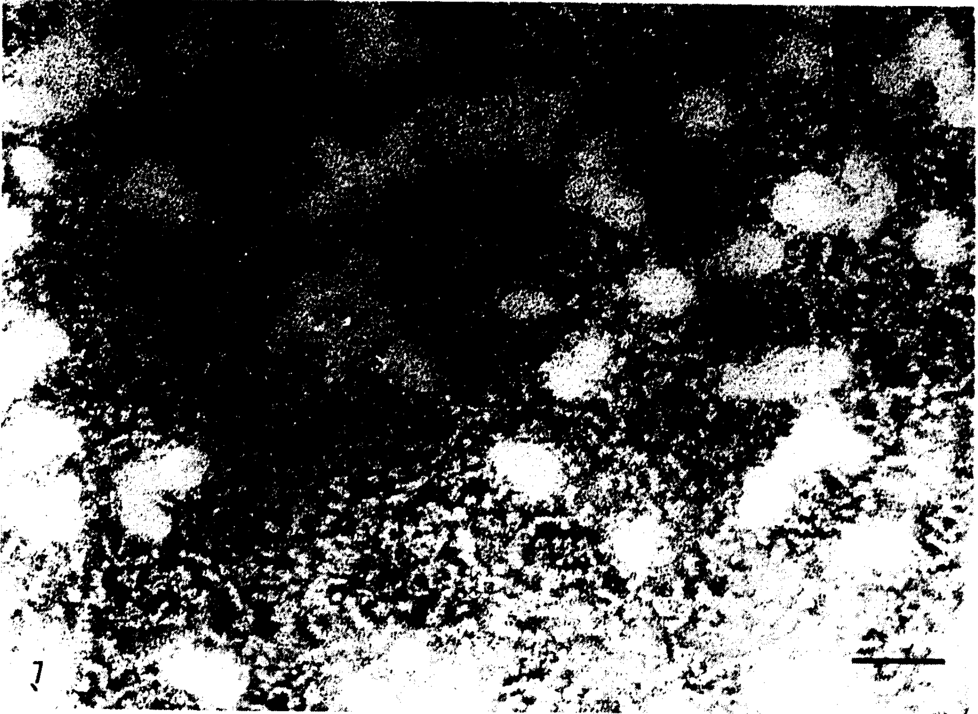
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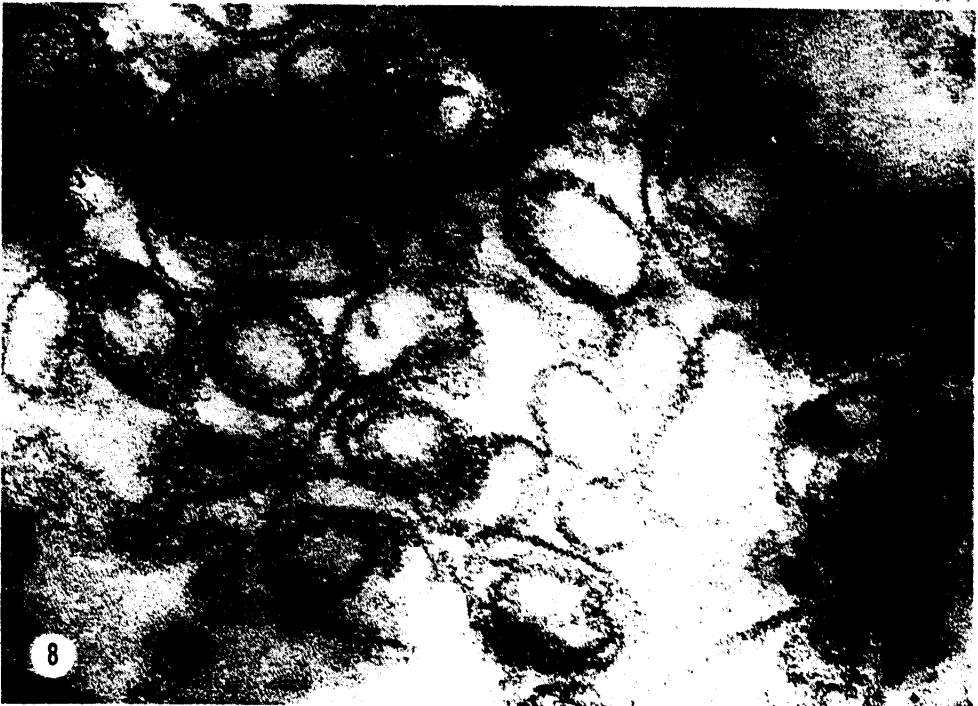
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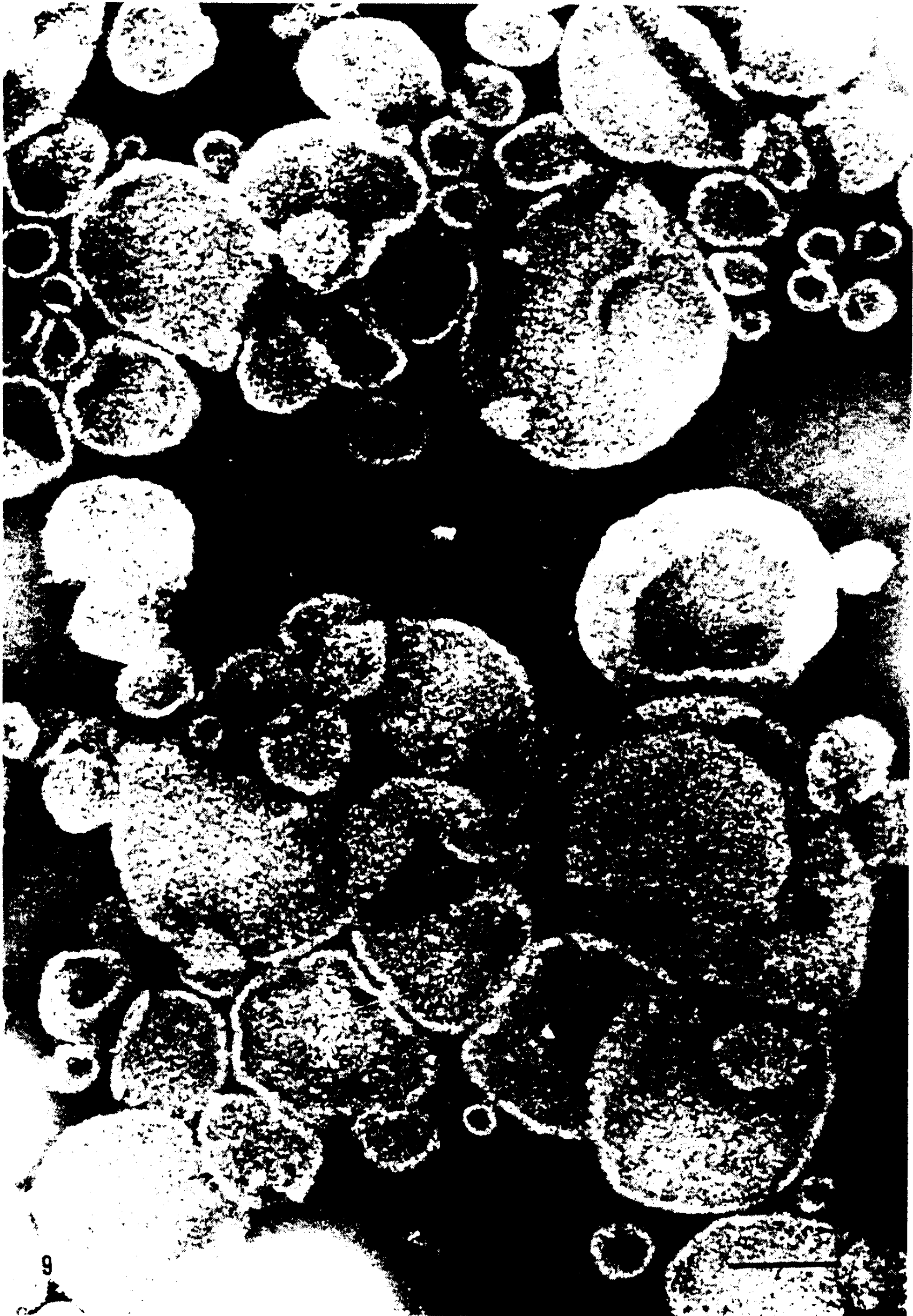
recovery to the vesicular membranes was about 54 %, and 50 % of the total cytochromes was recovered from solubilized mixture of complexes. The ratio of cytochrome concentration in the solubilized mixture was 1 : 0.98 : 0.34 of heme $a : b : c_1$ respectively, while in reconstituted membrane it was 1 : 0.78 : 0.25 respectively (Table 1). The results indicate that the solubilized complexes are incorporated into vesicular membrane homogeneously. $Q_6H_2$-cytochrome *c* reductase activity of the reconstituted membrane was 2.05 μ moles of cytochrome *c* reduced per mg of total protein; if converted per mg of complex III protein it was 4.25 μ moles of cytochrome *c* reduced per minute. In this case the membrane had an activity almost equal to the membranous complex III. These facts indicate that the conjugation or aggregation of the two kinds of complexes does not disturb $Q_6H_2$-cytochrome *c* reductase activity. Cytochrome oxidase activity of the reconstituted membrane, however, was inhibited by membrane formation, as shown in Table 2. The reconstituted membrane was inactive in oxidizing $Q_6H_2$ by oxygen in the absence of cytochrome *c*, but with the addition of only 0.1 mμ moles cytochrome *c* to the reaction medium, 0.71 μ moles $Q_6H_2$ was oxidized per minute per mg of total protein. Since the enzymatic activity of the membrane was accelerated twice as in the case of Mb. III+Mb. IV, it seems to be more effective for the electron transfer that the complexes are organized in the same membrane with the aid of a mobile component of cytochrome *c*. To test the effects of cytochrome *c* on the reconstitution of the membrane and overall activity, the following experiments were carried out. When 54 mμ moles of cytochrome *c* were added to the solubilized mixture of the complexes, cytochrome *c* were found to be bound to the membrane. The recovery of cytochrome *c* in the membrane was about 23 % of added cytochrome *c*. The overall enzymatic activity of the reconstituted membrane containing cytochrome *c* (Mb. (III+IV+c)) is shown in Table 3. It is important from the point of stoichiometry whether the ratio of components in the membrane reconstituted from complex III and complex IV was controlled by some fixed proportion of random aggregation or not. To examine this problem the complexes were mixed in different proportion by protein weight; that was (A) complex III with a ratio of 1 : 2 with complex IV; and (B) complex III with a ratio of 2 : 1 with complex IV. Fifty-four mμ moles of cytochrome

Fig. 7 The mixture of purified complex III and complex IV. Two kinds of particles are dispersed homogeneously.

Fig. 8 The membrane reconstituted from the mixture of purified complex III and complex IV. Fixed with $KMnO_4$ as described in the method. Vesicular membranes with trilamellar structure about 90 A in width are shown.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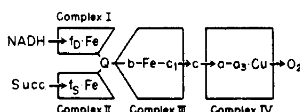


Fig. 10 Complexes I, II, III, and IV in the electron transfer chain of the mitochondrial inner membrane

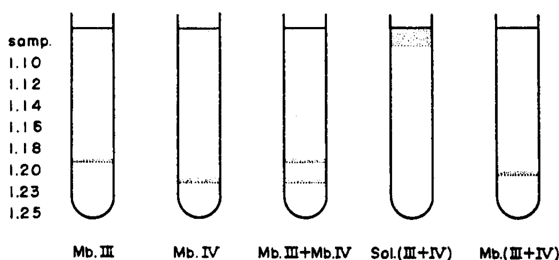


Fig. 11 Sucrose density gradient fractionations of the membrane reconstituted by solubilized complex III and/or complex IV

Table 3. Reduced coenzyme $Q_6$ oxidase activities of the membranes reconstituted from complex III, complex IV or the mixture of them

| Reconstituted membranes | $Q_6H_2$ oxidase activities* | |
|---|---|---|
| | + none | + cytochrome *c*** |
| Mb. III+Mb. IV | 0 | 0.35 |
| Mb. (III+IV) | 0 | 0.71 |
| Mb. III+Mb. (IV+c) | 0.06 | 0.38 |
| Mb. (III+IV+c) | 0.43 | 0.40 |

* Activities are expressed as $\mu$ moles of $Q_6H_2$ oxidized per minute per mg of protein as indicated.

** 0.1 m$\mu$ mole of cytochrome *c* was added to 2 ml of the reaction medium. Assay conditions are described in the text.

Mb. III+Mb. IV : The mixture of membranous complex III and membranous complex IV.

Mb. (III+IV) : The reconstituted membrane containing both complex III and complex IV in one single vesicle.

Mb. III+Mb. (IV+c) : The mixture of membranous complex III and membranous complex IV containing cytochrome *c*.

Mb. (III+IV+c) : The reconstituted membrane containing all of complexes III, IV and cytochrome *c* in one single vesicle.

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Fig. 9 The membrane recontituted from the mixture of purified complex III and complex IV. Negatively stained with PTA. There develop vesicular membranes with particulate structure. Two kinds of particles are observed on the surface: a) About 60 Å in diameter, 70—80 Å in center to center distance. b) About 90 Å in diameter, and 140 A in center-to center distance, however distributes from 70 Å to 140 Å evenly.

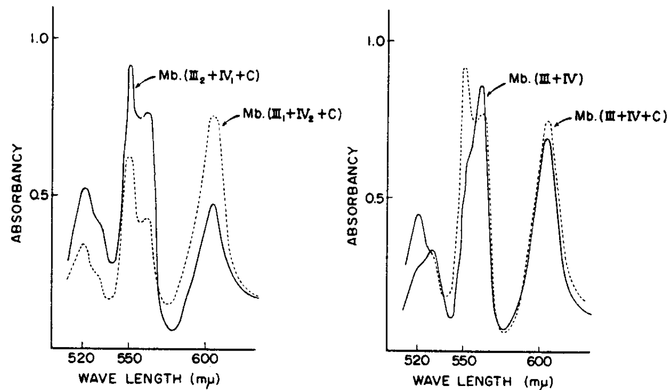


Fig. 12 Difference spectra of the reconstituted membranes
Mb. (III+IV): The membrane reconstituted from the mixture of solubilized complex III and complex IV. Mb. (III+IV+c): The reconstituted membrane from the mixture of solubilized complex III, complex IV andcy tochrome *c*. Mb. ($III_1+IV_2+c$): The membrane reconstituted from the mixture of solubilized complex III with a ratio of 1:2 with complex IV by weight of protein and cytochrome *c*. Mb. ($III_2+IV_1+c$): The membrane reconstituted from the mixture of complex III and complex IV with a ratio 2:1 by weight of protein and cytochrome *c*. The spectra were recorded by autspectrophotometer ETS-3T.

*c* were added to each case before dilution. The ratio of cytochrome components of the membrane obtained by centrifugation is shown in Table 2 and Fig. 12. The results of these experiments indicate that the aggregation of complexes into membranes is quite a random process and that membranes with any desired proportion of complexes can be obtained, and that cytochrome *c* also binds to the membranes reconstituted from the complexes.

## DISCUSSION

*Membrane formation by purified complexes.* The complexes of the electron transfer chain consist of 70 % of protein and 30 % of phospholipid by weight (5, 6). In that way the complexes are insoluble in an aqueous solution. A concentrated solution of the isolated complex III or complex IV seemed to be soluble in water. In electron microscopic observations they revealed dispersed particulate structure. This "solubilization" is a token of the presence of a residual amount of salts used in the preparation. When the concentrated solution was diluted to wash out the bound bile salts of the complexes, there occurred aggregation of the complexes by hydrophobic groups. The key point to be stressed is that the aggregation

of the particles does not develop in irregular three-dimentional directions, but only in regular two-dimentional membranous vesicles (Fig. 13). This

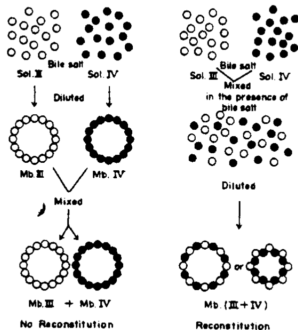


Fig. 13 Schematic illustration of the reconstitution of membranes. left: When the membranous complex III (Mb. III) and membranous complex IV (Mb. IV) were mixed, a vesicular membrane containing both complexes could not be reconstituted. right: When the solubilized complex III (Sol. III) and complex IV (Sol. IV) were mixed and then diluted, a vesicular membrane containing both complexes was reconstituted. (Mb. (III+IV)). Complexes aggregate into membranous structure next-to-next like mozaic patern.

was also confirmed by the fact that the surface of the reconstituted membrane was arranged into a regular particulate structure. Since the reconstituted membrane could not be broken to pieces by sonic irradiation, the membrane seems to be strong enough against physical forces. Judging from the results of the sucrose density gradient of the mixture of membranous complex III and membranous complex IV, it is clear that a complex being a constitutent of one vesicular membrane may not shift to the other membranes. It is similarly clear that the membrane may not fuse with one another by the specific interaction of each complex. These facts indicate that the hydrophobic binding of the reconstituted membrane is rather stable. The aggregation of complexes may be quite random because the ratio of concentration of the complexes is coincidental in the solubilized mixture at any proportion with a reconstituted membrane. The residual amount of bile salts in the complexes may be the only regulation in the process of the membrane formation. The role of cytochrome *c* on the reconstitution is not clear but it is certain that water soluble cytochrome *c* binds to the reconstituted membrane and that the affinity to the complex is fairly firm. The binding of the cytochrome *c* to the complex may be an electrostatic conjugation between the phospholipids and basic groups of cytochrome *c*. For that reason this binding is quite different from the aggregation of the complexes.

*Membrane formation and enzymatic activity.* It is natural for the enzyme system of the membrane that the molecules or complexes, which are themselves constituent of the membrane, should be organized according to some fixed order. In such concepts many models have been offered on the molecular structure of the biological membrane. McConnel *et al.*

(10) reported that cytochrome oxidase activity of the complex IV was enhanced by membrane formation. TZAGOLOFF *et al.* (12) indicated that both NADH-oxidase and succinic-oxidase were activated by the membrane constitution in the presence of cytochrome *c* as a mobile component. $Q_6H_2$-oxidase activities of the reconstituted membranes were active in Mb. (III+IV)+*c*, and Mb. (III+IV+*c*), and less active in Mb. III+Mb. (IV+*c*.). As a matter of course $Q_6H_2$-oxidase activity was inactive in the absence of cytochrome *c*. In the case of Mb. III+Mb. (IV+*c*), however, activity was very low, though there was cytochrome *c* in the membranous complx IV. These observations led us to interpret that the electron transfer from $Q_6H_2$ to oxygen through complex III, cytochrome *c* and complex IV is most effective in the case of membrane formation in which complexes are arranged next-to-next to mobile cytochrome *c* as a mediator. $Q_6H_2$-cytochrome *c* reductase activity of the membrane reconstituted from complex III and complex IV was almost equal to the membranous complex III. Furthermore the activity was proportional to the protein weight of complex III. These facts indicate that the active site of the complex III is not concealed by the membrane formation, and that the enzymatic active site and hydrophobic group of the complexes are located apart from each other. On the other hand, cytochrome oxidase activity was decreased by the membrane formation from complex III and complex IV. The fact suggests that the active site in the conjugation of complex III and complex IV, of the complex IV may be covered or modified by the complex III. On this point, however, more studies must be carried out. In the assay of $Q_6H_2$-oxidase activity, $Q_6$ easily aggregates into micelleous structure because of the insolubility in an aqueous solution, and it must be recognized that $Q_6$ may not fully play as a substrate. In contrast, cytochrome *c* is soluble in water. Such a water-soluble and insoluble factors make the reaction more complicated, and makes it difficult to analyze $Q_6H_2$ oxidase activity. This leaves many problems to be resolved about these reaction systems. According to the observations described above, it may be concluded as follows; each unit of the enzyme, which is itself the constituent of the membrane, must be organized next-to-next in one single membrane, especially in the case of coupling reactions, in order to accomplish the full activities of the system. The reorganization of solubilized components into the membrane, however, is not destined in favor of the overall enzyme reaction, but only by random force of hydrophobic groups of the components.

*Relationship between the natural mitochondrial membranes and the reconstituted membranes.* There has been a vast accumulation of information about the

molecular structure and electron transfer function of the inner membrane of mitochondria. We reported (8, 9) previously that, when the mitochondrial membrane was treated by bile salts, the surface of the membrane revealed various arrangements according to the concentration of the bile salts used for the treatment. These membranes were reported and named as "brown membrane" and "green membrane" (15, 16). The particulate structure of the membrane was closely related to the particle of the electron transfer complexes which were solubilized and purified by the treatment of bile salts. In other words, the particle of the isolated complex III was very similar to the particulate structure on the surface of the "brown membrane" which contained all the electron transfer components except oligomycin sensitive ATPase. The complex IV was similar to the particulate pattern of "green membrane" containing cytochrome oxidase at high concentration which was obtained on the process of purification of complex I+III and complex IV from the mitochondrial inner membrane. The structure of the brown membrane was arranged with uniform repeating units, measuring about 82 Å in diameter with center-to-center distance of about 100 Å (15). While the particulate structure of the "green membrane" revealed characteristic arrangement of the repeating unit measuring about 50—60 Å in diameter with center-to-center distance of about 70 Å (16). In the case of the membrane reconstituted by the complex III and complex IV (Mb. (III+IV)) the particulate structure seemed to be less clear and lacking in uniformity. But the reconstituted membrane seemed to have two kinds of particles measuring in center-to-center distance 70 Å and 110 Å respectively. This array of the particles corresponded to that of the membranous complex III and membranous complex IV. The feature of the surface of the reconstituted membrane from complex I+III and complex IV resembled the membrane from complex III and complex IV, but the disposition to the aggregation is stronger in the former than in the latter. By electron microscopic observation of the thin section specimen, the width of both of the reconstituted membranes was measured to be about 90 Å and that is approximately equal to the mitochondrial inner membrane. Since the protein weight of the complex III and IV occupies only about 25 % of the total protein of the inner membrane, it may be difficult to compare the structure of the reconstituted membrane with the inner membrane of the mitochondria directly. It may be concluded that the mitochondrial inner membrane is constituted of repeating units of the complexes of the electron transfer chain and some other lipoproteins all of which are arranged by something like a mosaic pattern (Fig. 13).

SUMMARY

In order to elucidate the molecular organization of mitochondrial inner membrane, biochemical and electron microscope observations were made on the formation of membrane structure and function by the purified complexes of the electron transfer chain of beef heart mitochondria. Purified complex III (CoQ-cytochrome *c* reductase) and complex IV (cytochrome oxidase) were soluble in the presence of bile salts. They were, however, aggregated to form membrane by washing out the bile salts. When the membranous complexes III and IV were mixed, both membranes were separate by density gradient centrifugation and the vesicle which contained both complexes could not be formed and $CoQH_2$-oxidase activity was hardly restored. When the mixture of the solubilized complexes III and IV were diluted to remove the bile salts, a membranous vesicle in which both complexes were assembled was formed. $CoQH_2$-oxidase activity was restored in accordance with the formation of the membrane. The membrane which contained any desired propotion of each complexes could be obtained. These facts indicate that the complexes of the electron transfer chain conjugate two-dimentionally each other and form the membrane to carry electrons from substrate to oxygen most efficiently.

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