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Studies on the reconstitution of the structure and function of the mitochondrial inner membrane. II. Dissolution and reconstitution of the mitochondrial inner membrane

Hideo Hayashi*

*Okayama University,

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Studies on the reconstitution of the structure and function of the mitochondrial inner membrane. II. Dissolution and reconstitution of the mitochondrial inner membrane*

Hideo Hayashi

Abstract

1) In order to study the molecular structure and electron transfer activities of mitochondrial inner membrane, dissolution and reconstitution of membranous structure and function of the inner membrane of beef heart mitochondria were carried out. 2) The inner membrane of mitochondria could be dissolved into some unit of particles 70-140 Å in diameter by the treatment with bile salts at the concentration 0.5 mg of deoxycholate per mg of protein, 0.5 mg of cholate per mg of protein and 74.5 mg of crystalline potassium chloride per ml of the suspension. 3) The dissolved unit particles readily reaggregated into a vesicular membrane simultaneously restoring over-all electron transfer activities by the removal of bile salts with dilution of the suspension.4) Isolated electron transfer unit particle fraction contammg all components of the electron transfer chain but no structural protein were soluble in aqueous solution due to some residual bile salts used in the preparation. The removal of bile salts by dilution led the dispersed particles to aggregate into membrane and restore their over-all enzymatic activities. 5) From these results and the results of the reconstitution of membrane from purified complexes as described in the previous paper, it may be concluded as follows: The mitochondrial inner membrane may consist of several kinds of repeating unit particles conjugating each other with adjacent particles. It is necessary for over·all enzymatic activities that some unit components aggregate into a single vesicular membrane. Structural proteins may play an important role in the constitution of the membranous structure and in the over-all enzymatic activities.

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

II. DISSOLUTION AND RECONSTITUTION OF THE MITOCHONDRIAL INNER MEMBRANE

Hideo HAYASHI

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

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In order to clarify the molecular structure of the mitochondrial inner membrane, we described in the previous report (1) about the formation of membranous structure and the restoration of over-all enzymatic activity by the purified complex III and complex IV of the electron transfer chain. Each of the complexes or the mixture of the complexes could form vesicular membrane with over-all enzymatic activity, but these are not all of the constituents of the mitochondrial inner membrane. Now, there arises a question what would be the role of the other proteins. The electron transfer components occupy 50 % of the total protein of the inner membrane of mitochondria and the remaining 50 % are said to be mainly structural protein (2). The isolation and properties of structural protein have been reported (3—5), but the role of structural protein as the constituent of the membrane has not been made clear (6). Biological membrane is said to be consisted of three main components; (a) protein with catalytic action, (b) protein holding membranous structure without catalytic action and (c) phospholipids or the other lipids. The mitochondrial inner membrane consists of all these components and the membrane has the activities of multi-enzyme systems as distinct markers. It is extremely important for the studies of the molecular structure of biological membrane to clarify the correlation of the electron transfer activities with the membranous structure in the mitochondrial inner membrane.

In order to resolve this problem, the author carried out the dissolution of inner membrane by the treatment with bile salts, and then the reconstitution of membranes with over-all enzymatic activities was carried out by the removal of bile salts.

MATERIALS AND METHODS

Beef heart mitochondria were isolated by the method described by CRANE *et al.* (7) with slight modification in the preparative medium of 0.25 M sucrose containing 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.4).

Mitochondrial inner membrane (electron transfer particle: ETP) was prepared by the treatment with sonic oscillation of beef heart mitochondria as described previously (1).

Solubilization of the membrane

ETP was suspended in 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4) (sucrose-Tris) at the protein concentration 20 mg per ml., 0.5 mg of deoxycholate (as 10 % solution, pH 8.0 adjusted with KOH) per mg of protein, and 0.5 mg of cholate (as 20 % solution, pH 8.0 adjusted with KOH) per mg of protein were added and quickly mixed, then 74.5 mg of crystalline KCl per ml of the solution was added. After complete dissolution of crystals, the solution showing optical clearness was centrifuged at 140,000×g for 60 minutes (Spinco Model L2 Rotor 50, at 45,000 rpm), and the clear supernatant was used for solubilized mitochondrial inner membrane (Sol. ETP). A small amount of dark pellet was not always obtainable.

Unit of solubilized electron transfer (ETUP)

ETUP was prepared by the method described by BLAIR *et al.* (8). The "floating layer" obtained by the procedure of preparing the so-colled unit of electron transfer was suspended in sucrose-Tris at the concentration 20 mg of protein per ml (Sol. ETUP).

Reconstitution of membranes.

Solubilized ETP or solubilized ETUP was diluted with sucrose-Tris to the concentration 0.5 mg of protein per ml and incubated at 0-4°C for 60 minutes. Then the diluted solution was centrifuged at 105,000×g for 60 minutes (Spinco Model L2 Rotor 30, at 30,000 rpm). The brown pellet was suspended in sucrose-Tris at the concentration 10 mg of protein per ml, which was used for the membrane reconstituted from Sol. ETP (MbETP) or that from Sol. ETUP (Mb. ETUP). To examine the effect of cytochrome *c* on the reconstitution, 54 mμ moles of cytochrome *c* were added to 1 ml of Sol. ETP or Sol. ETUP before dilution and the procedure of reconstitution was carried out as described above.

Reconstitution of oligomycin sensitive ATPase to the membrane

Solubilized ETP was dialysed against 10 mM Tris-HCl buffer (pH 7.4) for 60 minutes. Slight turbidity was seen during dialysis. Oligomycin sensitive ATPase (OSA particle) prepared by the method described by YAMAMOTO *et al.* (9), was added to the dialysate at the concentration 0.5 mg of protein per ml. The mixture was then diluted 30 times (its volume) with sucrose-Tris. After incubation for 60 minutes at 0-4°C, the mixture was centrifuged at 105,000×g for 60 minutes. The pellet was suspended in sucrose-Tris at the concentration 10 mg of protein per ml (Mb. (ETP+OSA)).

Sucrose density gradient fractionation and *assay of cytochromes* were carried out as described previously (1).

NADH-and succinic-oxidase activities

The activties were measured polarographically in the oxygen electrode (Gilson medical electronics oxygraph) (8). The oxygen contents of 2.0 ml of a suspension containing all the factors necessary for maximum activity were caluculated to be 0.83 μ atoms of oxygen at 37±1°C. The reaction medium containing 40 μ moles of phosphate buffer, pH 7.4, and 0.2 μ moles of EDTA, and 0.05-0.1 mg of enzyme protein were mixed and adjusted to 1.95 ml with the addition of deionized water. After 5-minute preincubation, the reaction was started by the addition of 0.05ml of 0.1 M NADH or 0.5 M succinate (pH 7.4). To test the effect of cytochrome *c* 0.02 ml of 1 % solution of cytochrome *c* was added to the reaction medium and adjusted to the final volume 1.95 ml and the reaction was started by the addition of substrate.

ATPase activity and oligomycin sensitivity were assayed by the method described by Seki *et al.* (10). Inorganic phosphate released was estimated by method of Takahashi (18).

Protein estimation was carried out by the biuret method of Gornall *et al.* (36).

Electron microscopic observation

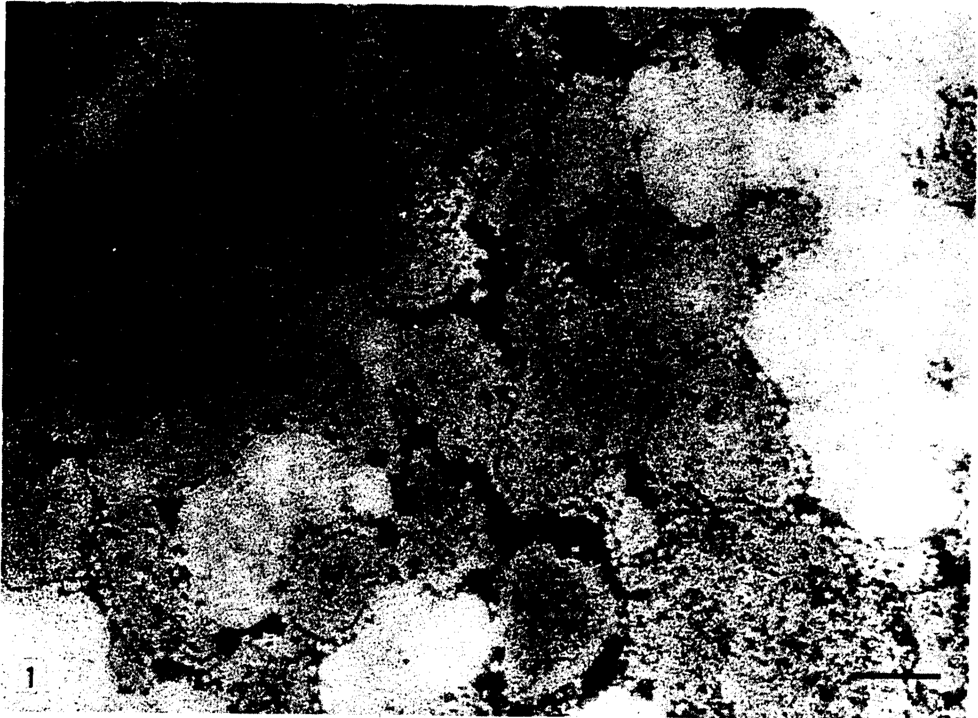
The specimens were examined by either negative staining with 1 % of PTA or $KMnO_4$-fixed positive staining.

RESULTS

Electron microscopy

The suspension of mitochondrial inner membrane became optically clear by the treatment of solubilization described in the methods. The membrane was completely solubilized and little amount of the pellet was obtained after the centrifugation at 140, 000×g for 60 minutes. In electron microscopic observation, Sol. ETP revealed isolated multiform particles, 70—140 Å in diameter. Some of the particles seemed to aggregate into granular structure and amorphous net-like structures (Fig. 2). There were several kinds of particles; such as isolated particles 100 Å in dimeter similar to head-pieces, OSA particles with unique structure of tripartite structure (head, stalk and base piece), rod-like petricles 70×30 Å in dimension, round-shaped perticles 90—120 Å in diameter and chain-like or netlike structure. But no vesiculer membrane could be found. This fact indicates that the membranes are completely dissolved into small unit of particles by the treatment with bile salts.

When the solution of dissolved membrane was diluted and then centrifuged, homogeneous brown pellet was obtained. The suspension of the pellet was visibly cloudy. Sonic oscillation of the suspension slightly decreased the cloudiness. The electron microscopic features of the suspension are shown in Fig. 3. There developed vesicular membranes 0.1—0.3 μ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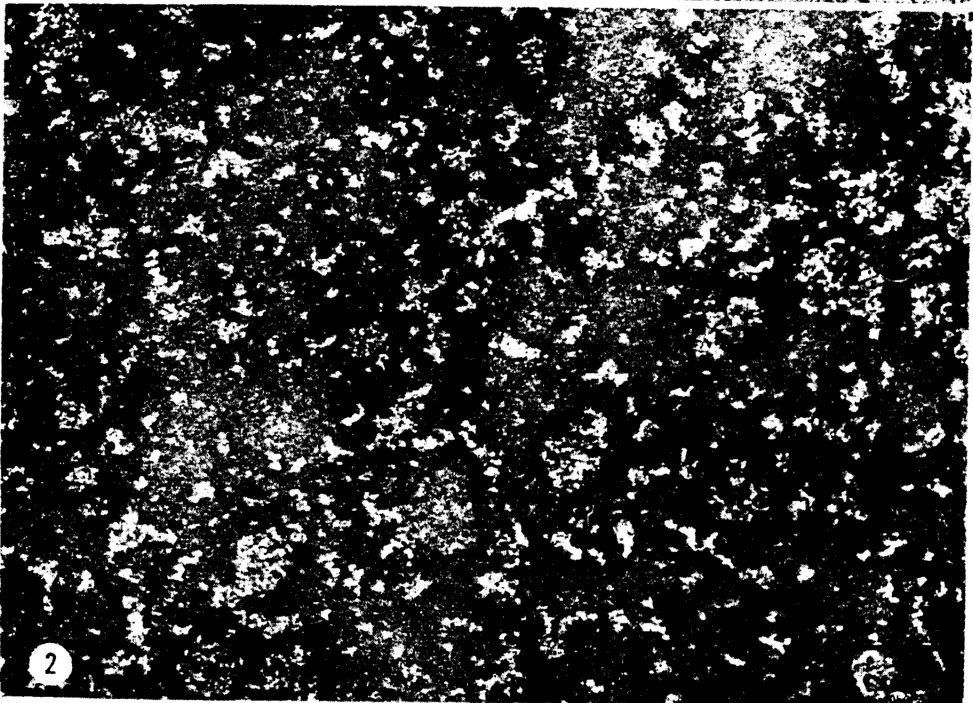
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in diameter in entire field of electron microscope. The surface of membrane showed of rather irregular particulate structure as compared with the membrane reconstituted from purified complexes. A few vesicles with the array of the head pieces could be found but not always. Apart from the vesicle, thread-like or rod-like aggregations connected with base pieces similar to OSA-particles (oligomycin sensitive ATPase particles (9)) could be seen. These features suggest that the membrane formation of electron transfer components and the aggregation of OSA particles differ in the manner of the reassembly. In other words, it is difficult for the electron transfer components and head pieces to organize in the same membrane by the direct dilution of the suspension of the solubilized membrane. In order to reconstitute the vesicular membrane with array of head pieces, an adequate amount of isolated OSA-particles was added to the solution at the transitional stage of the reconstitution of membrane obtained by dialysis of Sol. ETP as described in the methods above. The reconstituted membrane (Mb. (ETP+OSA)) is shown in Fig. 9. On the surface of the vesicle, where arranged head pieces about 110 Å in center-to-center distance. Mb. (ETP+OSA) with this feature coincides with the vesicles of ETP. By thin section specimen of Mb. ETP, there developed vesicular membrane with tri-lamellar picture about 90 Å in width, being also equal to the vesicle of ETP as shown in Fig. 4 and Fig. 5.

The fine structure of solubilized electron transfer components except structural protein (Sol. ETUP) is shown in Fig. 6. The feature seemed to resemble that of solubilized inner membrane. The particles varried in size and shape, being 70—110 Å in diameter, but neither net-like structure nor OSA-like particles could be found. Sol. ETUP also readily aggregates into vesicular membrane by the treatment of reconstitution.

The reconstituted membranes (Mb. ETUP) are about 0.1—0.3 μ in diameter and the surface of them seemed to be similar to that of Mb. ETP. The center-to-center distance measured 60—100 Å which was rather regular than Mb. ETP. Neither small aggregation of OSA-like structure nor the membrane arrayed with head pieces were seen.

Fig. 1 Vesicular membranes of mitochondrial inner membrane (ETP). Negatively stained with 1% of phosphotungustate pH 7.0. In this and subsequent plates the bar represents 1,000 Å.

Fig. 2 Dissolved mitochondrial inner membrane (Sol. ETP) by the treatment with deoxycholate, cholate and potassium chloride as described in the text. Negatively stained with 1% of PTA. Several kinds of particles and amorphous granular structure are shown.

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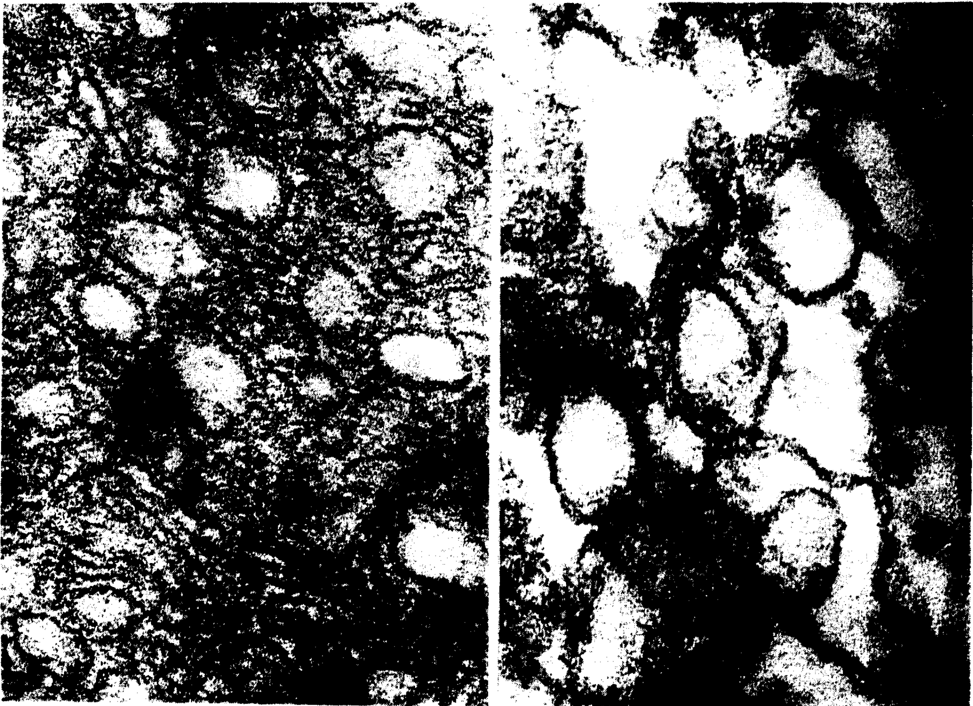

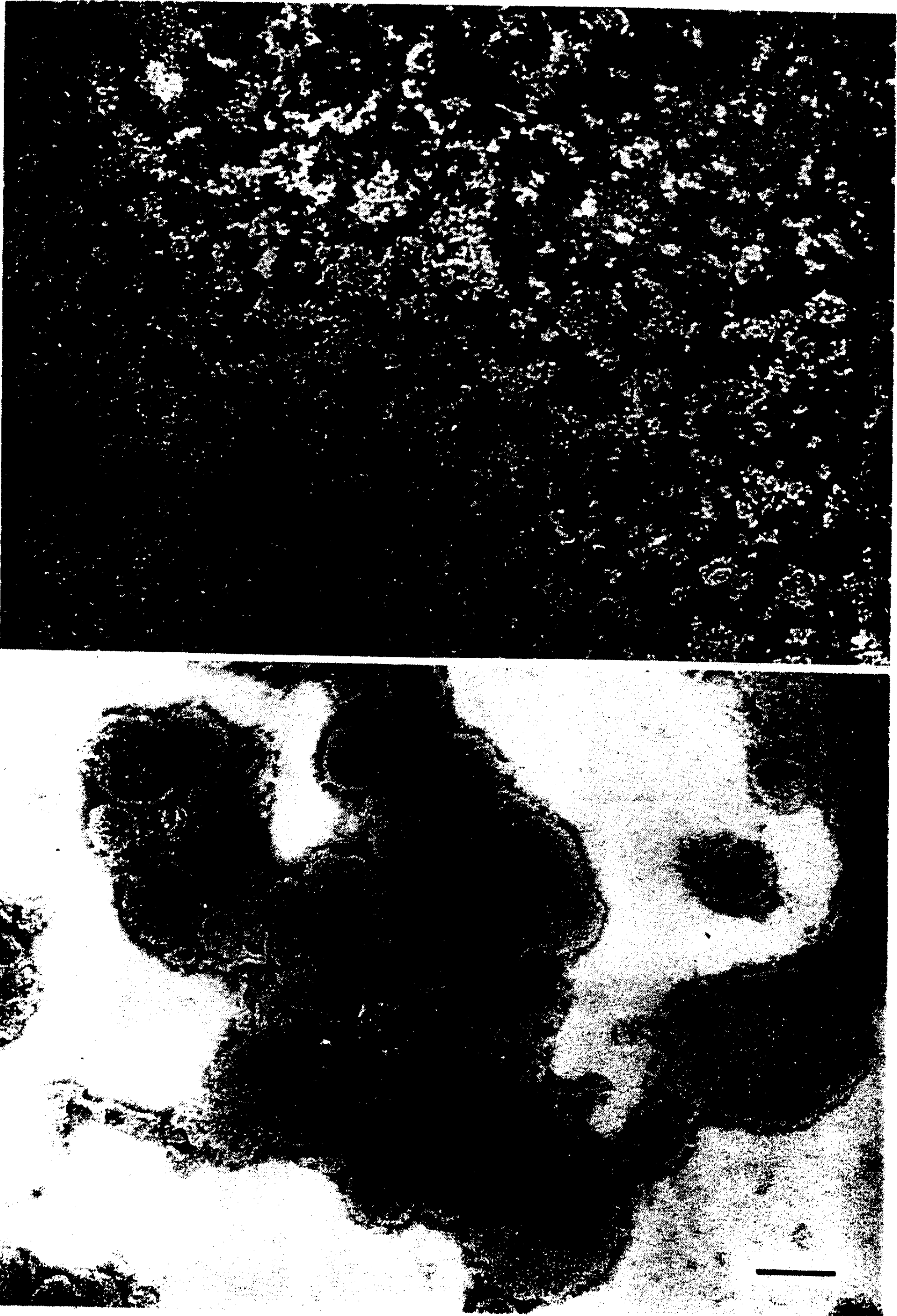

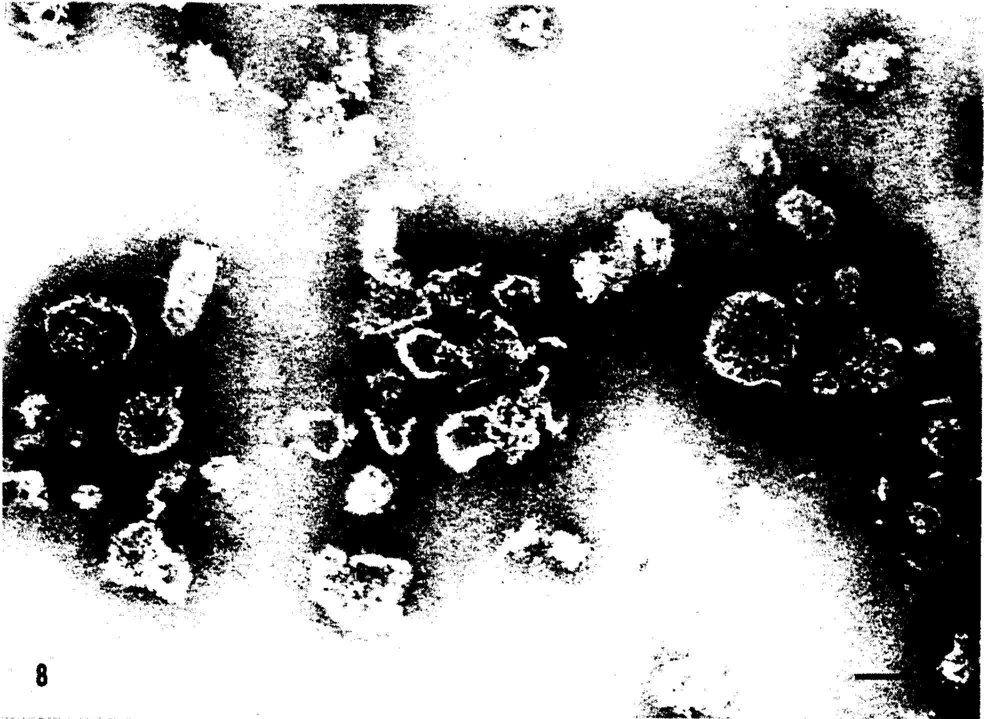
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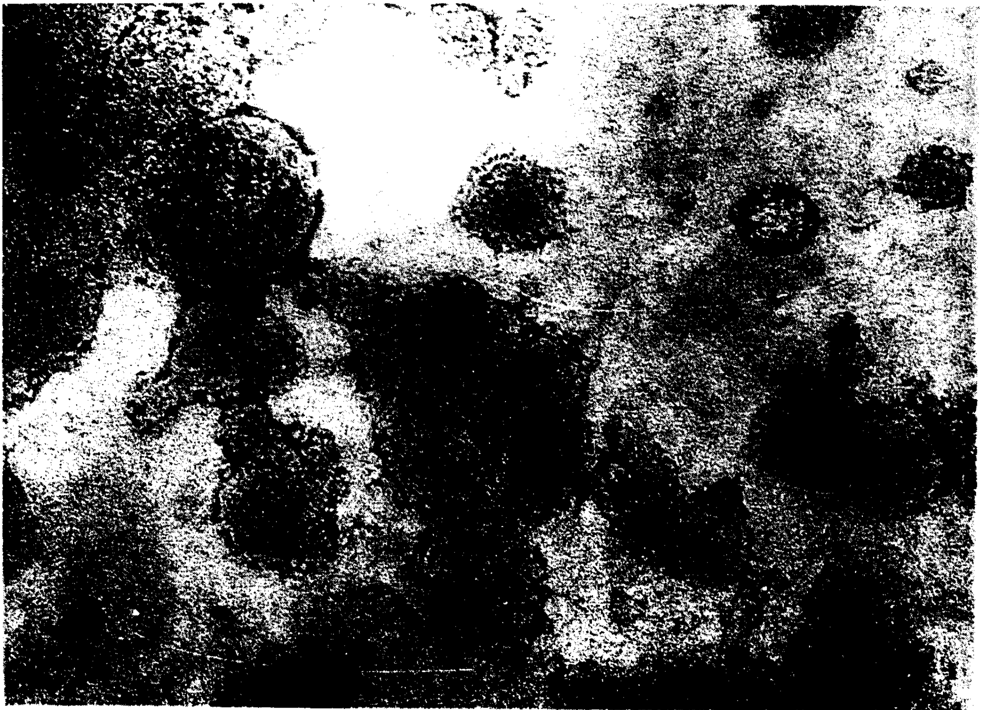

Sucrose density gradient fractionation

Since the components of the inner membrane differ from one another in their density, the components will separate into several bands on the sucrose density gradient centrifugation. In the reconstitued membrne, a if one kind of component aggregate into vesicle dominatly, the density of the vesicle will differ from the others. In consequence, the bands of gradient will separate into several densities. The results of the gradient centrifugation of original membrane (ETP), solubilized membrane (Sol. ETP), reconstituted membrene from Sol. ETP (Mb. ETP) and reconstituted membrane from Sol. ETUP (Mb. ETUP) are shown in Fig. 10. Original ETP was assembled into one band at the bottom of density 1.16, solubilized ETP separates into four bands; large amounts remaining at the top of the gredient and light colored bands being distributed at densities 1.14,

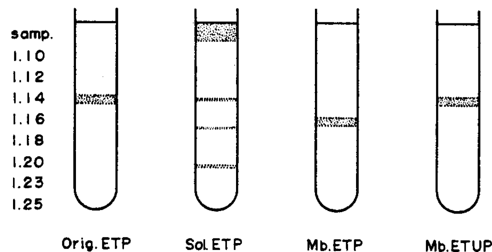

Fig. 10 Sucrose density gradient fractionation of the solubilized membrane and reconstituted membrane.

Fig. 3 The membrane reconstituted from the dissolved inner membrane of mitochondria. Negatively stained with 1 % of PTA. There develope vesicles. Dispersed particles can not be seen.

Fig. 4 Vesicular membrane of original inner membrane of mitochondria. Fixed by1.2 % of $KMnO_4$ in 0.02 M phosphate-buffer, dehydrated through a series of ethanol solution of graded concentation, embedded in Epon 812 and sectioned with a glass knife.

Fig. 5 The membrane reconstituted from dissolved inner membrane. Fixed with $KMnO_4$ as described above.

Fig. 6 Solubilized units of electron transfer. Negatively stained with 1 % of PTA. There revealed several kinds of particles and their aggregation.

Fig. 7 The vesicular membrane from solubilized components of the electron transfer chain. Negatively stained with 1 % of PTA. There develope vesicular membranes. Dispersed particles or aggregation of OSA-like particles cannot be found.

Fig. 8 Inner membrane disslolved under the condition for assay of enzymatic activity. The specimen for electron microscopic observation was prepared directly from the mixture of reaction medium with enzyme during the assay of NADH-oxidase activity.

Fig. 9 The vesicular membranes arranged by head pieces reconstituted from the mixture of membrane at the transitional stage of membrane formation with oligomycin-sensitive ATPase. Negatively stained with 1 % of PTA.

1.16 and 1.20 respectively. Mb. ETP and Mb. ETUP assembled into single band at density 1.16 and density 1.14 respectively. These facts indicate that all solubilized componets of inner membrane are organized homogenously into each vesicular membrane.

Cytochromes and protein recovery in the reconstituted membranes

Concentration of cytochromes and recovery of protein in the reconstituted membranes are shown in table 2. Sixty-two percent of protein of solubilized ETP was recovered into the reconstituted membrane (Mb. ETP). Cytochrome concentrations of the original ETP and Mb. ETP correspond to each other. The results show that small unit of solubilized particles of

Table 1 Concentration of cytochromes and recovery of protein in the membrane reconstituted from solubilized mitochondrial inner membrane

Reconstituted membranes	Concentrations of cytochromes*			Recovery of protein (%)
	a	b	$c+c_1$	
ETP	0.95	0.72	0.41	100
Mb. ETP	1.15	0.75	0.41	62
Mb. (ETP+c)	1.14	0.76	2.02	68
Mb. ETUP	1.52	1.12	0.63	65**

* Concentration of cytochromes is represented by mμ moles per mg of protein.
** The figure indicates recovery from solubilized unit of eleotron transfer.
ETP : Mitochondrial inner membrane.
Mb. ETP : The membrane reconstituted from dissolved inner membrane of mitochondria.
Mb. (ETP+c) : The membrane reconstituted from the mixture of dissolved membrane and cytochrome *c*.
Mb. ETUP : The membrane reconstituted from solubilized unit of electron transfer.

Table 2 NADH- and succinic-oxidase activities of dissolved inner membrane and rcconstituted membranes

	NADH-oxidase		Succinic-oxidase	
	+ none	+ cytochrome *c*	+ none	+ cytochrome *c*
ETP	1.16	1.67	0.42	0.81
Sol. ETP	0.58	1.25	0.15	0.43
Mb. ETP	0.60	1.45	0.24	0.79
Mb. (ETP+c)	0.99	1.33	0.48	0.76
Mb. ETUP	0.50	3.51	0.25	1.65

NADH-and succinic-oxidase activities are referred to μ atoms oxygen uptake per minute per mg of protein.
Sol. ETP : Dissolved or solublized inner membrane of mitochondria.
The other abbreviations are the same in Table 1.

inner membrane may reaggregate into vesicles homogenously by the drastic dilution of the solution. Cytochrome *c* added exogenously to Sol. ETP or Sol. ETUP, was organized also into the reconstituted membrane and did not inhibit the membrane formation of solubilized components.

Electron transfer activities of the reconstituted membranes

What is the correlation between the membrane formation and over-all enzymatic activities? In order to examine this problem, NADH and succinic-oxidase of the reconstituted membrane were assayed. In solubilized membrane, NADH- and succinic-oxidase were inactive without addition of exogenous cytochrome *c*, but on the addition of cytochrome *c* to the reaction medium the activities were detectable as shown in table 2. In the midst of the assay of enzymatic activity a portion of the reaction medium was examined in electron microscope after negative staining. In such medium, protein of Sol. ETP was contained 0.1 mg per 2 ml of the medium. There developed small vesicular membrane 0.1 μ in average diameter and several pieces of sheet-like membrane and rod-like aggregation of particles. Isolated particles were scarcely observed, but OSA-like particles and their aggregation were seen. These features led us to interpret the transitional stage of the reconstitution of the membrane.

Bovine serum albmin albmin (BSA) in high concentration (1—2 % of final concentration) inhibited the aggregation of the purified complexes, but it could not hinder the aggregation of the solubilized inner membranes. Cytochrome *c* in high concentration disturbed the aggregation of purified complexes, it had also no effect on the aggregation of Sol. ETP. The reconstitution of membrane could not inhibited by BSA or cytochrome *c*, in that way the electron transfer activities of Sol. ETP or Sol. ETUP were detectable in the condition of assay of activities where the dilution was attained enough to aggregation of solubilized components.

10^{-4}M KCN or 10^{-4}M NaN_3 inhibited the activities completely. The activities were also inhibited by deoxycholate at final concentration 0.075 % or by KCl at concentration of 37.5 mg per ml of medium. In such conditions, the reconstitution of membrane was attainable, and the original vesicular membranes were not disrupted.

NADH- and succinic-oxidase activities of Mb. ETP were fully restored with the addition of exogenous cytochrome *c*, to the same level of those of the original ETP. But it was inactive without addition of cytochrome *c*. The reconstituted membrane with cytochrome *c* (Mb. (ETP + c)) obtained by the mixture of Sol. ETP with cytochrome *c* was active without addition of exogenous cytochrome *c* though at slightly low level.

Sol. ETP and Mb. ETP showed high level of activities in the presence of cytochrome *c* just as in Mb. ETP and Sol. ETP. BSA or cytochrome *c* in high concentration did not inhibit the activities of Sol. ETUP. The concentration of cytochromes of Mb. ETUP is higher than Mb. ETP, because most of the structural protein was eliminated from ETP in case of ETUP, ETUP has more structural protein or core protein. In that way Sol. ETUP readily aggregate into membrane.

ATPase activities of the reconstituted membrane

ATPase activities of original ETP, solubilized ETP, reconstituted membrane from Sol. ETP and reconstituted membrane from the mixture of OSA with the membrane at transitional stage of reconstitution from Sol. ETP are shown in table 3. ATPase activity was detectable in all cases

Table 3 Adenosine triphosphatase activities of the membrane rcconstituted from dissolved inner membrane

Reconstituted membtanes	ATPase activities		Inhibition (%)
	+ none	+ oligomycin	
ETP	2.20	0.58	74
Sol. ETP	1.99	0.27	87
Mb. ETP	2.84	0.16	94
Mb. (ETP+OSA)	2.90	0.20	93

ATPase activities are expressed by μ moles Pi released per minnte per mgof protein.
Mb. (ETP+OSA): The reconstituted mcmbrane by the addition of oligomycin-scnsitive ATPase to the transitional membrane of Mb. ETP.
Thc other abbreviations are described in previous table.

and the values were almost equal. The results are coinsident with their structure, because in all of these cases OSA-like particles or structures were observed in electron microscope.

The structural and functional reconstitution of OSA particles in the membrane was accomplished in Mb. (ETP+OSA). In this instance, the membrane structure and respiratory function with ATPase are reconstituted corresponding to the original inner membrane of mitochondria.

The results described above may be summarized as follows:

1. The inner membane of mitochondria can be solubilized or completely dissolved into small pieces of several kinds of particles by treating with deoxycholate, cholate and potassium chloride.

2. The structural and functional reconstitution of the membrane can be accomplished by removing detergents and salts from the solubilized solution. But head pieces cannot be rearranged in the membrane by direct

dilution of the solution.

3. Solubilized components of all electron transfer chain obtained by removing the majority of structural protein can aslo reconstitute the membrane with full activities of electron transfer.

4. The membrane having head pieces with stalk and respiratory function with ATPase activity can be reconstituted by the addition of isolated oligomycin sensitive ATPase to the membrane at the transitional stage of reconstitution by the solubilized inner membrane components.

DISCUSSION

Regarding the molecular structure of the biological membrane two of main hypotheses have been offered. One is "theory of unit membrane" which explains that biological membrane may consist of tri-lamellar structure of protein-lipid-protein as described by ROBERTSON *et al.* (11) from the evidence of thin section specimens of membrane. The other is "theory of repeating unit" which states that biological membrane may be consisted of the mozaic organization of lipoprotein unit, as proposed by GREEN *et al.* (12) basing their opinion on the evidence of the reconstitution of membrane from partially purified electron transfer complexes. Now, discussions about this point will be given on the bases of the evidences obtained from this and previous communications.

Solubilization and reconstitution of inner membrane of mitochondria

The solubility of the components of inner membrane of mitochondria are different from one another at the concentration of bile salts used for treatment. In respect to these differeces many evidences have been reported as summarized in Table 4.

The head pieces of inner membrane could be detached from the membrane by sonic oscillation or high pressure by Nossal tube (16), and precipitated at high saturation of ammonium sulfate. Isolated head pieces, those are said to be coupling factor F_1, were soluble in water and could not form membranous aggregation. Oligomycin sensitive ATPase with triplet structure was solubilized from electron transfer membrane by the treatment with bile salts at low concentration (17). Salting out point for purification of OSA particle resembled that point of complex III (10). Purified OSA particles were readily connected with base pieces and formed ring-like structure which was quite different from electron transfer membrane (10).

Complex I (19) was solubilized from membrane at the same concentration of bile salts with complex III (20), but differed in the point of

Table 4 Key points of the solubilization, purification and reaggregation of the components of the inner nembrane of mitochondria

Components of inner membrane	Treatment for solubilization	Precipitation for purification	Feature of reaggregation
ATPase (coulping facor F_1) [16]	Nossal tube sonic oscillion	protamin 50% A. S. [c]	Water soluble. reconstitute to CF_0.
Oligomycin sensitive ATPase [9]	0.1 DOC[a] + 72 KCl[b]	0.5 DOC+16−29% A. A[d]	Thread-like polymer connected with base piece.
Complex I [2]	0.3 DOC+72 KCl	0.5 DOC+11−13.5 A. A further 0.4 Chol. +36% A. S	Not clarified.
Complex II [2.5]	0.3−0.5 DOC +72 KCl	0.3 DOC+10% A. S	Not clarified.
Complex III [5]	0.3 DOC+72 KCl	0.5 DOC+14.8−17 % A. A further 0.4 Chol. +47% A. S	Membrane with repeating unit about 90 A in center-to-center distance.
Complex IV [6]	0.5 DOC+ 74.5 KCl	0.5 DOC+16−18.7 % A. S. or 1 % Chol. +26−36% A. S	Membrane with repeating unit about 90 A in center-to-center distance.
structural [7] protein	2.0 DOC+1.0 Chol. +0.75SDS	2.0 DOC+1.0 Chol.+ 0.75 SDS+12% A. S	Amorphous aggregation. No membrane formation.
ETUP [8]	0.3 DOC+0.3 Chol.+10% A.S.	0.3 DOC+0.3 Chol. +50 % A. S	Membrane with repeating unit.
Mb. ETP	0.5 DOC+0.5 Chol.+74.5 KCl		Membrane with repeating unit.

a) Addition of deoxycholate mg per mg of protein
b) Addition of potassiun chloride mg per ml of solution
c) Final saturation of ammonium sulfate
d) Linal saturation of ammonium acetate

salting out. The biochemical properties of complex I were made clear by HATEEI *et al.* (21), but morphological characterization has not been clarified. About complex II (22), morphological properties remain obscure. By the treatment with bile salts complex II seemed to combine with complex IV fairly firm (24, 23). The role of complex I and complex II on the constitution of membrane has not been clarified yet. However, it is true that complex I+III or complex II+III forms the membrane structure (13).

Complex III was solubilized from membrane by the treatment with deoxycholate at concentration of 0.3 mg per mg of protein and 72 mg of KCl per ml of the solution (23). There remained still a membranous structure which was rich in complex IV (23, 25). At the concentration for solubilization of complex IV, there was hardly any membranous structure left (26). In a few cases we obtained thin membranous or net-like structure

with heme *a* of low concentration (23).

Inner membrane of mitochondria could be dissolved into several pieces of units with bile salts as described above and also reported by FERNANDEZ-MORAN *et al.* (27). The treatment with cholic acid alone was not effective on the dissolution of the membrane. The treatment with deoxycholate alone did not dissolve membrane but all of deoxycholate, cholate and salts were necessary for the dissolution of membrane. For the preparation of ETUP, all of them were also necessary (8).

On the process of purification of each complex, much of insoluble proteins were eliminated. The greater was the removable of insoluble proteins, the higher was the solubility of the complexes, making it difficult to reaggregate into membranous structure. On the other hand, the insoluble proteins were not solubilized even by the addition of detergents at high concentration and they aggregate readily into amorphous net-like structure without formation of membrane (27). These insoluble proteins corresponded to structure proteins in some properties.

Characteristic membranes were reconstituted from purified complex III or complex IV or the mixture of them (1). Neither the structural protein alone nor the mixture with phospholipids constituted membranous struture (27). In the presence of electron transfer complex, however, they aggregated more readily into membrane with full activities than purified complex alone. These facts show that the structural protein may play an important role giving some hydrophobic group to enzymatic active components to hold the membrane firm.

Electron microscopic observation of thin section specimen revealed no difference between the reconstituted membrane and original membrane, both of which demostrated tri-lamellar image by the fixation of $KMnO_4$. The results suggest that the biological membrane shoud be consisted of some units of lipoproteins bound to each other hydrophobically (Fig. 11). But there remains a problem whether the solubilized units of membrane constitute the membrane in its intact form or not ; in other words, whether the particu-

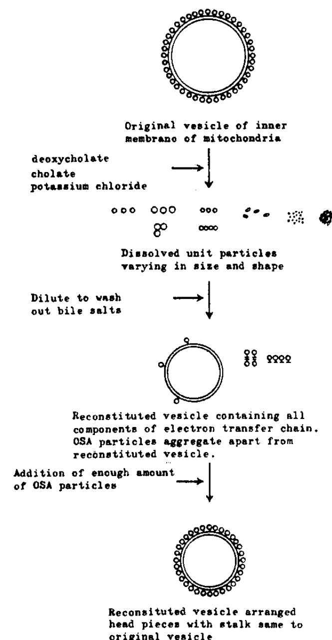

Fig. 11 Schematic illustration of the dissolution and reconstitution of mitochondrial inner membrane

late units apparing in electron microscopic observation aggregate into the membrane without modification of particle structure or with modification of submolecular conformational changes.

The reconstitution of head pieces to a membrane (CF_0) and simultaneous restoration of oligomycin sensitivity were reported by KAGAWA *et al.* (28). But in their study the relationship between ATPase and electron transfer membrane was not mentioned. In this communication, we described the reconstitution of membrane having both electron transfer activities and oligomycin sensitive ATPase activity.

Enzymatic activities and reconstitution of membrane

Reconstitution of NADH- or succinic-oxidase activity by purified complexes were reported by HATEFI *et al.* (29). TZAGOLOFF *et al.* (13) showed that the over-all enzymatic activities were accomplished by the membrane formation of the complexes with mobile factors of coenzyme Q and cytochrome *c*. But the membrane with activities of both NADH- and succinic-oxidase have not been reconstituted. In our studies, the membrane with both activities are reconstituted from the solubilized inner membrane. It is made clear that the reconstitution of the electron transfer activities corresponds to the reconstitution of the memane structure. But it must be emphasized that the activities were inhibited under the condition where membrane was reconstituted or membrane was not disrupted, as in the case with the medium containing 0.075 % of deoxycholate or 0.5 M of potassium chloride. These evidences seem to indicate that the reconstitution of membrane is one thing and the restoration of over-all enzymatic activities is another thing; that is to say, the reconstitution of membrane is neccesary but not enough for over-all enzymatic activity, and in order to accomplish over-all enzymatic activities much more delicate reorganization of components may be required.

The activity of the reconstituted membrane by complex III and complex IV was detected only at low level for the concentration of components (1). It was true with the reconstituted membrane by complex I+III and complex IV (13). On the other hand the activities of the membrane reconstituted from solubilized inner membrane restore almost fully to the level of original membrane. This fact suggests that the structural proteins are necessary to carry out the over-all enzymatic activities effectively.

Relationship of mitochondrial inner membrane to the other membrane

The inner membrane of mitochondria can be dissolved into some unit particles and the particles can be reconstituted reversibly into membranous structure. RAZIN *et al.* (14) reported that the cell membrane of *Mycoplasma*

laidlawii could be solubilized by the treatment with sodium dodecylsulfate showing mono-peak at the sedimentation pattern of analytical centrifugation 4.8 in its S value, and that the solubilized membrane could reconstitute membrane corresponding to original membrane by dialysis against hypotonic solution containing divalent ion Mg^{++} or Ca^{++} (15). MADDY (30) reported the solubilization of the plasma membrane of erythrocyte by the treatment with butanol. ZWAAL *et al.* (31) described also the solubilization of human erythrocyte membrane by the the treatment with n-pentanol.

These facts suggest that the cellular membranes should be constituted by some unit of particles conjugating with each other in the direction of the second dimension. GREEN *et al.* (12) regarded it a universal structure of biological membrane. In many biological membranes there are arrayed some uniform repeating units in electron microscopic observation; such as microsomes (32), liver cell membranes (33), ascites carcinoma cell membrane (34) and microvilli of intestinal epithelial cell (35) etc. The repeating units on the microvilli of intestinal epithelial cell were identified as disaccharidase and leucine aminopeptidase. But in other membranes the correlation of morphological unit to functional unit has not been elucidated. That is to say, it remains obscure whether or not the repeating unit of natural membrane is in itself the unit of activity and at the same time the unit of constituent.

The biological membrane may hold the key point of "life" itself. Therefore, it is an urgent problem to clarify the molecular structure and functions of the biological membrane along with the physical and chemical properties by the method of analysis and reconstitution.

SUMMARY

1) In order to study the molecular structure and electron transfer activities of mitochondrial inner membrane, dissolution and reconstitution of membranous structure and function of the inner membrane of beef heart mitochondria were carried out.

2) The inner membrane of mitochondria could be dissolved into some unit of particles 70—140 Å in diameter by the treatment with bile salts at the concentration 0.5 mg of deoxycholate per mg of protein, 0.5 mg of cholate per mg of protein and 74.5 mg of crystalline potassium chloride per ml of the suspension.

3) The dissolved unit particles readily reaggregated into a vesicular membrane simultaneously restoring over-all electron transfer activities by the removal of bile salts with dilution of the suspension.

4) Isolated electron transfer unit particle fraction containing all components of the electron transfer chain but no structural protein were soluble in aqueous solution due to some residual bile salts used in the preparation. The removal of bile salts by dilution led the dispersed particles to aggregate into membrane and restore their over-all enzymatic activities.

5) From these results and the results of the reconstitution of membrane from purified complexes as described in the previous paper, it may be concluded as follows: The mitochondrial inner membrane may consist of several kinds of repeating unit particles conjugating each other with adjacent particles. It is necessary for over-all enzymatic activities that some unit components aggregate into a single vesicular membrane. Structural proteins may play an important role in the constitution of the membranous structure and in the over-all enzymatic activities.

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