Acta Medica Okayama

Volume 21, *Issue* 2 1967 *Article* 4

APRIL 1967

Purification and fine structure of reduced coenzyme Q-cytochrome C reductase in the mitochondrial membrane

Shuji Seki* Osamu Hatase†

Takuzo Oda‡

*Okayama University,

†Okayama University,

‡Okayama University,

Purification and fine structure of reduced coenzyme Q-cytochrome C reductase in the mitochondrial membrane*

Shuji Seki, Osamu Hatase, and Takuzo Oda

Abstract

For the purpose of revealing the molecular organization of the mitochondrial membrane the authors attempted to clarify the fine structure of reduced coenzyme Q-cytochrome c reductase and also studied how the CoQH2-cyt. c reductase is arranged in the mitochondrial membrane by systematic analyses of fractions from the purification process of CoQH2-cyt. c reductase. 1. Purified CoQH2-cyt. c reductase contained high concentration of cyt. b (9.5 mμmoles per mg protein) and cyt. Cl (4.5 mμmoles per mg protein), and was almost free from cyt. c, a, flavoproteins, primary dehydrogenases and ATPase. The enzyme complex also showed a high specific activity (48 μmoles of cyt. c reduced per mg protein per min at 30°). 2. CoQH2-cyt. c reductase was composed of particles of about 120 Å in diameter with irregular form, some time exhibiting electron opaque cores. In the loose aggregates of the particles, the size of each particle was about 95 Å in diameter. 3. An intimate correlation was observed between the particles of CoQH2cyt. c reductase and those on the surface of the NADH-cyt. c reductase fraction. 4. Regular arrays of uniform particles (about 82 Å in diameter with a center to center distance of about 100 Å) were observed on the surface of the submitochondrial membrane (brown membrane) obtained from beef heart mitochondria by treatment with deoxycholate (0.1 mg / mg protein) and KCl (72 g/l). The correlation between these particles and CoQH2-cyt. c reductase was discussed.

---

*PMID: 4229456 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 21, 79—89 (1967)

PURIFICATION AND FINE STRUCTURE OF REDUCED COENZYME Q-CYTOCHROME C REDUCTASE IN THE MITOCHONDRIAL MEMBRANE

Shuji SEKI, Osamu HATASE, Hideo HAYASHI and Takuzo ODA

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

Received for publication, March 8, 1967

For the purpose of revealing the biochemical functions of mitochondria, it is important to know the molecular organization, especially how the energy transducing system is organized in the mitochondrial membrane. Up to the present time we have studied the correlation of ultrastructure to biochemical function in the inner membrane of mitochondria[1,2a,b]. The present communication deals with the purification of reduced coenzyme Q-cytochrome c reductase (QH_2-cyt. *c* reductase) from beef heart mitochondria and studies of its fine structure. A discussion will also be given on the structural arrangement of the QH_2-cyt. c reductase in the NADH-cyt. c reductase fraction and in the inner membrane of mitochondria.

MATERIALS AND METHODS

Preparation of QH_2 : QH_2 was prepared by reduction of coenzyme Q with sodium hydrosulphite according to the method of GREEN *et al.*[3].

Assay method for cytochromes : The concentrations of cytochromes were estimated from the difference spectra between reduced and oxidized forms. The extinction coefficients used were as follow : for cyt. b, $\varepsilon = 20\ mM^{-1}\ cm^{-1}$ ($\Delta_{562} - \Delta_{575m\mu}$) ; cyt, c_1, $\varepsilon = 19\ mM^{-1}cm^{-1}$($\Delta_{554} - \Delta_{540m\mu}$).

Protein determination : Protein was estimated by the biuret method of GORNALL *et al.*[4]. This method seriously over-estimates the protein content due to the contribution of cytochromes to absorbancy at 540 mμ. When the protein concentration was corrected according to the method of YONETANI[5], the values for the cytochromes are 1.25 times the values obtained by the direct analysis[6].

Assays of QH_2-cyt. c reductase, NADH-cyt. c reductase, NADH-ferricyanide reductase, and succinate-cyt. c reductase activities : Assay procedures of HATEFI *et al.*[7] for the enzymatic activities of NADH-cyt. c reductase ($NADH_2$: cytochrome c oxidoreductase, EC 1. 6. 2. 1) and succinate-cyt. c reductase (succinate : cytochrome c oxidoreductase, EC 1. 3. 99. 1) were used with a slight modification. QH_2-cyt. c reductase activity was assayed by the

method of GREEN and BURKHARD[5] with a slight modification. All these enzyme activities were assayed at 30°.

Purification of QH_2-cyt. c reductase: QH_2-cyt, c reductase was prepared by following the method of HATEFI *et al.*[6] with some modification. Beef heart mitochondria, prepared from slaughter house materials according to the method of CRANE *et al.*[8] with a slight modification, were suspended in 0.25 M sucrose, and stored several days at −20°. The mitochondrial suspension was thawed at room temperature, chilled to 0°, and centrifuged at 20,000g for 20 min. All subsequent operations were carried out at 0°—4°. The supernant fluid was discarded and the residue was suspended in a Tris-sucrose-histidine solution of the following composition : Tris-chloride, 0.05 M, pH 8.0, sucrose, 0.66 M, histidine, 0.001 M. The protein concentration of the suspension was adjusted to 23 mg per ml. Then potassium deoxycholate (DOC, 10% w/v, pH 10, 0.3 mg/mg protein) and solid KCl (72 g/1 of total suspension) were added while stirring. After the KCl was thoroughly dissolved, the suspension was centrifuged for 30 min at 105,000 g. In all centrifugal fractionations each fraction was described as (S_n, R_n) ; S, supernatant, R, residue, n, fraction No. The clear red supernatant fluid (S_1) was collected by decantation. A quarter volume of distilled water was slowly added to the S_1, stirring constantly and the suspension was centrifuged for 40 min at 105,000g (S_2, R_2). The clear supernatant fluid was collected and dialyzed against 0.01 M Tris-chloride, pH 8.0, for 3 hr. The dialysate thus obtained was centrifuged at 105,000g for 10 min (S_3, R_3). The brownish-red residue (R_3) was suspended in a small volume of Tris-sucrose-histidine solution and stored overnight at −20°. After thawing, R_3 was thoroughly homogenized in a glass homogenizer fitted with a teflon plunger, and diluted with Tris-sucrose-histidine solution to a protein concentration of 10 mg/ml. Then potassium deoxycholate (0.5 mg/mg protein) was added. The enzymatic activity of R_3 was determined after the addition of DOC. To R_3 already containing DOC, 16.4 ml per cent (v/v) of a 50% saturated solution of ammonium acetate (AmAc) was slowly added with constant stirring. After standing in an ice bath for 15 min, the suspension was centrifuged at 105,000 xg for 30 min, (S_4, R_4). The subsequent fractionations with ammonium acetate were conducted as mentioned above. To S_4 through S_9, the following ml percentages of 50% saturated AmAc were added respectively : S_4, 6.5 ; S_5 3.2 ; S_6, 3.2 ; S_7, 3.5 ; S_8, 3.5 ; S_9, 7.0 ; to yield R_5 through R_{10} and S_{10}. To S_{10} 7.0 ml % (v/v) of saturated ammonium acetate was added and the mixture was fractionated, (S_{11}, R_{11}).

Since it was difficult to adjust accurately the ammonium acetate solution to 50% saturation, these fraction fluctuated up or down slightly. For this reason it was necessary to observe carefully the color, solubility, and yield of each

fraction, and refer to the following fractionations. Prior to fractionations, 50% saturated solution of ammonium acetate was prepared from the stock solution of saturated ammonium acetate with crystals at 0°. The residue of each fraction was suspended in a small volume of Tris-sucrose-histidine solution and then used for the assays of various enzymatic activities and electron microscope observations. The remaining samples were stored at −20°. The R fractions gave a spectrum of solubilities in Tris-sucrose-histidine, with early R fractions being insoluble, later ones more soluble, and final ones above R_9 completely soluble in concentrated solution. When the residues were suspended in Tris-sucrose-histidine solution to a protein concentration of about 10 mg/ml, R_6 and R_7 were almost clear solution, while R_9, R_{10} and R_{11} were clear solution. R_9 and R_{10} were stored at 0° for several hours without any turbidity. R_9 and/or R_{10}, which contained highly active QH_2-cyt. c reductase, were suspended in Tris-sucrose-histidine solution to a protein concentration of 10 mg/ml. Neutral potassium cholate (10% w/v, 0.4 mg per mg protein) was added to the sample. Then ammonium sulphate fractionations were conducted as follows. Cold saturated ammonium sulphate (neutralized) was added to a concentration of 65 ml per 100 ml of protein suspension. After standing in an ice bath for 15 min, the cloudy mixture was centrifuged at 105,000 g for 10 min (R_{10}-S_1, R_{10}-R_1). To R_{10}-S_1, 6.9 ml of ammonium sulphate per 100 ml of supernatant was added, and the mixture was centrifuged as before (R_{10}-S_2, R_{10}-R_2). To the supernatant (R_{10}-S_2), 8 ml of ammonium sulphate per 100 ml of supernatant were added and the mixture was centrifuged (R_{10}-S_3, R_{10}-F_3). QH_2-cyt. c reductase (R_{10}-F_3) was collected near the surface of the liquid in the centrifuge tubes as a dark red, oleaginous material. Infranatant liquid was a clear, pale pink substance and no precipitate could be detected. The infranatant liquid was carefully withdrawn with a capillary tube and R_{10}-F_3 was dissolved in Tris-sucrose-histidine solution. R_{10}-R_1 was a light yellow substance which partially dissolved in Tris-sucrose-histidine solution. R_{10}-R_2 was a light pink substance which completely dissolved in Tris-sucrose-histidine solution.

Preparation of NADH-CoQ reductase : The R_7 which was obtained from previous fractionations was the most active preparation of NADH-cyt. c reductase. The fraction was used for the preparation of NADH-CoQ reductase according to the method of HATEFI *et al.* [9].

Electron microscopy : Negative staining, especially floating method described by ODA[10], was used. A droplet of the samples suspended in the Tris-sucrose-histidine solution was spread on the surface of a grid coated with a thin film of collodion. After a few minutes, excess fluid was removed with filter paper and the grid was immediately floated on the surface of a drop of 1% potassium phosphotungstate, pH 7.0. After some tens of seconds the grid was

picked up, excess fluid drained off with filter paper, and dried in a vacuum dessicator. After coating the specimens with carbon, they were examined in the JEM 7 electron microscope. Micrographs were taken at 80 KV on Fuji film (FG), at an electron optical magnification of 40, 000 to 50, 000.

RESULTS

Protein recovery and enzymatic activities of each fraction are illustrated in Tables 1 and 2. These tables also give an outline of color change during the

Table 1 Purifications of $NADH_2$-cyt. c reductase and QH_2-cyt. c reductase from beef heart mitochondria. About the purification-procedures and assay-methods for oxido-reductases refer to the text.

Fractions	Preparation precedures	Protein recovery (%)		Color of samples* G	Y	R	Oxido-reductase activities** $NADH_2$- Cyt. c reductase	$NADH_2$- Ferricyanide reductase	Succinate- Cyt. c reductase	Succinate- Ferricyanide reductase	Q_2H_2 cyt. c reductase
S_1	0.25 vol. H_2O	100		±	+	|||	3.6		0.04		
R_2		8		|||		2.4		0		
S_3	Dialysis	30					1.5	20	0.12	1.3	
R_3	DOC & AmAc***	38	100	+	+	|||	3.7	78	0.18	5.7	
R_4	(7.1%)****		7.3	+	+	+					
R_5	(9.7%)		8.2		+	+					
R_6	(11.0%)		6.6		+	++	4.3	73	0	3.1	
R_7	(12.3%)		11.9	+	++	++	5.6	129	0	0	11
R_8	(13.5%)		7.8		+	|||	4.0		0	2.3	8.3
R_9	(14.8%)		5.4			||||	2.9	74	0.12	8.7	43
R_{10}	(17.0%)		10.4			||||		24	0.07	3.7	31
R_{11}	(29.0%)		1.3			++		0			

* Color of samples : G, green ; Y, yellow ; R, red ** Oxido-reductase activities are expressed as μmoles of cyt. c or ferricyanide reduced per mg protein per min at 30°. *** Addition of 0.5 mg deoxycholate per mg of protein in a protein concentration of 10 mg per ml and ammonium acetate in final saturation as indicated. **** PH of the solution must be checked and adjusted at 8.0 with 5N KOH after the first addition of AmAc.

preparation. The greater part of NADH-cyt. c reductase activity was recovered in R_3. The enzyme complex was purified during the procss of fractionation and the fraction R_7 shows the highest activity of the enzyme complex (5.6 μmoles of cyt. c reduced per mg protein per min at 30°) and also shows the highest activity of ferricyanide-cyt. c reductase (129 μmoles of ferricyanide reduced per mg protein per min at 30°).

The fraction R_9 shows the highest enzymatic activity of QH_2-cyt. c reduc-

tase (43 μmoles of cyt. c reduced per mg protein per min at 30°) as indicated in Table 1. R_{10} is next most active after R_9. The specific activity of this enzyme complex in R_7 is about one-third that of R_{10}. For this reason QH_2-cyt. c reductase was purified from R_9 and/or R_{10}. The purification process of QH_2-cyt. c reductase from the R_{10}, which showed the highest total activity and a high specific activity of the enzyme complex, is shown in Table 2. The preparation

Table 2 Further purification of QH_2-cyt. c reductase from the crude preparation of the enzyme complex (R_{10}) by the fractionation with ammonium sulphate in the presence of cholate.

Fractions	Preparation Procedures	Protein recovery (%)	Color of samples		Q_2H_2-cyt. c reductase*
			White tone	Red tone	
R_{10}	{Cholate, amm. sulf. **	100		##	20
R_{10}-R_1	(39%)	32.4	####	±	5.3
R_{10}-R_2	(43%)	28.4	#	##	21
R_{10}-F_3	(47%)	21.2		####	48

* QH_2-cyt. c reductase activity is expressed as μmoles of cyt. c reduced per mg protein per min at 30°.

** Addition of 0.4 mg cholate per mg of protein and ammonium sulfate in final saturation as indicated.

of purified QH_2-cyt. c reductase (R_{10}-F_3) thus obtained shows a high specific activity of the enzyme complex (48 μmoles of cyt. c reduced per mg protein per min at 30°) and is completely or almost completely free of NADH-dehydrogenase, succinic dehydrogenase, NADH-cyt. c reductase, succinate-cyt. c reductase, as well as cyt. c oxidase activity.

Composition of QH_2-cyt. c reductase: The cytochrome concentrations in the purified QH_2-cyt. c reductase (R_{10}-F_3), estimated from the difference spectra between the oxidized and reduced forms, were as follows: cytochrome b, 9.5 mμmoles; cyt. c_1, 4.5 mμmoles per mg protein. Cyt. c and cyt. a were absent. As has been previously mentioned, the QH_2-cyt. c reductase was completely free from succinate- as well as NADH-cyt. c reductase activities. The enzyme complex also does not show any ATPase activity.

Electon microscope observation: Electron microscope observations of materials from each step through the fractionations have been performed by negative staining. In this instance, the main findings with respect to QH_2-cyt. c reductase are presented. Fig. 1 is an electron micrograph of the highly purified QH_2-cyt. c reductase. The particles of the enzyme complex are about 120 Å in diameter and show somewhat irregular form, probably due to the orientation of the faces of the particles. In the same photograph a structure, which appears

to be a rod-like arrangement of the particles, can be seen in some places. It is difficult to see the particles of such structure in the intact mitochondrial membrane. The enzyme complex is observed also in an aggregated form as illustrated in Figs. 2 and 3. Fig. 3 is an electron micrograph of the enzyme complex which was frozen and thawed before sampling. In the aggregates, each particle shows an almost uniform structure (round or polygonal) and uniform size (about 95 Å in diameter). Some of these particles exhibit an electron opaque core. The fact that a transitional state between the isolated complex and the aggregated complex can be seen (as illustrated in Fig. 2), indicates that the isolated enzyme complex in Fig. 1 is the unit or building block from which the aggregates shown in Fig. 3 arise.

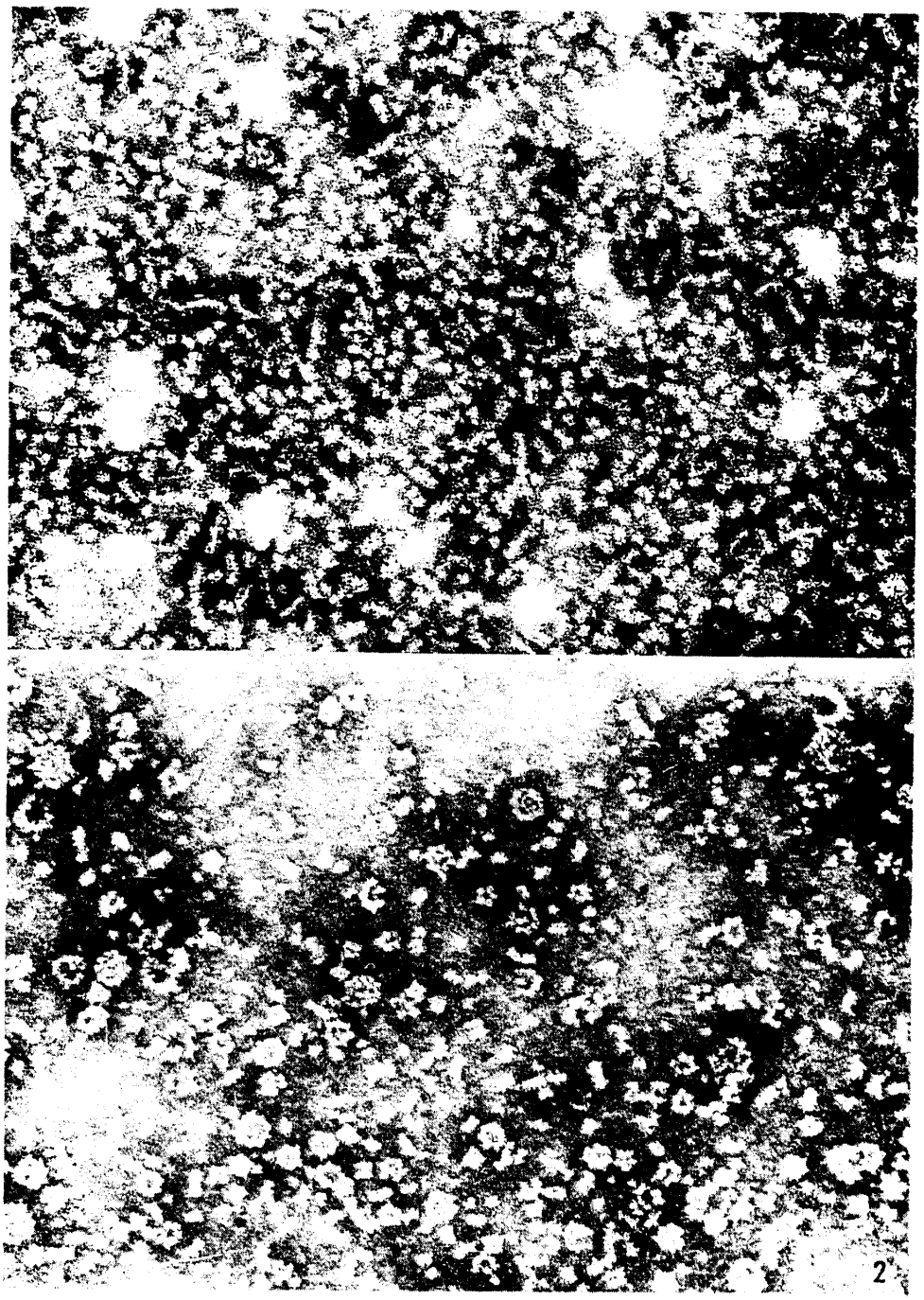
There are seen two major patterns in R_{10} (Fig. 4). The one is aggregates of particles which is similar to that of the QH_2-cyt. c reductase previously mentioned. The other is the structure in which several head pieces are arranged with stalks on thread-like base-pieces. As a result of systematic analysis through the process of purification of QH_2-cyt. c reductase from R_{10}, the former was collected mostly in R_{10}-F_3, while the latter was collected in R_{10}-R_1 and R_{10}-R_2. The pattern of R_9 is much the same as that of R_{10}.

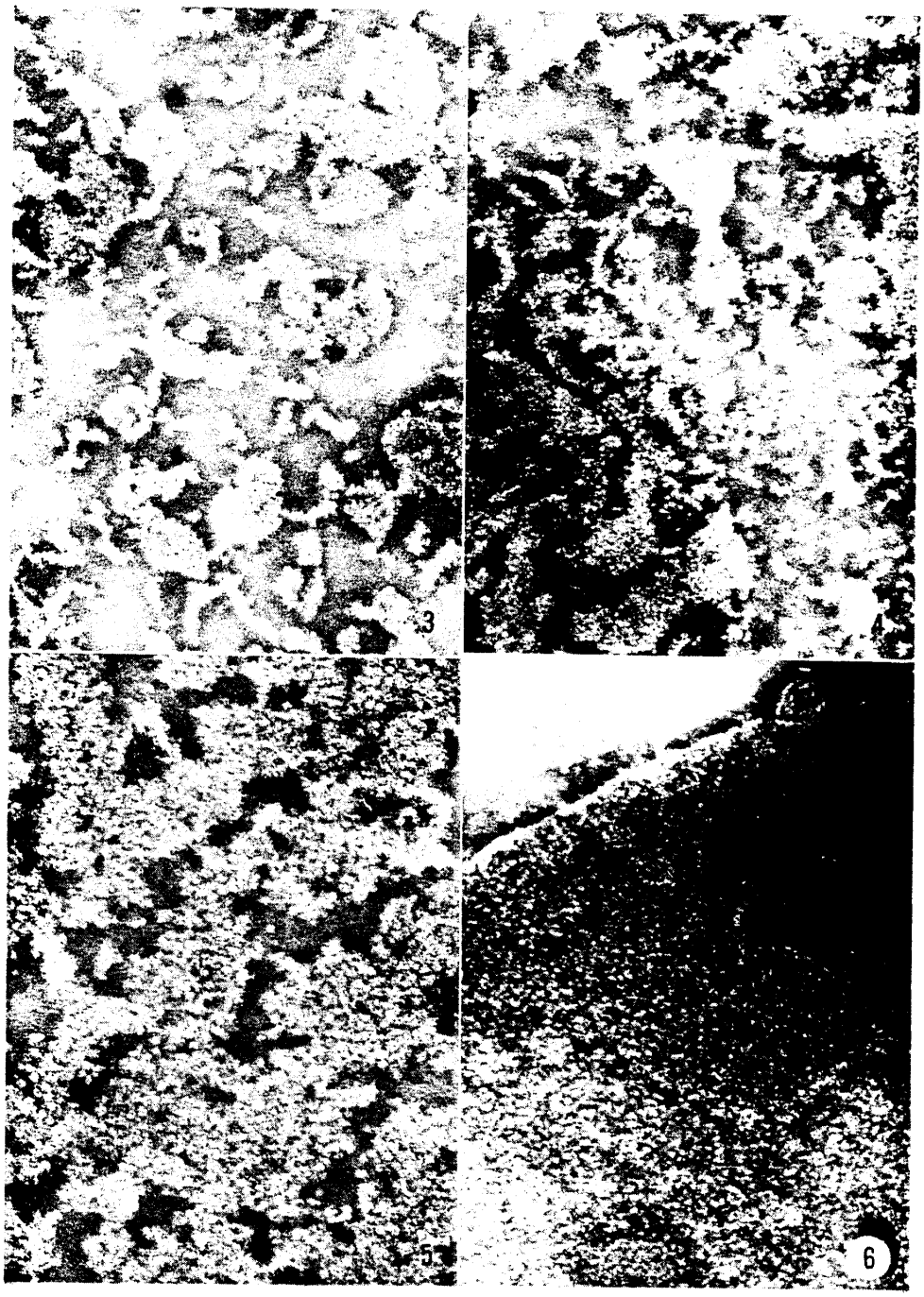
The major pattern of the fraction of NADH-cyt. c reductase (R_7) was similar to the aggregates of QH_2-cyt. c reductase (Fig. 5). When NADH-CoQ reductase and QH_2-cyt. c reductase from the fraction (R_7) were isolated and purified, the fine structure of the partially purified NADH-CoQ reductase exhibited thin membraneous fragments and filamentous structures, while QH_2-cyt. c reductase isolated from the fraction is the same as that previously mentioned. The fact that the pattern of NADH-CoQ reductase mentioned above could not be found in R_7 probably means that in R_7 the NADH-CoQ reductase is covered or intermingled with QH_2-cyt. c reductase.

It is important to know how the QH_2-cyt. c reductase is arranged and organized in the mitochondrial membrane. In practice, regular arrays of head-pieces covering the surface of ETP and the inner surface of the crista of mitochondria interfere the observation of QH_2-cyt. c reductase. When mitochondria were treated with deoxycholate (0.1 mg/mg protein) and KCl (72 g/l), the cristae were divided into two major components. One is a submitochondrial

Fig. 1 QH_2-cyt. c reductase negatively stained with phosphotungustate (PTA) immediately after purification from beef heart mitochondria. Most of the enzyme complexes have an isolated particulate form with average diameter of 120 Å. Final magnification was x140, 000.

Fig. 2 QH_2-cyt. c reductase purified and neagatively stained as described under the methods. The enzyme complexes have an isolated particulate form in some places and loosely aggregated form in other places. Between these two forms transitional states are observed. Final magnification was x112, 000.




3
6

membrane (brown membrane) containing electron transfer components and depleted of the head-pieces, stalks and the thread-like base-pieces. The other is a part of the elementary particle consisting of several head-pieces connected with stalks to thread-like base-pieces. On the surface of the former (brown membrane) possibly containing QH_2-cyt. c reductase and other terminal electron transfer components, there were observed regular arrays of small particles (about 82 Å) in diameter with a center to center distance of about 100 Å (Fig. 6). These particles might be the QH_2-cyt. c reductase as will be discussed later. Concerning the latter, possibly oligomycin sensitive ATPase, a report will be made in a separate paper.

DISCUSSION

As described previously, in order to understand the biochemical functions of mitochondria, it is important to study the molecular organization of the mitochondrial membrane. Concerning this problem, there are many reports[11,12,13,14,15,16]. However, up to the present few reports dealing with the arrangement or organization of the components of the respiratory chain of mitochondria are available[12].

As regards the molecular structure of QH_2-cyt. c reductase, FERNANDEZ MORAN *et al.*[12] reported that QH_2-cyt. c reductase appeared as an electron opaque series of particles approximately 40 to 50 Å in diameter. However, the electron micrograph presented in their paper did not show any precise structure of the enzyme complex nor any relation to the mitochondrial membrane. The photograph merely shows the pattern of the aggregated form of the complexes.

In the present paper the authors elucidated the molecular structure of the complex and studied how the complexes are arranged and organized in the mitochondrial membrane. Concentrations of cyt. b and c_1 of the QH_2-cyt. c reductase

Fig. 3 QH_2-cyt. c reductase which was frozen and thawed before negative staining. The enzyme complexes are observed mostly in an aggregated form. Final magnification was x112, 000.

Fig. 4 An electron micrograph of R_{10} (a crude fraction of QH_2-cyt. c reductase). There are seen two major patterns in this fraction. The one is aggregates of particles similar to that of the QH_2-cyt. c reductase. The other is the structure in which several head pieces are arranged with stalks on the thread-like base pieces. Final magnification was x112, 000.

Fig. 5 The fraction of NADH-cyt. c reductase (R_7) negatively stained with PTA. The major pattern of the fraction is similar to the aggregates of QH_2-cyt. c reductase. Final magnification was x140, 000.

Fig. 6 A submitochondrial membrane, brown membrane, obtained from beef heart mitochondria by treatment with DOC (0.1 mg/mg protein) and KCl (72 g/1). On the surface of the submitochondrial membrane, which are depleted of the head pieces, stalks and thread-like base pieces from the inner membrane of mitochondria, there are observed regular arrays of small particles (about 82 Å in diameter with center to center distance of about 100 Å). Final magnification was x112, 000.

presented in this paper are 9.5 mμmoles per mg protein and 4.5 mμmoles per mg protein, respectively, which are higher than the values reported by HATEFI *et al.*[6] Although it is difficult to compare the specific activity of the complex with those of others since the assay conditions are somewhat different, the fact that the complex shows a high specific activity suggests that the cyt. b and c_1 are in a complex form. As has been described above, the size and form of the purified enzyme complexes slightly differ from that of the particles in the aggregates, but a transitional state was observed between them. There are many possibilities to explain why the form of QH_2-cyt. c reductase changes in its isolation from that in the aggregates or in the mitochondrial membrane. The change could result from the difference of angles at which faces of the particles are observed, or that the particles are flattened by the surface tension during the process of negative staining. As has been described above, the particles observed in NADH-cyt. c reductase fraction may be considered to be QH_2-cyt. c reductase itself.

On the surface of the brown membrane obtained from mitochondria by treatment with DOC (0.1 mg/mg prot.) and KCl (72 g/1), electron microscope observation reveals regular arrays of particles, which frequently exhibit electron opaque cores. These particles are similar to those of NADH-cyt. c reductase and the aggregates of QH_2-cyt. c reductase, only except that they are a little smaller than those of QH_2-cyt. c reductase. When negatively stained particles are observed by electron micoscopy, in general, the particles arranged closely on the surface of a membrane are observed to be a little smaller than the same particles in the isolated form or in loose aggregates. The surface structure of the cytochrome oxidase-rich submitochondrial membrane[2a,2b], which was obtained from beef heart mitochondria after extraction of cyt. b, c, c_1 and NADH-dehydrogenase, distinctly differs from that of the brown membrane. These facts suggest that the particles on the surface of the brown membrane are QH_2-cyt. c reductase.

SUMMARY

For the purpose of revealing the molecular organization of the mitochondrial membrane the authors attempted to clarify the fine structure of reduced coenzyme Q-cytochrome c reductase and also studied how the $CoQH_2$-cyt. c reductase is arranged in the mitochondrial membrane by systematic analyses of fractions from the purification process of $CoQH_2$-cyt. c reductase.

1. Purified $CoQH_2$-cyt. c reductase contained high concentration of cyt. b (9.5 mμmoles per mg protein) and cyt. c_1 (4.5 mμmoles per mg protein), and was almost free from cyt. c, a, flavoproteins, primary dehydrogenases and

ATPase. The enzyme complex also showed a high specific activity (48 μmoles of cyt. c reduced per mg protein per min at 30°).

2. $CoQH_2$-cyt. c reductase was composed of particles of about 120 Å in diameter with irregular form, some time exhibiting electron opaque cores. In the loose aggregates of the particles, the size of each particle was about 95 Å in diameter.

3. An intimate correlation was observed between the particles of $CoQH_2$-cyt. c reductase and those on the surface of the NADH-cyt. c reductase fraction.

4. Regular arrays of uniform particles (about 82 Å in diameter with a center to center distance of about 100 Å) were observed on the surface of the submitochondrial membrane (brown membrane) obtained from beef heart mitochondria by treatment with deoxycholate (0.1 mg/mg protein) and KCl (72 g/l). The correlation between these particles and $CoQH_2$-cyt. c reductase was discussed.

ACKNOWLEDGEMENT

This research was supported by PHS research grant GM 10538, NIH, U. S. A. Our sincere thanks are due to Mr. T. ITAYA for his skillful technical assistance.

REFERENCES

1. ODA, T., SEKI, S. and HAYSHI, H. : *J. Electron Microscopy* 14, 354, 1963
2-a ODA, T. and SEKI, S. : Proc. of 6th Internatl. Congr. for Electron Microscopy, ed. by UYEDA, R. 2, 369, 1966
2-b ODA, T., SEKI, S., YAMAMOTO, G,, HAYASHI, H., HATASE, O., WAKABAYASHI, A. and KOSHIBA, K. : Proc. 18th Symp. Enzyme Chem, Sappro, p. 136—138, 1966 (in Japanese)
3. GREEN, D. E. and BURKHARD, R. K. : *Arch. Biochem. Biophys.* 92, 312, 1961
4. GORNALL, A. G., BARDAWILL, C. J. and DAVID, M. M. : *J. Biol. Chem.* 177, 751, 1949
5. YONETANI, T. : *J. Biol. Chem.* 236, 1680, 1961
6. HATEFI, Y., HAAVIK, A. G. and GRIFFITHS, D. E. : *J. Biol. Chem.* 237, 1681, 1962
7. HATEFI, Y., HAAVIK, A. G. and JURTSHUK, P. : *Biochim. Biophys. Acta* 52, 106, 1961
8. CRANE, F. J.., GLENN, J. L. and GREEN, D. E. : *Biochim. Biophys. Acta* 22, 475, 1956
9. HATEFI, Y., HAAVIK, A. G. and GRIFITHS, D. E. : *J. Biol. Chem.* 237, 1676, 1962
10. ODA, T. : Protein, Nucleic Acid, and Enzyme, 11, 73, 1966 (in Japanese)
11. FERNÁNDEZ-MORÁN, H. : *Circulation* 26, 1039, 1962
12. FERNÁNDEZ-MORÁN, H., ODA, T., BLAIR, P. V., and GREEN, D. E., : *J. Gell Biol.* 22, 63, 1964
13. STASNY, J. T. and CRANE, F. L. : *J. Cell Biol.* 22, 49, 1964
14. GREEN, D. E., WHARTON, D. C., TZAGOLOFF, A., RIESKE, J. S. and BRIERLEY, G. P. : In "Oxidases and Related Redox Systems", ed. by King, T. E., Mason, H. S. and Morrison, M. Vol. 2, 1032. John Wiley and Sons, Inc., New York, 1965
15. RACKER, E., TYLER, D. D., ESTABROOK, R. W., CONOVER, T. E., PARSONS, D. F. and CHANCE, B. : In "Oxidases and Related Redox Systems". ed. by King, T. E., Mason, H. S., and Morrison, M., Vol. 2, 1077. John Wiley and Sons, Inc., New York, 1965
16. KAGAWA, Y. and RACKER, E. : *J. Biol. Chem.* 241, 2475, 1966