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Abstract

1. A cytochrome oxidase-rich submitochondrial membrane (green membrane) was obtained from beef heart mitochondria after extraction of flavoproteins, cytochrome b, Cll C, etc. by treating with deoxycholate and potassium chloride. 2. The green membrane was formed by self assembly from the membrane fragments (flat sheets), which derived from the cristae membrane of mitochondria and had essentially the same particulate structure as the green membrane. 3. The green membrane exhibited regular arrays of small particles on the surface, measuring approximately 50 to 60 A in diameter with center to center distance of about 70 A. These particles sometime were arranged in a woven structure on the surface. 4. Both the configuration of the particles and the regularity of the arrangement were influenced by detergents and temperature. 5. Green membranes as well as beef heart mitochondria and electron transfer particles commonly retained membrane-structure after sonication and exhibited higher specific activity of cytochrome oxidase than that of purified cytochrome oxidase, if the activity is calculated on the basis of heme a concentration (sec1 / 10 m,lJ.moles of heme a/3 ml). The results suggest that the active sites of cytochrome oxidase are arranged on the surface of these membranes. 6. From these results and other experimental findings, an intimate correlation between cytochrome oxidase and the particles observed on the green membranes is suggested.

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CYTOCHROME OXIDASE IN A CYTOCHROME OXIDASE-RICH SUBMITOCHONDRIAL MEMBRANE

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Since the discovery of cytochromes by MACMUNN (1886) (1) in muscle tissue and its designation by KEILIN (1925) (2), cytochrome oxidase (E. C. 1. 9. 3. 1. cytochrome c: oxygen oxidoreductase), a component of cytochromes, was extensively studied by KEILIN and subsequent investigators. However, owning to the difficulties of its purification, the research was restricted on its spectral properties and kinetics in early days. Since the fifties of this century, studies on solubilization and purification of cytochrome oxidase were actively done, and at the end of the decade it became possible to obtain a reliable preparation of purified cytochrome oxidase and consequently to study not only the kinetics but also its molecular weight, compositions, and molecular structure in high resolution. Subsequently much knowledge has been gained by many investigators, but even now an important question still remains unanswered. Namely, whether the cytochrome oxidase is one enzyme, as proposed by OKUNUKI's group (3) and WAINIO's group (4), or two, as pointed out initially by KEILIN *et al.* (5), which has been supported by many investigators. Even for the purpose to solve the question, it is important to make clear the molecular structure of cytochrome oxidase.

Another important problem to be clarified on cytochrome oxidase is its arrangement or organization in the mitochondrial membrane in its functional states and resting state. Research for the organization of cytochrome oxidase in the mitochondrial membrane is valuable not only for the understanding of its biochemical function and molecular structure but also for clarifying structural and functional modifications of cytochrome oxidase occurring in the processes of the purification and the storage of cytochrome oxidase. Up to date some informations concerning molecular organization of cytochrome oxidase in the mitochondrial membrane have been obtained by indirect method, but little evidence has been presented by direct morphological studies. The studies must be done side by side with the studies on purified cytochrome oxidase. Up to present we have

studied the molecular organization of the mitochondrial membrane by the method of systematic microdisection followed by biochemical analysis and electron microscopical observations (6, 7, 8, 9).

The present communication deals with the purification procedures, biochemical properties, and molecular organization of a cytochrome oxidase-rich submitochondrial membrane (green membrane, GM) isolated from beef heart mitochondria by treating with deoxycholate and potassium chloride. As a result of the experiments the author was able to find regular arrays of small particles on the green membrane, which were intimately correlated with cytochrome oxidase. Correlation between the ultrastructure and enzymatic properties of the green membrane and its systematically degraded products by using ultrasonic oscillator or various detergents, was also studied.

MATERIALS AND METHODS

Mitochondria: Mitochondria were prepared from beef heart muscle by almost the same method as described in the previous paper (10), with the exception that in the present procedure layering process on a 0.34 M sucrose solution was avoided and crude mitochondrial fraction obtained by centrifuging the 700 x g supernatant at 7000 x g for 10 min was washed and purified 2~3 times with a 0.25 M sucrose solution.

Electron transfer particles (ETP): As reported in the previous paper (10), beef heart mitochondria (BHM) suspended in a 0.25 M sucrose solution were sonicated with an ultrasonic oscillator of Kaijo Denpa Co. Ltd. (7 ψ tip, 20 kc, maximum out put, 1 min per ml of mitochondrial suspension), and centrifuged at 26,000 x g for 10 min to remove unbroken mitochondria, and further centrifuged at 140,000 x g for 30 min. The resulting pellet, ETP, was suspended in a 0.25 M sucrose solution and stored at $-20°$ to be used.

Cytochrome oxidase-rich submitochondrial membrane: Early step of the purification of cytochrome oxidase described by FOWLER *et al.* (11) was modified. BHM or ETP were suspended in Tris-sucrose-histidine solution (Tris-HCl buffer, 0.05 M, pH 8.0; sucrose, 0.66 M; histidine, 0.001 M) to a protein concentration of 23 mg per ml. All subsequent operations were conducted at 0~4°.

Potassium deoxycholate (from 10 % w/v solution of pH 8.0, DOC) was added at a concentration of 0.3 mg/mg protein to the mitochondrial suspension (or 0.4 mg/mg protein to the ETP suspension). Then 72 g per 1 of solid KCl was added and the mixture was stirred until the KCl was thoroughly dissolved. The suspension was centrifuged for 30 min at 105,000 x g. The clear, red supernatant fluid was decanted. The residue consisted of three layers, fluffy, light colored top layer, green middle layer and dark brown bottom layer. The deep green, middle layer (GR_I), constituting the bulk of the pellet, was carefully

separated from the other two layers and suspended in the Tris-sucrose-histidine solution. When the separation of the green layer was well done and the concentration of heme *a* of the preparation became sufficiently high, the preparation needed no further purification. In case of need, the GR_1 fraction suspended in the Tris-sucrose-histidine solution by homogenization with a Teflon homogenizer was centrifuged at 105,000 x g for 20 min. After the clear supernatant solution having been decanted, the deep green, middle layer (GR_2) was carefully separated from the other two layers, and suspended in the Tris-sucrose-histidine solution by homogenization.

Since the fraction (sufficiently purified GR_1 or GR_2) exhibited a deep green color due to its high content of cytochrome oxidase and a characteristic membraneous structure by an electron microscopic observation, the component of the fraction was designated as cytochrome oxidase-rich submitochondrial membrane or green membrane (GM). As reported previously, the clear red supernatant contained high concentration of oligomycin-sensitive ATPase, flavoproteins and cyt. *b*, *c*, c_1, and from the clear red supernatant NADH-cyt. *c* reductase, QH_2-cytochrome *c* reductase, NADH-dehydrogenase and oligomycin-sensitive ATPase were purified (12, 13, 8, 9).

Even when the preparation conducted at the conditions described above, good preparation could not constantly be obtained, because the extent of extraction of cytochromes with deoxycholate and potassium chloride was critically affected by the properties of the mitochondria preparation (its purity and lipid content), protein concentration and the temperature and pH during the treatment. Accordingly, for the purpose to obtain constantly a highly purified preparation of green membranes, a test experiment was performed. Three tubes each containing 1 ml of mitochondria suspension (23 mg protein/ml) were added with 0.25 mg, 0.30 mg and 0.35 mg of DOC, respectively. After KCl (72 mg/ml) having been added further in each tube and thoroughly dissolved, these suspensions were centrifuged. Appropriate DOC-concentration for the preparation of the green membranes from the mitochondria suspension was decided from colors of the residue and supernatant of the test experiment.

Determination of cytochrome contents: Cytochrome contents of the sample obtained during the preparation of the green membranes were determined by the difference spectra between the reduced form and oxidized form as described previously (10). Concentrated suspensions of mitochondria or their subfragments were diluted with 0.1 M phosphate buffer (pH 7.4) to an appropriate concentration (for example, 2~3 mg protein per ml of beef heart mitochondria), and the diluted suspension was poured into three cuvettes, 3 ml in each. Base line was recorded with the voluntary two cuvettes. To one of the three cuvettes a bit of solid ferricyanide (a half of an ear pick) was added, to another one 0.02 ml of 1 M KCN, pH 7.4, and 0.03 ml of 1 M ascorbate, pH 7.4, and to the last one a bit of sodium dithionite (about 1 mg/ml). About ten minutes later, the difference spectrum of each of hydrosulfite reduced form versus ferricyanide oxidized form, hydrosulfite reduced form versus ascorbate reduced form and ascorbate reduced form versus ferricyanide oxidized form, was recorded with

EPS-3T form of the Hitachi auto-recording spectrophotometer or MPS-50 form of the Shimazu auto-recording spectrophotometer. The quantities of cyt. *a*, cyt. *b* and cyt. $c+c_1$ were calculated from the respective spectrum using millimolar extinction coefficient ($mM^{-1}cm^{-1}$) as follows: cyt. *a* 16 ($\triangle_{605}-\triangle_{630}$), cyt. *b* 20 ($\triangle_{563}-\triangle_{575}$), cyt. $c+c_1$ 19 ($\triangle_{553}-\triangle_{540}$).

Assay of cytochrome c oxidase activity: According to the assay procedure reported by SMITH (14) and modified by ORII *et al.* (15), oxidation rate of reduced cytochrome *c*, initial concentration of which was fixed at 15 μM (0.1 ml of 450 μM cytochrome *c* in 3 ml of the reaction mixture), was recorded as the decrease in the absorbance at 550 mμ with lapse of time using EPS-3T form of the Hitachi autorecording spectrophotometer.

Beef heart muscle cyt. *c* (Sigma Chemicals, Type IV) was dissolved in a sodium chloride-EDTA solution (0.1 % w/v sodium chloride, 0.001 M EDTA) at a concentration of 20 mg per ml. Cytochrome *c* was reduced by adding a few milligrams of sodium hydrosulfite to the solution, and excess sodium hydrosulfite and its degradation products were removed by passing the solution through a Sephadex G-25 column (coarse, 12 mm x 250 mm) which has been equilibrated with the sodium chloride-EDTA solution. Colored portion (reduced cyt. *c*) was collected, stored at 0° and used within 24 hr.

Concentration of cyt. *c* and reduction rate were determined spectrophotometrically using millimolar extinction coefficient of 18.5 mM^{-1} cm^{-1} ($\triangle_{550}$ mμ, reduced-oxidized). Total concentration of cyt. *c* was adjusted to 4.5×10^{-4} M by adding the sodium chloride-EDTA solution. Reaction medium containing 1.5 ml of 0.15 M phosphate buffer, pH 6.0, 1.4 ml of deionized water and 0.1 ml of 4.5×10^{-4} M reduced cytochrome *c*, was equilibrated at reaction temperature of 25°. Absorbance at 550 mμ was determined (phosphate buffer for a blank). The reaction was initiated by rapid mixing of 0.01—0.05 ml of an appropriately diluted sample with the reaction mixture and scanning was started simultaneously. The first order velocity constant, k (see^{-1}), was calculated from the equation of SMITH (14). The specific activity was expressed as k (sec^{-1}) per mg of protein per 3 ml of the reaction mixture (sec^{-1}/mg protein/3 ml).

Protein determination: Protein content was determined by the biuret method of GORNALL *et al.* (16) for the particulate preparations (BHM, ETP, GM, etc.) and a modification of the biuret method described by YONETANI (17) for purified cytochrome oxidase. When the protein content of the purified enzyme was determined by the biuret method of GORNALL *et al.*, the protein content was seriously overestimated due to the contribution of the cytochromes to absorbance at 540 mμ. In YONETANI's method the assay medium contained hydrogen peroxide (0.03 ml of 30 % H_2O_2 per 3 ml) besides biuret reagent for the purpose to destroy hemes.

Electron microscopic observation: Samples were negatively stained as described previously (12) using 1 % phosphotungustic acid adjusted to pH 7.0 with KOH. Some samples were routinely fixed with OsO_4 or $KMnO_4$, dehydrated, embedded in Epon 812, sectioned, and stained with uranyl acetate.

Specimens were observed and photographied with a HU-11C (Hitachi Ltd.)

or JEM-7 (Japan Electron Optics Ltd.) electron microscope. Micrographs were taken at 75 KV (HU-11C) or 80 KV (JEM-7) on Fuji film (FG), at an electron optical magnification of 40,000 to 50,000. The magnification was checked by simultaneous observation of Cu-phthalocyanin crystals.

RESULTS

Cytochrome contents of the green membranes : Cytochrome contents of the green membranes (BHM-GM) and various fractions obtained during the purifiction processes of the green membranes are illustrated in Table 1. The greater part of cyt. *b*, *c* and c_1 are extracted in the red supernatant (S_1), and the green residue (GR_1) contains a high concentration of cyt. *a* but a low concentration of cyt. *b* and cyt. $c+c_1$. Further purified green membranes (GR_2) contained about 2 mμ moles of cyt. *a* per mg protein.

Table 1. Cytochrome concentrations and recovery of cytochromes in the subfractions of beef heart mitochondria treated with deoxycholate* and potassium chloride**.

Sample	Protein (%)	Cyt. *a* mμmoles/ mg prot.	Recovery (%)	Cyt. *b* mμmole/ mg prot.	Recovery (%)	Cyt. $c+c_1$ mμmole/ mg prot.	Recovery (%)
Beef heart mitochondria	100	0.775	100	0.595	100	0.482	100
Green residue (GR_1) — Upper & middle layer	33.7	1.73	75.0	0.352	20.0	0.278	19.3
Green residue (GR_1) — Bottom layer	2.8	1.72	6.2	0.187	0.9	0.212	1.2
Red superuatant (S_1) — R_2	3.6	2.26	11.0	0.408	2.5	0.251	1.8
Red superuatant (S_1) — S_2	55.0	0.018	1.3	0.805	74.5	0.690	77.7

*, ** Concentrations of deoxycholate and potassium chloride are 0.3 mg/mg prot. and 72 g/l, respectively.

Table 2. Cytochrome concentrations and recovery of cytochromes in the subfractions of electron transfer particles treated with deoxycholate* and potassium chloride**. Electron transfer particles were obtained from beef heart mitochondria by severe sonication and differential centrifugation.

Fraction	Protein (%)	Cyt. *a* mμmoles/ mg prot.	Recovery (%)	Cyt. *b* mμmoles/ mg prot.	Recovery (%)	Cyt. $c+c_1$ mμmoles/ mg prot.	Recovery (%)
Electron transfer particle	100	1.55	100	1.57	100	1.01	100
Green residue (ETP-GR_1)	31.8	3.30	70.3	0.360	7.3	0.202	6.4
Red supernatant (ETP-S_1)	59.0	0.539	21.1	2.14	80.6	1.43	82.6

*, ** Concentrations of deoxycholate and potassium chloride are 0.4 mg/mg protein and 72 g/l, respectively.

Since ETP has already been concentrated to some extent in regard to cytochrome oxidase for the reason of extraction of soluble and sonically dissociable proteins, green membranes obtained from ETP (ETP-GM) contains higher concentration of cyt. *a* than that of BHM-GM as illustrated in Table 2. Heme *a* concentration of the green membrane (ETP-GM) was 3 to 4 mμ moles/mg prot., and the concentration of cyt. *b* or $c+c_1$ was as low as about 1/10 of the heme *a* concentration.

Fig. 1 illustrates the difference spectra between the hydrosulfite reduced form versus the ferricyanide oxidized form of cytochromes of the green membranes purified from beef heart mitochondria.

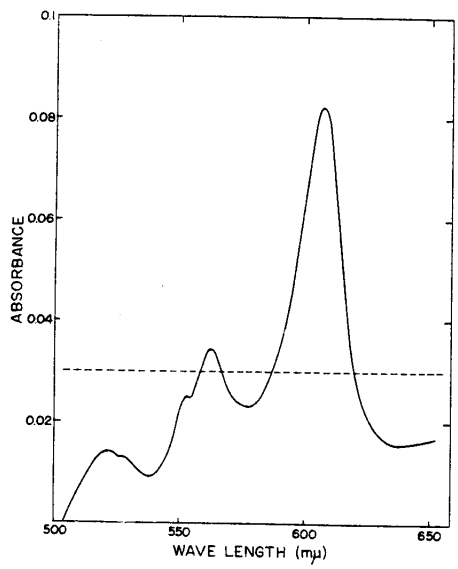


Fig. 1 Difference spectrum of cytochromes of the green membranes obtained from beef heart mitochondria by treatment with K-deoxycholate (0.3 mg/mg protein) and KCl (72 g/1). Concentration of cytochromes: cyt. *a*, 2.1 mμmoles/mg protein; cyt. *b*, 0.27 mμmole/mg protein.

Electron microscopic observation of the green membrane : When the purified green membranes negatively staind with PTA were observed on electron micrographs, membranes of various size exhibiting a folded-bag form were

Fig. 2 Cytochrome oxidase-rich submitochondrial membrane (green membrane, GM) obtained from beef heart mitochondria. On the surface of the membrane there are observed regular arrays of small particles, measuring approximately 50 to 60 Å in diameter, with center to center distance of about 70 Å. These particles are sometime arranged in woven structure as observed in the middle part of the photograph. PTA negative staining, × 193,000.

2

observed and the surface of the membranes presented a fine granular structure, as illustrated in Fig. 2. Fig. 3 illustrates a high magnification photograph of the green membrane distinctly presenting fine granularity. The small distinct particles observed on the surface of the green membranes have a diameter of 50 to 60 Å and these particles are regularly arranged on the surface with a center to center distance of about 70 Å. In some place these particles are arranged in a woven structure as observed in Fig. 2. In this instance the center to center distance between the two particles among neighboring particles was about 70 Å just as in the case described previously.

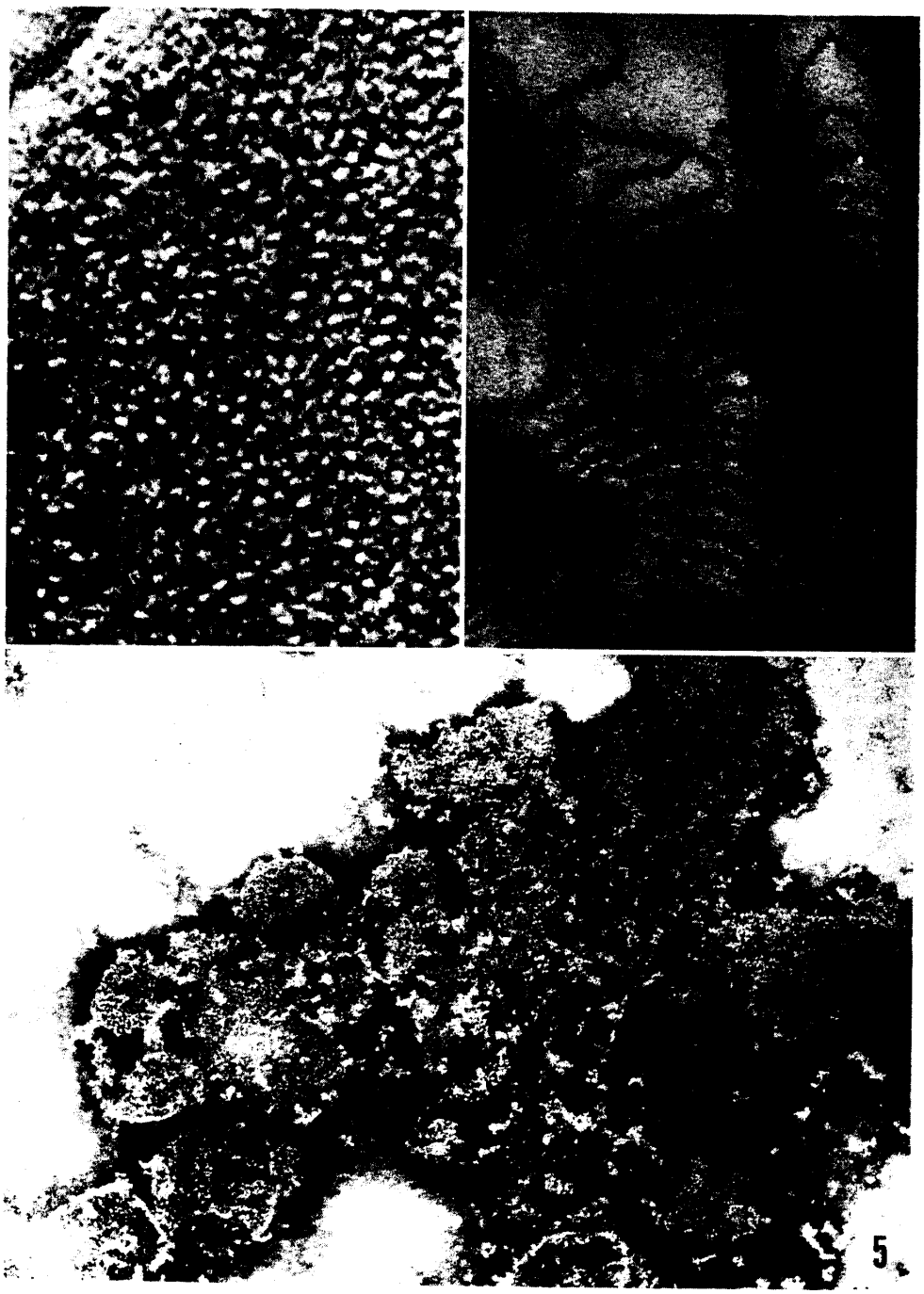
The green membranes, fixed with 0.5% $KMnO_4$, embedded in Epon 812 and sectioned with Porter Blum ultramicrotome, show a lammellar membrane structure as illustrated in Fig. 4 and in some place trilaminal figure was observed.

When the purification process of the green membranes was minutely examined, it was revealed that the green membranes were not always directly derived from the cristae membrane of mitochondria, but they were formed by self assembly of relatively small membrane fragments derived from the cristae membrane. Namely, the BHM or ETP were treated with DOC (0.3 mg/mg prot. or 0.4 mg/mg prot.) and KCl (72 g/l), and the resulting suspension negatively stained before centrifugal fractionation was observed with the electron microscope. The sample revealed relatively small membrane fragments and dispersed small particles (Fig. 5). The small membrane fragments were much smaller than the green membranes and were not vesicles but sheets having irregular margins. They presented the fine granular surface structure just as that observed on the green membranes. The dispersed small particles were removed in the red supernatant by centrifugation and the small membrane fragments were recovered in the green residue (GR_1). When the residue was suspended in the Tris-sucrose-histidine solution, large vesicles and sheets were formed by self assembly of the small membrane fragments due to dilution of DOC concentration with the Tris-sucrose-histidine solution.

Fig. 3 Higher magnification of a part of a green membrane. PTA negative staining, × 440,000.

Fig. 4 Green membrane fixed with potassium permanganate, embedded in Epoxy resin and sectioned with Porter Blum ultramicrotome. × 120,000.

Fig. 5 Beef heart mitochondria (23 mg prot./ml) dissolved with deoxycholate (0.3 mg/mg protein) and potassium chloride (72 g/l). Irregular form of sheets, exhibiting fine granular surface structure just as that of the green membrane, are observed. The green membtane is constructed of the sheets through the process of self assembly during the purification steps of the green membrane. PTA negative staining, × 108,000.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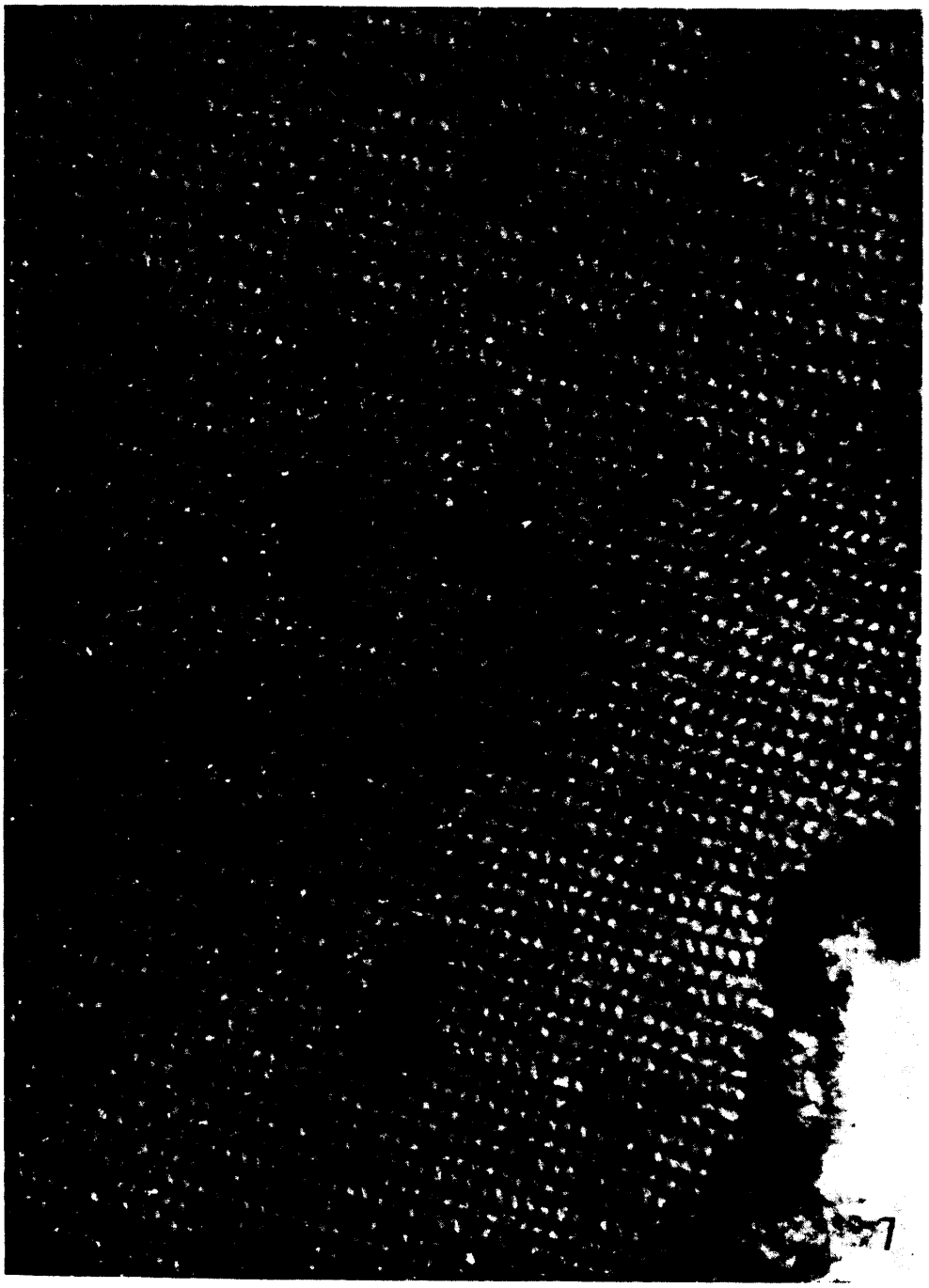
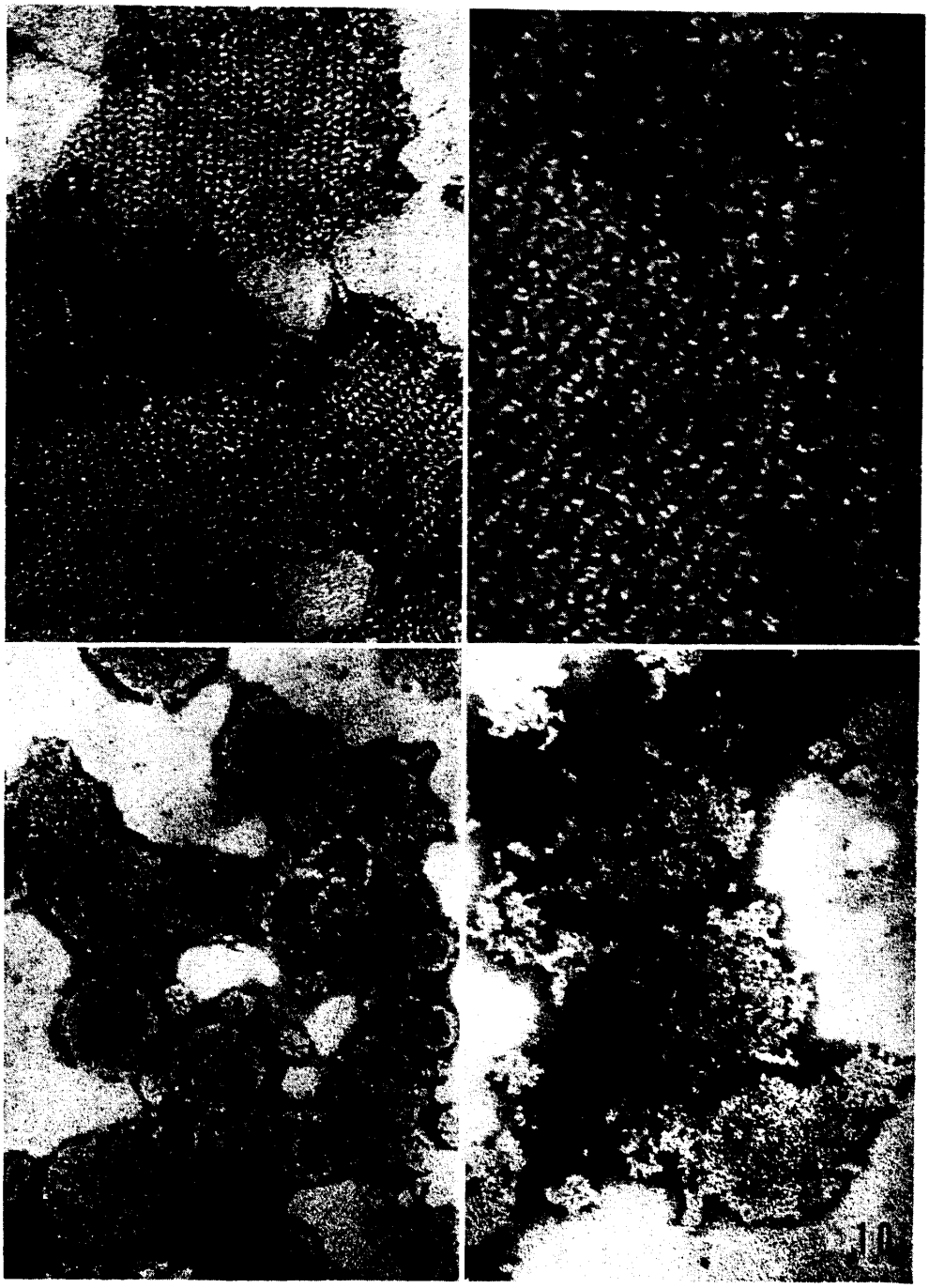
The membranes so formed are the green membranes and their fundamental structure is the same as the small membrane fragments derived from BHM or ETP.

Green membranes treated with DOC : The green membranes suspended in the Tris-sucrose-histidine solution were treated with deoxycholate of a relatively low concentration (0.3 mg DOC/mg prot. or 1 %) and centrifuged at 105,000 x g for 30 min. The supernatant was carefully aspirated, and the fluffy layer and packed pellet were suspended in a small volume of the Tris-sucrose-histidine solution. By these treatments cytochrome oxidase was not solubilized but recovered in the residue, and the structure of the green membranes was somewhat modified. Rounded membrane contour was lost and various sized sheets were formed. The sheets had fundamentally the same surface structure as that of the green membranes, but the small particles regularly arranged on the sheets were more distinct than that of the green membranes and the woven structure was more frequently observed. Center to center distance among neighboring particles is about 70 Å equally in x and y axes (warp and woof) in some cases as illustrated in Fig. 6, and is about 65 Å in x axis (warp) and 75 Å in y axis (woof) in the other cases as illustrated in Fig. 7. Even in the latter cases the mean distance in both axes is about 70 Å. As observed in Fig. 7, on the most part of warps there are observed regular arrays of small particles, but on some other part of warps the particulate structure is obscure. Both types of the warps are arranged mutually. It is clear that the particles observed on the green membrane are identical with the particles observed on the DOC-treated green membrane (sheet). Woven arrangement of the particles is more prominent on the DOC-treated green membrane than on the original green membrane. Frequently transitional forms are observed between them. Center to center distance of the particles are identical in both preparations. When the green membranes were treated with 1 % DOC, there were observed not only the sheets with woven structure, but also the sheets on which the particles were randomly arranged and from which some of them were detached (Fig. 8).

Cytochrome oxidase activity of the green membrane : As reported by SMITH *et al.* (18, 19) and others (20, 3), cytochrome oxidase activity is inhibited with anionic detergents such as cholate, deoxycholate, etc. For the purpose to reveal the relationship between the structure and function, it is neces-

Fig. 7 Sheets obtained from the green membrane by the same method as in Fig. 6. Small particles are regularly arranged in a woven structure. Center to center distance of warps and woops is 63 Å and 75 Å in an average, respectively. On a part of some warps particulate structure is obscure. PTA negative staining, × 380,000.





sary to obtain full activity of a preparation after removing the inhibitory effect of the detergents used during the preparation. It is well known that the inhibitory effect of cholate or deoxycholate on cytochrome oxidase activity can be overcome by the addition of non-ionic synthetic detergents such as Emazol, Tween and Triton (21, 22). For this purpose Tween 20 (polyoxyethylene sorbitan monolaurate) was used in the experiment. In addition, Tween 20 does not cause any morphological changes of the preparations such as destruction of a membrane structure. Making use of these properties of Tween 20, the relationships between morphology and enzymatic activity of the green membranes treated with ultrasonic oscillator and various detergents were investigated. Oxidase activity was determined by the addition of these pretreated samples (0.01～0.08 ml) in the reaction medium as described previously. At the same time samples were negatively stained and observed with an electron microscope. When the concentrated solution of the green membranes was diluted with distilled water, their morphological structure did not change and the specific activity was 0.48 sec^{-1}/mg prot. /3 ml as illustrated in Table 3. When the same preparation of the green membranes was sonicated by ultrasonic oscillator (mixmum output, 7ψ tip, 30 sec/ml) after dilution with distilled water, they were broken down to small membranes preserving essentially the same structure as the original membranes (Fig. 9). The specific activity of the sample was 4.4 sec^{-1}/mg prot. /3 ml, which was about ten times higher than that of the sample before sonication. The green membranes diluted with 0.25 % Tween 20—0.05 M phosphate buffer, pH 7.4, did not show any morphological change, but exhibited slightly higher specific activity (0.60 sec^{-1}/mg prot. /3 ml) than that of the green membranes diluted with distilled water. When the green membranes diluted with Tween 20-phosphate buffer were sonicated and diluted again with Tween 20-

Fig. 6 Cytochrome oxidase-rich sheets obtained from green membrane by treating with 1 % DOC. On the surface of the sheets the small particles identical to the particles on the green membrane are regularly arranged in a woven structure and some of them are released from the margin of the sheets. Center to center distance of warps and woops is equal, about 70 Å. PTA negative staining, × 201,000.

Fig. 8 A sheet obtained from green membrane by the same method as in Fig. 6. On the margin of the sheet, arrangement of the particles becomes loose and some of them are released from the sheet. PTA negative staining, × 490,000.

Fig. 9 Fraction of purified green membrane diluted with distillled water and sonicated with ultrasonic oscillator. Size of each membrane is markedly diminished, but membraneous structure is still preserved. PTA negative staining, × 107,000.

Fig. 10 Green membranes diluted with 0.25 % Tween 20—0.05 M phosphate buffer, pH 7.4, and sonicated with ultrasonic osscillator. Small sheets with irregular forms are observed. PTA negative staining, × 107,000.

Table 3. Cytochrome oxidase activity of green membranes (GM) modified with various pretreatments. GM suspended in T-S-H solution (40 mg prot./ml) were diluted with various solutions (distilled water, 0.25 % Tween 20—0.05 M phosphater buffer, 1 % cholate-0.1 M phosphate buffer, 1 % deoxycholate, 1 % SDS or 1 % deoxycholate-72 g KCl/1), and some of the diluted samples were sonicated for 30 sec/ml with ultrasonifier (Kaijo Dempa, Co, Ltd., 7ψ tip) and diluted again with distilled water or 0.25 % Tween 20—0.05 M phosphate buffer (pH 7.4). The reaction was initiated by addition of 0.02—0.05 ml of the diluted samples. Incubation was conducted at 25°.

Diluted	Treatment	Diluted again	Specific* activity
x 15 c̄ distilled water (D.W.)	——	——	0.48
x 15 c̄ D.W.	Sonication	x 5 c̄ D.W.	4.35
x 30 c̄ Tween 20	——	——	0.60
x 30 c̄ Tween 20	Sonication	x 5 c̄ Tween 20	7.65
x 5 c̄ cholate (pH 7.5)	——	x 10 c̄ Tween 20	0.74
x 5 c̄ cholate (pH 7.5)	Sonication	x 20 c̄ Tween 20	5.80
x 6 c̄ SDS	——	x 10 c̄ Tween 20	2.40
x 5 c̄ DOC (pH 8.0)	——	x 20 c̄ Tween 20	1.66
x 5 c̄ DOC. KCl	——	x 30 c̄ Tween 20	10.7

* sec^{-1}/mg prot./3 ml

phosphate buffer, the green membranes were broken down to small membrane fragements (a kind of sheet) and their specific activity increased to 7.7 sec^{-1}/mg prot./3 ml, which was about ten times higher than the activity of the green membranes before sonication and was about two times higher than that of the green membranes sonicated in distilled water. When the green membranes were treated with 1 % cholate, the membrane structure was partially destroyed and the activity of the sample diluted with 0.25 % Tween 20-phosphate buffer after treating with cholate was 0.74 sec^{-1}/mg prot./min. When the green membranes were treated with 1 % DOC and diluted with 0.25 % Tween 20-phosphate buffer, sheet-like membrane fragments were formed as described previously and their activity was 1.66 sec^{-1}/mg prot./3 ml. When the green membranes (19 mg prot./ml of suspension) were treated with DOC (0.3 mg/mg prot.) and KCl (72 g/1), the membranes were decomposed into small irregular particles containing cytochrome oxidase and relatively large net structures, main components of which were colorless protein. The activity of the samples treated with DOC and KCl and diluted with 0.25 % Tween 20-phosphate buffer was 11 sec^{-1}/mg prot./3 ml, which was comparable to the activity of purified cytochrome oxidase (11 sec^{-1}/mg prot./3 ml) assayed at the same time.

DISCUSSION

There are many reports dealing with cytochrome oxidase, but studies on the molecular arrangement or organization of cytochrome oxidase in the mitochondrial membrane or submitochondrial membranes have been scarcely done. Up to the present time we have studied the correlation of ultra-structure to biochemical function in the mitochondrial membrane, and have reported on the fine structure of QH_2-cyt. *c* reductase, NADH-cyt. *c* reductase, cytochrome oxidase and oligomycin sensitive ATPase and molecular organization of these enzymes in the mitochodrial membrane (6, 7, 8, 9, 12, 13, 23). In the present paper the purification procedures of the cytochrome oxidase-rich submitochondrial membrane (greeu membrane) and its fine structure and biochemical properites are reported.

Heme *a* content of the green membranes isolated from beef heart mitochondria (BHM-GM) is about 2 mμmoles/mg prot. (about 3 times of that of the original BHM) and that of the green membrane isolated from ETP (ETP-GM) is 3 to 4 mμmoles/mg prot. (about 5 times of that of the original BHM). Since the heme *a* concentration of purified active cytochrome oxidase is 10 to 12 mμmoles/mg prot. (24), cytochrome oxidase protein occupies about one fifth of the protein of the green membrane in the case of BHM-GM and about one third of the protein of the green membrane in the case of ETP-GM. It is thought that the greater part of proteins of the green membrane (ETP-GM) besides cytochrome oxidase is structural protein, but the problem will be discussed elsewhere (26). On the surface of the green membrane there were observed regular arrays of small particles measuring approximately 50 to 60 Å in diameter with center to center distance of about 70 Å. These particles were frequently arranged in a woven structure. When the green membranes were treated with 1 % DOC, the sheet-like membrane fragments with woven surface structure were markedly increased and occupied the majority of the products. The configuration of these particles was modified from globular to cylindrical form by the effects of detergents, temperature and probably by the effects of pH's. The fact that the particles are frequently arranged in woven structure indicates that the particle itself or its environment has a symmetric structure. When the green membranes are treated with 1 % DOC, besides the sheet-like membrane fragments on which the particles are regularly arranged, there are observed the particles arranged at random on some membrane fragments and several particles released from the fragments. It seems that these experimental results give a counter evidence for the opinion that the particles indeed represent lipid and not oxidase

protein as asserted by JACOBS *et al.* (25). These facts are further confirmed by the experiment concerning the purified cytochrome oxidase and reconstructed membrane from them, as will be reported elsewhere (26). Namely, when cytochrome oxidase is extracted from the green membranes, the particles regularly arranged on them are simultaneously extracted from them and collected in the purified cytochrome oxidase fraction probably as a dimeric form of the particles, and net-like structures remaining after the extraction of cytochrome oxidase are then collected in the gray-colored residue. The membrane reconstructed from purified cytochrome oxidase and phospholipids exhibit a similar surface structure as that of the green membranes (23, 9, 26, 27), but the membranes reconstructed from NADH-cyt. *c* reductase (or QH_2-cyt. *c* reductase), structural protein and phospholipids, or submitochondrial membranes such as ETP and brown membrane, clearly differ from the green membranes (12, 23). These results indicate that there are an intimate correlation between the particles observed on the green membranes and cytochrome oxidase, and phospholipids are one of the essential components for the membraneous organization of cytochrome oxidase and the regular arrangement of the particles are possibly provided not only by phospholipids but also by the structural proteins.

Finally, oxidase activity of the green membranes is compared with that of beef heart mitochondria (BHM), electron transfer particles of BHM, and purified cytochrome oxidase, assayed at the same time under the same condition (Table 4). For the purpose to compare readily, the oxidase activity is expressed as a unit of sec^{-1}/10 mμ moles of cytochrome oxidase/3 ml besides the ordinary unit of sec^{-1}/mg prot. /3 ml. ORII and OKUNUKI (15) reported a high specific activity of 17.1 sec^{-1}/mg prot. /3ml at 25° using purified cytochrome oxidase treated with 0.5 M guanidine hydrochloride. We obtained also a high specific activity of 23.6 sec^{-1}/mg prot. /3 ml. But almost all the values of specific activity of cytochrome oxidase reported until now are about 5 sec^{-1}/mg prot. /3 ml (15). Specific activity, converted to oxidase protein, of sonicated preparations of BHM, ETP and green membranes(GM) is higher than that of purified cyt. oxidase. Especially, the specific activity of the green membranes treated with DOC (0.3 mg/mg prot.) and KCl (72 g/l) is about 2.5 times higher than the maximum activity of purified cytochrome oxidase hitherto obtained. Relatively low specific activity of the green membranes before sonic treatment compared to that of BHM and ETP is due to a higher aggregation. YONETANI already reported that the turn-over number of cytochrome oxidase was gradually decreased with the advance of its purification. And he

Table 4. Cytochrome oxidase activity of beef heart mitochondria, electron transfer particles, green membranes and purified cytochrome oxidase. Specific activities are expressed as unit of sec^{-1}/mg prot./3 ml* at 25° and of sec^{-1}/10 mμmoles of heme *a*/3 ml**.

Sample	Suspended in	Treatment	Specific activity*	Concentration of heme *a***	Specific activity**
BHM	D. W.	—	0.82	0.66	12.4
BHM	D. W.	Sonication	1.8	0.66	27.3
ETP	D. W.	—	2.4	0.94	25.5
ETP	D. W.	Sonication	3.1	0.94	33.0
GM	D. W.	—	0.48	2.1	2.3
GM	D. W.	Sonication	4.4	2.1	21.0
GM	Tween 20+	—	0.61	2.1	2.9
GM	Tween 20	Sonication	7.7	2.1	36.7
GM	DOC·KCl·Tween 20‡	—	10.7	2.1	51.0
Cytochrome oxidase	Tween 20	—	10.3	10.0	10.3
Cytochrome oxidase	Tween 20	—	23.6	10.0	23.6

*** mμmoles/mg protein; + 0.25 % Tween 20—0.05 M phosphate buffer.

‡ Green membranes were dissolved with 1 % deoxycholate (pH 8.0) and KCl (72 g/l), and then diluted 30 times with 0.25 % Tween 20—0.05 M phosphate buffer (pH 7.4) as discribed in Table 3.

Abbreviations: BHM, beef heart mitochondria; ETP, electron transfer particles; GM, green membranes; D. W., distilled water.

presumed that the loss of activity was due to the removal from the preparation of a lipid or lipid-like cofactor associated with the cytochrome *b* complex. However, considering that the lipid-free cytochrome oxidase obtained by JACOBS *et al.* (29) still retained a high specific activity when the activity was assayed after dilution of the sample with 0.25 % Tween 20-phosphate buffer and that a part of purified enzyme was already in polymerized states which gave a relatively low activity and the process of polymerization proceeded gradually accompanied by decrease of activity (28, 26), it seems that the relatively low activity of the purified enzyme is not due to a lipid or lipid-like cofactor but due to the polymerization of the enzyme. Decreases in the enzyme activity associated with polymerization is probably dependent on the masking of active site or its conformational change. It is also obvious that the lipid-depleted enzyme is liable to aggregation and concomitant decrease in the enzyme activity. But even in this instance the fact does not imply that the activity depends directly on lipid.

It is also noteworthy that cytochrome oxidase arranged in the submitochondrial membrane such as sonicated beef heart mitochondria, ETP,

and green membranes (vesicle or sheet) readily reacts with such a macromolecule as reduced cyt. *c* and exhibits higher specific activity than the purified cytochrome oxidase. These facts indicate that the active sites of cytochtome oxidase are arranged on the surface of these membranes.

SUMMARY

1. A cytochrome oxidase-rich submitochondrial membrane (green membrane) was obtained from beef heart mitochondria after extraction of flavoproteins, cytochrome *b*, c_1, *c*, etc. by treating with deoxycholate and potassium chloride.

2. The green membrane was formed by self assembly from the membrane fragments (flat sheets), which derived from the cristae membrane of mitochondria and had essentially the same particulate structure as the green membrane.

3. The green membrane exhibited regular arrays of small particles on the surface, measuring approximately 50 to 60 Å in diameter with center to center distance of about 70 Å. These particles sometime were arranged in a woven structure on the surface.

4. Both the configuration of the particles and the regularity of the arrangement were influenced by detergents and temperature.

5. Green membranes as well as beef heart mitochondria and electron transfer particles commonly retained membrane-structure after sonication and exhibited higher specific activity of cytochrome oxidase than that of purified cytochrome oxidase, if the activity is calculated on the basis of heme *a* concentration (sec^{-1}/10 mμmoles of heme *a*/3 ml). The results suggest that the active sites of cytochrome oxidase are arranged on the surface of these membranes.

6. From these results and other experimental findings, an intimate correlation between cytochrome oxidase and the particles observed on the green membranes is suggested.

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