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Abstract

For the purpose to reveal the correlation between molecular structure and biochemical functions of cytochrome oxidase the author studied purified cytochrome oxidase by using high resolution electron microscope and biochemical methods. 1. Cytochrome oxidase was purified from the cytochrome oxidase-rich submitochondrial membrane (green membrane), obtained from beef heart mitochondria, by three different methods; modification of the method of OKUNUKI et ai., method of FOWLER et ai. and modification of the method ofJACOBS et ai. All the preparations showed a high specific activity under appropriate conditions and consisted mainly of small particles measuring approximately 80 to 90 A. in diameter. 2. The particle, measuring approximately 80 to 90 A. in diameter, took a cylindrical form measuring about 70 A. in diameter at the base and 95 A. in height in an appropriate condition. Many experimental results indicate that the particle is the smallest, fundamental unit of the active cytochrome oxidase. For this reason it was designated as the unit particle of cytochrome oxidase (abbreviated as UPCO). 3. The molecular weight of the unit particle, calculated from its volume and average density (1.24) of lipoproteins (3: 7), was about 270,000. The value was roughly twice the minimum molecular weight of 128, 000 calculated from the heme a content. Accordingly, it is considered that the unit particle contains two heme a molecule and two copper atoms. 4. It was suggested electron microscopically that the particle collected in the 22.6 S position by sucrose gradient ultracentrifugal analysis was a dimer of the unit particle of cytochrome oxidase and also that the particle collected in the 5. 7 S position was a half of the unit particle of cytochrome oxidase. 5. It was also suggested that the particle observed on the green membrane was a subunit of cytochrome oxidase, containing one heme a and one copper atom, and the unit particle of cytochrome oxidase was constituted of two of the particles observed on the green membrane. Namely, the results indicate that the molecular state of cytochrome oxidase on the green membrane apparently differs from that of the purified cytochrome oxidase.

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FINE STRUCTURE AND BIOCHEMICAL PROPERTIES OF PURIFIED CYTOCHROME OXIDASE

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Solubilization and purification of mammalian cytochrome oxidase were first achieved with beef heart muscle preparation by ammonium sulfate fractionation in the presence of cholate by STRAUB (1) and by YAKUSHIJI and OKUNUKI (2). Later the purification procedure was improved and developed by many subsequent investigators and nowadays several purification methods have been established. Such preparations of cytochrome oxidase purified by these methods are commonly highly pure and have almost identical fundamental properties. Using the purified preparations, various properties of cytochrome oxidase have been made clear. Nevertheless, information on molecular states of cytochrome oxidase has been scarcely obtained. CRIDDLE *et al.* (3) reported that a purified preparation of cytochrome oxidase was monomerized by treating with sodium dodecyl sulfate in an appropriate concentration and the molecular weight of the monomerized sample determined by Ehrenberg's modification of the Archibald method was about 72, 000. By using ultracentrifugal anaysis TAKEMORI *et al.* (4) obtained a molecular weight of 530, 000 from a homogeneous, monodispersed preparaton of cytochrome oxidase in the presence of the non-ionic detergent, Emazol 1130. Since the minimum molecular weight of cytochrome oxidase calculated from its iron content is 100, 000, they thought that their preparation of cytochrome oxidase was pentamer. TZAGOLOFF *et al.* (5) gave an average molecular weight of 290, 000 for the preparation of cytothrome oxidase treated with 0.1 % taurocholate by using light scattering technique. ORII *et al.* (6) studied the molecular states of cytochrome oxidase by treating with various concentrations of sodium dodecyl sulfate or guanidine hydrochloride by the methods of sedimentation analysis and sucrose gradient ultracentrifugal analysis of MARTIN and AMES. By these methods they obtained three components, 16.6 S, 5.7 S and original 22.6 S components, from cytochrome oxidase. And they determined the molecular weights of both 16.6 S and 5.7 S components to be 290, 000—330, 000 and 67, 000, respectively, on the

basis of a value of 530, 000 for the original 22.6 S component presented by TAKEMORI *et al.* (4). As analyses of the heme *a* content of purified cytochrome oxidase gave a minimum molecular weight of 128, 000, they supposed that the components of 22.6 S and 16.6 S corresponded to the tetramer and dimer, respectively, of the hypothetical species of 128, 000, and that a half the numbers of the 5.7 S component contained heme *a*. And they concluded that the smallest active unit was the dimer containing two heme *a* molecules and two copper atoms.

As pointed out in the previous paper (7) in relation to the molecular state of cytochrome oxidase, the important problem whether the cytochrome oxidase is one enzyme or two remains to be solved. HORIE and MORRISON (8) reported that a cytochrome *a* preparation free of a_3 was prepared with or without the use of cyanide. However, opposing opinions have been presented by ORII and OKUNUKI (9) and WAINIO *et al.* (10).

We have already reported about the molecular organizations and biochemical properties of oligomycin-sensivive ATPase, reduced coenzyme Q-cytochrome c reductase and cytochrome oxidase in the mitochondrial or submitochondrial membrane (11～14). The present communication deals with the studies on the correlation between molecular structure and biochemical properties of cytochrome oxidase purified from beef heart mitochondria by three different methods. As the results it was clarified that the smallest unit of cytochrome oxidase generally took a somewhat ellipsoid-like configuation of about 80～90Å in diameter and in an appropriate condition it took a cylindrical configuration of about 70 Å in diameter at the base and 95 Å in height. And it was also suggested that the particle observed on the cytochrome oxidase-rich submitochondrial membrane (green membrane) reported in the previous paper (7) was a subunit of the cytochrome oxidase and the smallest unit of cytochrome oxidase was composed of two of the particles observed on the green membrane.

MATERIALS AND METHODS

Preparation of mitochondria: Beef heart mitochondria (BHM) were prepared from fresh beef heart muscle by the method described in previous papers (15, 7).

Preparation of electron transfer particles: Electron transfer particles (ETP) were prepared from beef heart mitochondria by the method of sonication and differential centrifugation, as described in the previous paper (15).

Preparation of cytochrome oxidase-rich submitochondriat membrane: Cytochrome oxidase-rich submitochondrial membrane (green membrane, GM) was prepared as outlined below. The precise method was described in the previous paper (15).

Beef heart mitochondria or electron transfer particles were suspended in Tris-sucrose-histidine solution (Tris-Cl buffer, 0.05 M, pH 8.0; sucrose, 0.66 M; histidine, 0.001 M) and the protein concentration was adjusted to 23 mg protein per ml. Deoxycholate (DOC, 10 % w/v, pH 8 adjusted with KOH) was added at the concentration of 0.3 mg per mg protein to the mitochondrial suspension or 0.4 mg per my protein to the suspension of electron transfer particles. Then potassium chloride was added in the concentration of 72 g per l of total suspension and the mixture was stirred until KCl was thoroughly dissolved. The suspension was centrifuged at $105,000 \times g$ for 30 min. The clear, red snpernatant was carefully decanted. From the three-layered pellet the deep green, middle layer (GR_1) occupying the bulk of the pellet, was carefully separated from the other two layers and suspended in the Tris-sucrose-histidine solution by homogenization with Teflon homogenizer. And the suspension was recentrifuged at $105,000 \times g$ for 20 min and the dark green, middle layer (GR_2) of the pellet was collected and suspended again in a small quantity of the Tris-sucrose-histidine solution. The component of the GR_2 fraction was designated as cytochrome oxidase-rich submitochondrial membrane or green membrane based on the color of the residue and its membraneous structure.

Purification of cytochrome oxidase: Cytochrome oxidase was purified from the green membranes according to the following three methods. All subsequent operations were conducted at 0~4°. An outline of the purification procedures 1 and 2 is given in Fig. 1.

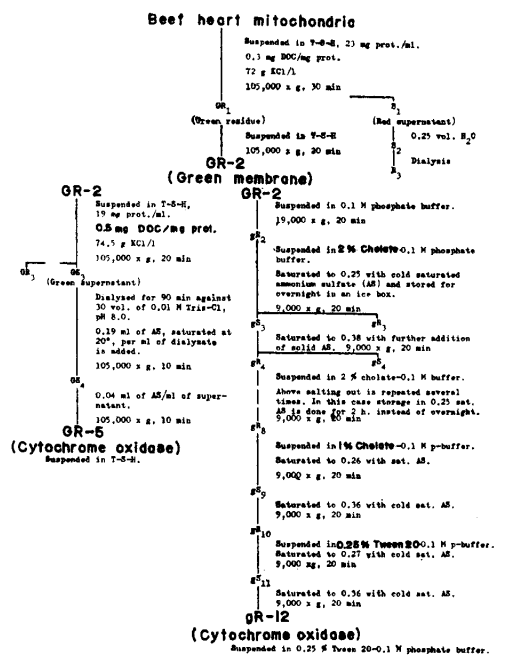

Fig. 1. Procedures for the purification of cytochrome oxidase.

1. Slight modification of the method of OKUNUKI *et al.* (16): The green membranes (CR_2) were suspended in 0.1 M phosphate buffer and centrifuged at 19,000 x g for 20 min. The supernatant was decanted and the residue was suspended in about ten volumes of 0.1 M phosphate buffer. The 0.1 M phosphate buffer, pH 7.4, containing 10 per cent of cholate, was added to the suspension until the final concentration of cholic acid became 2 per cent and then cold saturated ammonium sulfate solution adjusted to pH 7.4 with ammonium hydroxide was added to bring the solution to 0.25 saturation with ammonium sulfate and the pH was adjusted to 7.4 with 1 N NaOH. After keeping overnight in an ice bath, the massive precipitate formed was removed by centrifugation (9,000 × g, 20 min). The deep reddish green supernatant (gS_3) containing the major amount of cytochrome oxidase was made up 0.38 % saturation with solid ammonium sulfate (77 g/l of the supernatant). After the ammonium sulfate was completely solubilized by stirring and the pH was adjusted to 7.4, the mixture was allowed to stand for 10 min in an ice bath and centrifuged at 9,000 × g for 20 min (gS, gR_4). The residue (gR_4) was dissolved in 2 per cent cholate-buffer (2 % cholate-0.1 M phosphate buffer, pH 7.4) and the ammonium sulfate fractionation between 0.25 and 0.38 saturation was repeated for several times; (gS_5, gR_5), (gS_6, gR_6), (gS_7, gR_7), (gS_8, gR_8). By these treatements cytochrome oxidase was purified from the green membrane by removing colorless proteins and cytochrome *b*, c_1 as the residue at the saturation of 0.25 with ammonium sulfate and solubilized cytochrome c_1 and other solubilized proteins as the supernatant at the saturation of 0.38. Salting out took more than two hours when it was conducted at the saturation of 0.25 or 0.27 with ammonium sulfate, and about 5 to 10 min when it was conducted at the saturation of 0.38 or 0.35. The partially purified fraction of cytochrome oxidase (e. g. gR_9) obtained by the procedures described above, was dissolved in 1 per cent cholate-buffer, and the suspension was made up to 0.27 saturation with the cold saturated ammonium sulfate solution adjusted to pH 7.4 and centrifuged just like before (gS_9, gR_9). The supernatant was made up to 0.35 saturation with the saturated ammonium sulfate solution and centrifuged (gS_{10}, gR_{10}). The residue (gR_{10}) was dissolved in 0.25 per cent Tween 20-phosphate buffer (0.05 % sodium phosphate buffer, pH 7.4, containing 0.25 % Tween 20). The solution was made to 0.27 saturation with the saturated ammonium sulfate solution and adjusted to pH 7.4, with 1 N sodium bydroxide. After being left standing 15 min, the mixture was centrifuged (gS_{11}, gR_{11}). The supernatant (gS_{11}) was made up to 0.35 saturation by addition of the saturated ammonium sulfate solution(gS_{12}, gR_{12}). The reddish green precipitate obtained by these treatments was the purified preparation of cytochrome oxidase. The preparation was dissolved in a small volume of the 0.25 per cent Tween 20-Na-phosphate buffer and stored in an ice bath for periods of up to a week before the use. When necessary to store for a long time, the purified preparation of cytochrome oxidase dissolved in 1 % cholate-buffer was stored at −20° or less.

For the purpose to obtain a preparation of uniform molecular state of cytochrome oxidase, the purified preparation of cytochrome oxidase was treated as follows according to the method of ORII and OKUNUKI (6). Cytochrome oxidase

dissolved in a small volume of the 0.25 per cent Tween 20-Na-phosphate buffer was passed through a Sephadex G-100 column which had been equilibrated with the 0.25 % Tween 20-phosphate buffer. Colored portion was collected, and made to 0.38 saturation with the saturated ammonium sulfate solution and centrifuged. The precipitate dissolved in a minute volume of the same medium was passed through the same column which was regenerated. Eluated cytochrome oxidase fraction was centrifuged for 40 min at 198,000×g at 5°. The upper one-third of the solution in the tubes was sucked off and discarded. Then the condensed cytochrome oxidase-portion was decanted and stored in an ice bath for several days before the use.

2. A rapid method of FOWLER *et al.* (17): The green membranes were suspended in the Tris-sucrose-histidine solution and the protein concentration was adjusted to 19 mg protein per ml. Potassium deoxycholate (10 % w/v, pH 10.0, 0.5 mg/mg protein) was stirred into the suspension and solid KCl (74.5 g/l of suspension) was added while stirring. After the salt being completely dissolved, the mixture was centrifuged at 105,000×g for 20 min (GS_3, GR_3). The green-colored supernatant (GS_3) was collected by decantation and poured into size-24 Visking cellulose casing, and dialyzed for 90 min against 30 vol. of 0.01 M Tris-chloride buffer, pH 8.0. During this time, little increase in the turbidity of the dialyzate was observed. In using an impure preparation of green membranes as a starting material for the prepartion of cytochrome oxidase, a relatively high increase in the turbidity was observed. Such an impure preparation of green membrane was not used, because cytochrome oxidase purified from this proved to be only of a poor purity and gave a low yield. To the dialysate ammonium sulfate solution (0.19 ml/ml of dialysate) saturated at 20° and adjusted to pH 7.4 with ammonium hydroxide, was added while stirring. After a 10 min-incubation, the mixture was centrifuged at 105,000 x g for 10 min (GS_4, GR_4). The copious precipitate (GR_4) was discarded. Ammonium sulfate solution (0.04 ml/ml of supernatant) was added to the supernatant (GS_4) and the mixture was centrifuged (GS_5, GR_5). The reddish green precipitate (GR_5), cytochrome oxidase fraction, was dissolved in a small volume of Tris-sucrose-histidine solution and stored in an ice bath or stored at −20°.

3. Modification of the method of JACOBS *et al.* (18): Cytochrome oxidase was purified from the green membranes by treating with Triton x 100 according to the modification of the method of JACOBS *et al.*, who purified lipid-free soluble cytochrome oxidase from membraneous cytochrome oxidase prepared from rat liver mitochondria. The green membranes suspended in 0.2 M neutral potassium phosphate buffer were centrifuged. To the pellet 4 volumes of a solution of 5 % (v/v) Triton x 100 dissolved in 0.2 M neutral potassium phosphate buffer were added while stirring rapidly. After 2-hour incubation at 0°, the suspension was centrifuged at 105,000×g for 30 min. The clear green supernatant was decanted into a messcylinder and 7 volumes of water were added while stirring rapidly. The diluted supernatant was charged on a column of DEAE-cellulose which was equilibrated with a solution of 0.5 % (v/v) Triton × 100 in 0.025 M neutral potossium phosphate. Cytochrome oxidase formed a dark green band at the top.

The column was subsequently washed with 10 x holdup volume of a solution of 0.5 % (v/v) Triton x 100 in 0.025 M neutral potassium phosphate and then the column was eluted with a solution of 1 % (v/v) Triton × 100 in 0.2 M neutral potassium phosphate. Cytochrome oxidase eluted as a dark green band, was collected and stored at 0° or −20°. In some experiments solvent of the preparation was replaced in place of the 1 % (v/v) Triton x 100 in 0.2 M neutral potassium phosphate to the 0.25 % Tween 20 in 0.05 M sodium phosphate by the method of ammonium sulfate fractionation, as described previously.

Sucrose gradient ultracentrifugal analysis: Sucrose gradient ultracentrifugal analysis of purified cytochrome oxidase was conducted according to the method which was originally described by MARTIN and AMES (19) and applied to cytochrome oxidase by ORII and OKUNUKI (6). A linear 5 to 20 % sucrose gradient containing 0.05 M sodium phosphate buffer at pH 7.4, 0.25 % Tween 20 and 1 mM EDTA was made in a tube of 5 ml capacity. The gradient was stored for 4 hours or more before the use. Cytochrome oxidase (5×10^{-5} M) having uniform molecular state, prepared by the method of Sephadex G-100 column treatment as described previously, was treated with sodium dodecyl sulfate at an appropriate concentration at 20° for 10 min, according to ORII and OKUNUKI (6). Two-tenth ml of the sample was layered on the top of the gradient and the tubes were spun at 36,000 r. p. m. for 15 hours at 10° in a Spinco preparative ultracentrifuge, Model L-2, using SW 39 rotor. After centrifugation, samples were collected in 5-drop fractions by perforating the bottom of the tubes. For the purpose to facilitate comparisons of the results total number of drops was corrected to 100. The sample of every other tube, which was diluted by adding 2 ml of 0.25 % Tween 20-phosphate buffer, was used for spectrophotometry at 280 mμ. The other samples were used for the assay of cytochrome oxidase activity and electron microscopic observation. Purified cytochrome oxidase which was reported to have a value of $S_{20,w}$ of 22.6 S by ORII and OKUNUKI (6) was used as a standard.

Determination of cytochrome contents: Cytochrome contents of turbid samples obtained during the preparation of cytochrome oxidase were determined by the difference spectra between the reduced form and oxidized form as reported previously (7, 15). Cytochrome concentration of purified cytochrome oxidase was determined by the direct spectrum (phosphate buffer for a blank). Millimolar extinction coefficient (ε $mM^{-1}cm^{-1}$) of the reduced form of heme *a* was assumed to be 16.5 (A_{605}-A_{630}).

Assay of cytochrome c oxidase activity: As already reported (7), the activity of cytochrome oxidase was measured spectrophotometrically according to the method of SMITH (20). The initial concentration of reduced cytochrome *c* was fixed at 15 μM and the oxidation rate of reduced cytochrome *c* was recorded as the decrease in the absorbance at 550 mμ with lapse of time using EPS-3T form of the Hitachi autorecording spectrophotometer. The first order velocity constant, k (sec^{-1}), was calculated from the formula of SMITH (20). 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) (21, 22).

Protein determination: Protein contents were determined by the biuret method of GORNALL *et al.* (23) for the particulate preparations (e.g. BHM, ETP, GM, etc.) or a modification of the biuret method reported by YONETANI (22) for the purified cytochrome oxidase.

Electron microscopic observation: Fine structure of purified cytochrome oxidase in various conditions and partially purified preparations obtained during the purification steps was observed by the negative staining technique with phosphotungustic acid (pH 7.0) with a HU-11C (Hitachi Ltd.) electron microscope, as described in a previous report (13). Electron optical magnification was checked by simultaneous observation of Cu-phthalocyanin crystals.

RESULTS

1. *Cytochrome oxidase purified from the green membranes according to the modification of the method of OKUNUKI et al. (16)*

a. *Biochmical properties:* An example of cytochrome content and cytochrome recovery in the fractions obtained during the purification of cytochrome oxidase from the green membranes (GM), which were purified from electron transfer particles (ETP), are illustrated in Table 1. In the

Table 1. Cytochrome concentrations and recovery of cytochromes in the purification steps of cytochrome oxidase from green membranes (ETP-GR2)

Fraction*	Protein (%)	Cyt. *a*		Cyt. *b*		Cyt. $c+c_1$	
		mμmoles/ mg prot.	Recovery (%)	mμmole/ mg prot.	Recovery (%)	mμmole/ mg prot.	Recovery (%)
ETP-GR2	100	3.40	100	0.366	100	0.183	100
gR_3	21.8	0.318	2.0	0.332	19.8	0.102	12.1
gS_4	16.6	4.2	20.5	—	—	0.588	53.4
gR_5	7.5	0.436	1.0	0.754	15.4	—	—
gR_7	0.5	0.244	0.0	0.338	0.5	—	—
gR_8	0.3	1.48	0.1	0.091	0.1	—	—
gR_{10}**	18.9	9.20	51.1	—	—	—	—

* A little amount of cytochromes was distributed among the fractions (gS_6, gS_8 and gS_{10} which are not described in this table.

** gR_{10}: fraction of cytochrome oxidase

Table "gR_3" was a light gray-colored, insoluble precipitate which was remained after extraction of cytochrome oxidase from the green membranes. Fractions "gR_5" and "gR_7" had similar fundamental properties to the "gR_3". It was thought that the main component of these fractions was structural protein. The integrated proteins of the fractions, of which main component was thought to be structural protein, such as gR_3, gR_5 and gR_7

occupied about a half of the protein content of the original green membranes (ETP-GM). Fraction "gS_4", which was a supernatant fraction after sedimenting cytochrome oxidase, contained a relatively high concentration of cytochrome oxidase. The quantity of remaining cytochrome oxidase in the gS_4 fraction depended on the purity of the green membranes, the lower the purity of the green membranes, the higher was the remaining cytochrome oxidase in the gS_4 fraction. In such an instance the cytochrome oxidase was recovered in the gR_4 fraction by increasing the concentration of ammonium sulfate. It is clearly indicated in the Table that cytochrome *b* and c_1 were removed from the cytochrome oxidase fraction as the residues at 0.25 or 0.27 saturation with ammonium sulfate and the rest of cytochrome c_1 was removed as the supernatants at 0.38 or 0.35 saturation. The fraction "gR_{10}" was a cytochrome oxidase fraction before replacement of the solvent from 1 % cholate-buffer to 0.25 % Tween 20-phosphate buffer. In general the heme *a* concentration of purified preparations of cytochrome oxidase was about 10 mμmoles per mg protein. Cytochrome oxidase protein contributes about one-third of the total protein of the green membranes as estimated by comparing the cytochrome concentration of the green membranes with that of purified cytochrome oxidase.

The specific activity of purified cytochrome oxidase markedly differs in accordance not only with preparations but also with storage and media in which cytochrome oxidase was dissolved. As a result, the specific activity of purified cytochrome oxidase ranged widely from 3 to 24 (sec^{-1}/mg protein/3 ml, 25°). Fig. 2 illustrates an example of assay of cytochrome oxidase activity. The specific activity of purified cytochrome oxidase was

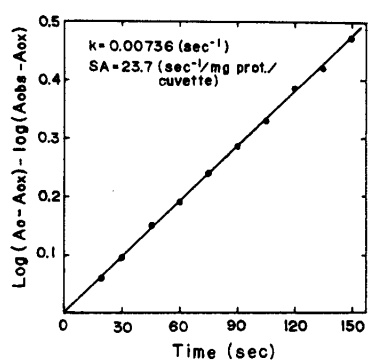

Fig. 2. Spectrophotometric assay of cytochrome oxidase. The assay cuvette contained 225 μmoles phosphate buffer (pH 6.0), 0.15 μmole EDTA, 0.15 mg sodium chloride, and 45 mμmoles ferrocytochrome c in a total volume of 3.0 ml. The reaction was initiated by addition of 0.31 μg of cytochrome oxidase. Incubation was conducted at 25°.

not necessarily proportional to the heme *a* concentration but intimately correlated to molecular states as will be discussed later. The specific activity was markedly influenced by the conditions of the enzyme-storage. When cytochrome oxidase was dissolved and stored (as short time as possible) in 1 % cholate-buffer at 0° and the activity was assayed directly after dilution (more than ten-fold) with 0.25 % Tween 20-phosphate buffer or after replacement from the 1 % cholate-buffer to 0.25 % Tween 20-phosphate buffer, a relatively high specific activity was exhibited. On the contrary, when cytochrome oxidase was dissolved and stored in 0.25 % Tween 20-phosphate buffer, the activity was relatively high for a few hours but fell gradually with lapse of time as illustrated in Table 2. When sodium

Table 2. Effects of storage of cytochrome oxidase dissolved in 0.25 % Tween 20—0.05 M sodium phosphate buffer and of sodium dodecyl sulfate in a reaction mixture on cytochrome oxidase activity

Days after preparation	Concentration of SDS	Relative activity (%)
0	—	100
0	5×10^{-4} %	102
3	—	46
3	5×10^{-4} %	74

dodecyl sulfate was added in the reaction medium at the concentration of 5×10^{-4} %, no effect was observed on the activity of the cytochrome oxidase prepared directly before, but the decreased activity of the cytochrome oxidase dissolved and stored in 0.25 % Tween 20-phosphate buffer for 3 days was partially recovered with SDS. As reported by Orii and Okunuki (6) similar effect of SDS on the activity was observed when the cytochrome oxidase was preincubated with SDS (Table 3).

Table 3. Effect of incubation of cytochrome oxidase with SDS on cytochrome oxidase activity. 0.9×10^{-5} M cytochrome oxidase was incubated with SDS for 30 min at 20°.

Concentration of SDS (%)	0	0.01	0.05	0.1	0.2	0.5	1.0
Relative activity (%)	100	110	113	140	150	93	83

The molecular state of cytochrome oxidase was made uniform in the Tween 20-phosphate buffer by treating with a Sephadex G-100 column as described previously. Sucrose gradient ultracentrifugal analysis was conducted on the cytochrome oxidase itself, and cytochrome oxidase treated with 0.5 % and 1.0 % SDS at 20° for 10 min. As observed in Fig. 3,

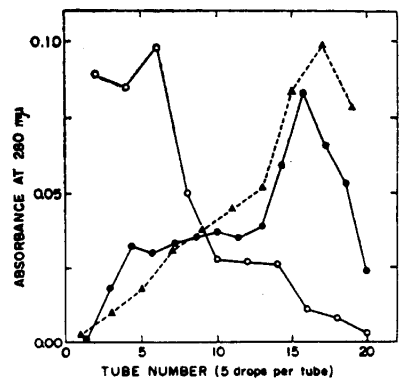

Fig. 3. Density gradient analysis of cytochrome oxidase treated with SDS. Open circles conjugated with solid line indicate the centrifugal pattern on untreated uniform preparation of cytochrome oxidase. Solid circles connected with solid line and solid triangle connected with broken line indicate the centrifugal patterns of cytochrome oxidase treated with 0.5 % and 1.0 % SDS, respectively. Experimental details are given in the text.

although the each peak was not as steep, since the volume of the sample (5×10^{-5} M) layered on the sucrose gradient was 0.2 ml, the centrifugal pattern was essentially the same as that reported by ORII and OKUNUKI and each peak was also identical. The untreated cytochrome oxidase showed only one peak in the tube of No. 6, to which the value of 22.6 S was assigned according to ORII and OKUNUKI (6). With 1.0 SDS the original peak disappeared and a distinct peak appeared in the tube of No. 17 (5.7 S position). With 0.5 % SDS a shoulder was observed in tube No. 10 (about 17 S position) in addition to the main peak at the 5.7 S position. Activity was found both in the original peak (22.6 S position) and in the shoulder at the tube No. 10 (about 17 S position), but activity was scarcely detected at the 5.7 S position.

b. *Electron microscopic observation :* Electron microscopic observations of various preparations of cytochrome oxidase were carried out by the negative staining technique with phosphotungustate (pH 7.0).

Main structural element of the cytochrome oxidase, which was purified from the green membrane by ammonium sulfate fractionation in the presence of the cholate-buffer and dissolved in 1 % cholate-buffer, is small particles with somewhat ellipsoid-like form, measuring approximately 80 to 90 Å in diameter (Fig. 4). Adding to these particles, irregularly formed aggregates with various size are observed on the photographs.

When the solvent was replaced in place of the cholate-buffer to 0.25 % Tween 20-phosphate buffer, fine structures of the cytochrome oxidase

are observed as in Fig. 5. The smallest unit of the cytochrome oxidase observed in Fig. 5 is the somewhat ellipsoid-like particles measuring about 80 to 90 Å in mean diameter $\left(\frac{\text{long axis} + \text{short axis}}{2}\right)$.

When cytochrome oxidase is observed in detail on the photographs, it is made clear that the particles took a cylindrical configuration in an appropriate condition. Dimeric or polymeric forms of the particles are also observed on the same photograph. Fig. 6 illustrates the typical patterns of the smallest unit of the cytochrome oxidase and its dimers, polymers and subunits. The pattern which is the most conspicuous and is frequently observed is a rectangle, measuring about 70 × 95 Å (width × length), as illustrated in Fig. 6a. The fact that the rectangular patterns are frequently arranged in a chain-form (Fig. 6a, b, c. d) confirms that the particle exhibiting the rectangular partern is a unit of cytochrome oxidase. In case of negative staining, it is thought that the projections of the particles whose positions are at random are shown in the photographs. It is thought from the rectangular pattern of the particles that a length or diameter of the base of the particles is about 70 Å. Besides the rectangular pattern, the pattern smallest in size exhibiting a circular or square form and measuring about 70 Å in diameter $\left(\text{Mean} = \frac{66\ (\text{short axis}) + 72\ (\text{long axis})}{2} = 69\ \text{Å}\right)$, was observed in the same photographs (Fig. 5, 6e, f, g). This circular pattern is less frequently observed than the rectagular pattern and both circular and rectangular patterns are thought to result from observing identical particles at different angles. Namely, it seems that the cylindrical particle measuring about 70 Å in diameter at the base and 95 Å in height is the fundamental unit of cytochrome oxidase. The results will be comfirmed by the following experimental results.

Besides the cylindrical particles, a rod- or chain-like pattern, measuring about 90 to 100 Å in width, is observed in the cytochrome oxidase fraction (Fig. 5). As observered in Fig. 6 some rods clearly indicate their organizing units, e. g. the chain-like arrangement of the cylindrical particles. The rods were formed from the purified cytochrome oxidase with lapse of time during the storage and they depolymerized to the cylindrical particles on the treatment with sodium dodecyl sulfate at an appropriate concentration. These facts combined with their constant width of about 90 to 100 Å indicate that the rods are formed by chain-like arrangements of the cylindrical particles. The majority of the rods, however, do not show clearly their organizing unit, probably due to the tight, linear arrangement of the particles. When cytochrome oxidase, dissolved in 0.25

% Tween 20-phosphate buffer, was stored at 0° for several days, the rod-like and amorphous polymerization increased and subsequently the oxidase activity decreased (Table 2, Fig. 7).

As described above, the cylindrical particle, measuring about 70 Å in diameter at the base and 95 Å in height, is the prominent, smallest component of highly active preparations of cytochrome oxidase, and is the organizing unit of the rods or amorphous polymers. Considering these points the cylindrical particle is found to be the smallest unit of the active cytochrome oxidase. By reason of this, in the following the cylindrical particle will be called "unit particle" of cytochrome oxidase and will be abbreviated as UPCO.

In Fig. 5 there were also observed the particles of dimerized unit. The properties of the particles were made clear by the sucrose gradient ultracentrifugal analysis. Fig. 8 illustrates an electron micrograph of the fraction of the peak (22.6 S position) obtained with the untreated, uniform cytochrome oxidase by the sucrose gradient ultracentrifugal analysis. The major pattern in the photograph is somewhat like ellipse or rectangle, measuring approximately 90×150 Å (short axis $\times$ long axis) in diameters. The other pattern in the photographs, which is less frequently observed, is somewhat like ellipse, measuring approximately 75×95 Å in diameters. Since the particles observed in the photographs are the particles located at an identical position on the sucrose gradient ultracentrifugal analysis, the patterns observed above are thought to be due to dimers seen at different angles. If the particle is supposed to be roughly of an elliptic cylinder, based on the configuration of the particle as a dimer of the UPCO as illustrated in Fig. 15, the size of the particle measures approximately 75×150 Å (x axis $\times$ y axis) at the base and 95 Å in height. The electron micrographs revealed that the particle was a dimer of the unit particle of cytochrome oxidase. A constriction, observed on the middle part of some particles, is considered to be a contact point of two of the unit particles of cytochrome oxidase. The electron micrographs of material from the 5.7 S position, obtained from the cytochrome oxidase treated with 1.0 % SDS by the sucrose gradient ultracentrifugal analysis, show somewhat ellipsoid-like or cylindrical particles which are smaller than the unit particles (Fig. 9). These particles must be subunits of cytochrome oxidase. Regarding the subunit particle as a cylinder, the size of the particle was approximately 55 Å in diameter at the base and 70 Å in height. Besides the subunits, relatively large, amorphous particles, probably formed by the aggregation of the subunits after the centrifugal analysis, are observed.

2. *Cytochrome oxidase purified from the green membranes according to the method of* FOWLER *et al.*

Heme *a* concentration of the preparation was 8 to 9 mμmoles per mg protein, which value was slightly lower than that of the preparation previously stated. But the specific activity of the preparation was 17 (sec^{-1}/mg prot./3 ml) in the highest case. The solution of the purified cytochrome oxidase dissolved in Tris-sucrose-histidine solution was optically clear. But on the electron micrographs the preparation was not frequently observed as isolated particles but was generally observed as sheets (Fig. 10). When the preparation was observed on an electron micrograph after treating with 0.2 % SDS, there were observed both rods of about 80 Å in width and isolated particles of about 80 Å in diameter (Fig. 11). When cytochrome oxidase was extracted from a highly purified preparation of the green membranes by treating with DOC (0.5 mg/mg protein) and KCl (74.5 g/l), there were observed, even though it was rare, isolated particles and their aggregates, having identical size and structure to those of the unit particle of cytochrome oxidase (Fig. 12).

3. *Cytochrome oxidase prepared from the green membrane by treating with Triton* x *100*

Heme *a* concentration of the preparations of cytochrome oxidase, purified from the green membranes (ETP-GM) by treating successively with Triton × 100 and DEAE-cellulose column chromatography, was about 8 mμmoles per mg protein. The catalytic activity of the preparation is shown in Table 4. The activity of the preparation diluted with the solution of 0.2 M phosphate buffer (pH 7.4) containing 1 % Triton × 100 or with

Table 4. Oxidase activity of the cytochrome oxidase fraction obtained from the green membrane (ETP-GR_2) by the method of extraction with 5 % Triton × 100-phosphate buffer and fractionation through DEAE-cellulose column.

The assay cuvette contained 225 μmoles phosphate buffer (pH 6.0), 0.15 μmole EDTA, 0.15 mg sodium chloride, and 45 mμmoles ferrocytochrome c in a total volume of 3.0 ml. The reaction was initiated by addition of 0.01 ml of an appropriately diluted solution of cytochrome oxidase. Incubation was conducted at 25°.

Diluted with*	Specific activity sec^{-1}/mg prot./3 ml
1 % Triton × 100—0.2 M phosphate buffer	2.5
Distilled water	1.7
5 % solution of phospholipid (asolectin)	14.7
0.25 % Tween 20—0.05 M phosphate buffer	20.3

* A concentrated solution of cytochrome oxidase was diluted with those media before assay of enzymatic activity.

distilled water was relatively low, but when the preparation was diluted with a solution of phospholipid or 0.05 M phosphate buffer containing 0.25 % Tween 20, the activity was about 6 or 8 times higher than the value of the preparation diluted with the solution of Triton × 100—0.2 M phosphate buffer.

Electron microscopic examination of the preparation diluted with the 1 % Triton × 100—0.2 M phosphate buffer has failed to show any structure. The preparation of electron microscopic specimens was very difficult owning to the solubilization of the supporting collodion film by Triton × 100 in that system. But in the condition of the preparation exhibiting high activity such as dilution of the preparation with phospholipid or as replacement of the solvent from the Triton × 100-phosphate buffer by the 0.25% Tween 20-phosphate buffer, electron microscopic observation was possible with rapidly prepared specimens. In the case of the preparation diluted with a solution of phospholipid, a membrane structure was observed as reported elsewhere (25). The preparation from which the Triton × 100-phosphate buffer was replaced to the Tween 20-phosphate buffer showed particles of 80 to 90 Å in diameter, similar to the unit particle of cytochrome oxidase, the polymers and amorphous aggregates on the electron micrograph (Fig. 13).

DISCUSSION

Minimum molecular weight of cytochrome oxidase calculated from heme *a* content or iron content on the basis of the protein counterpart generally gave a value between 100, 000 and 150, 000. Contrary to this, various values of the molecular weight of cytochrome oxidase have been obtained by physicochemical methods. As described previously TAKEMORI *et al.* (4), TZAGOLOFF *et al.* (5), and CRIDDLE and BOCK (3) gave values of 530, 000, 290, 000 and 72, 000, respectively. ORII and OKUNUKI (6) studied the molecular states of cytochrome oxidase after treating with SDS and guanidine hydrochloride at various concentrations, and they obtained 16.6 S and 5.7 S components from the original preparation (22.6 S component) by sedimentation analyses of SDS-treated preparations. As the results they suggested that the 16.6 S component (M. W. =330, 000) was the smallest active unit containing two heme *a* molecules and two copper atoms, and that it had half the molecular weight of the 22.6 S component (M. W. =530, 000) and had four times the particle weight of the 5.7 S component (M. W. =67, 000).

In the present paper, the author studied enzymatically and morpho-

logically the molecular states of cytochrome oxidase under various conditions. The aims of the present experiment were to solve the following problems. 1. What is the smallest unit of cytochrome oxidase, molecular structure and molecular weight? 2. What are the structures of the polymers and subunits, and how is the polymerization brought about? 3. What is the molecular state of cytochrome oxidase *in situ* and how does modification occur during the processes of purification? 4. How is cytochrome oxidase organized in the mitochondrial membrane or in the submitochondrial membrane? As already reported, the particles observed on the green membranes obtained from beef heart mitochondria undergo configurational changes from globular structure to cylindrical structure by means of DOC-treatment, etc. As for the possibility of the configurational changes also occurring in the purified cytochrome oxidase, it is unreasonable to expect uniform configuration of cytochrome oxidase. But the electron micrographs of the three active preparations of cytochrome oxidase purified from the green membranes by the three different methods (1. Modification of the method of OKUNUKI *et al.* 2. Method of FOWLER *et al.* 3. Modification of the method of JACOBS *et al.*) equally showed somewhat ellipsoid-like or cylindrical particles measuring about 80 to 90 Å in diameter and their linear polymers in appropriate conditions. Since the particles are the main component of the highly active preparation of cytochrome oxidase and are the organizing unit of polymers, the particle is thought to be the smallest fundamental unit of the active cytochrome oxidase. For this reason it was designated as the "unit particle" of cytochrome oxidase (abbreviated as UPCO). When the fine structure of the UPCO was observed more precisely with the preparations of cytochrome oxidase dissolved in the Tween 20-phosphate buffer, the UPCO sometimes took a cylindrical form, measuring about 70 Å in diameter at the base and 95 Å in height.

The particles collected in the 22.6 S position of the sucrose gradient ultracentrifugal analysis, which were the main component of the cytochrome oxidase uniformly conditioned by the Sephadex treatment, were observed as nearly an elliptic cylindrical form, measuring approximately 75×150Å (x axis $\times$ y axis) at the base and 95 Å in height. For the reason that there were sometimes observed a constriction on the particle and estimated volume of the particle was twice that of the UPCO, the particle was thought to be a dimer of the UPCO. This possibility was also suggested by ORII and OKUNUKI (6) according to their ultra-centrifugal analyses. The rods, which were frequently observed on the photographs of the purified cytochrome oxidase, were considered to be linearly arranged polymers of the UPCO. Transitional forms from UPCO to rod-shape

polymers were shown. The particles collected in the 5.7 S position of the sucrose gradient ultracentrifugal analysis of the cytochrome oxidase treated with 1 % SDS were smaller than the UPCO and regarding as a cylinder the particle size was about 55 Å in diameter at the base and 70 Å in height. As already reported by ORII and OKUNUKI (6), considering the active cytochrome oxidase to be divided into some subunits of the same particle weight, the UPCO was thought to be composed of two of the subunit collected in the 5.7 S position.

It is somewhat difficult to determine the molecular weight of a particle from its specific gravity and form and size (volume), observed on the electron micrographs of the particle negatively stained with PTA, for the reason that the particle is not neccessarily compact and there is a possibility of contraction due to dehydration of the particle during the preparation, etc. But in practice GREEN *et al.* (26) and MELLENA *et al.* (27) reported that the molecular weight of the particle, calculated from its volume and specific gravity, coincided comparatively well with those estimated by physicochemical methods.

Assuming the particle observed on the electron micrograph to be a compact particle, the approximate particle weight in grams is given by the following formula (26).

$$\text{M. W.} = N_A \times V \times \rho$$

In the formula, N_A is Avogradro's Number (6.02×10^{23}), V is the volume in cm^3 of the particle and ρ is the specific gravity in g/cm^3 of the particle. Regarding that lipid (mainly phospholipid) accounts for 30 per cent of the dry weight of cytochrome oxidase and the average densities of proteins and phospholipids are 1.33 and 1.02, respectively, the average value for ρ should be equal to $(1.33 \times 0.7 + 1.02 \times 0.3)$, i.e., 1.24. When the molecular weight of the unit particle of cytochrome oxidase is calculated from its volume $(3.14 \times 35^2 \times 95 \times Å^3)$ and the specific gravity (1.24) according to the formula, molecular weight of 270,000 is given. The value was slightly lower than that of ORII *et al.* (6) and was roughly twice the minimum molecular weight of 128,000 calculated from the heme *a* content. Similarly, weight of the particle collected in the 22.6 S position was about 620,000 and that of the subunit collected in the 5.7 S position was about 120,000.

For the solution of the problems, what the most natural molecular state of cytochrome oxidase is and how much the molecular state is modified during its purification process, first of all the molecular organization of cytochrome oxidase in mitochondrial or submitochondrial membranes has to be made clear, and then the purification process of cytochrome

oxidase must be studied systematically. As already reported (7), there were observed regular arrays of small particles on the green membranes. When the green membranes were treated with DOC at a low concentration, some of the particles arranged at random and were released from the sheets as illustrated in Fig. 14. The size of the particles measured 46×86 Å (width $\times$ length) in average. In the particles arranged at random there were observed round plane figures measuring 46 Å average in diameter. The round plane figure was thought to be a figure at another plane of the same particle. Assuming the particle to be a cylinder, its size was 46 Å average in diameter at the base and 86 Å average in height. Particle weight estimated roughly according to the previous method was about 120,000. This value is about half of the molecular weight of UPCO, and is fairly identical with the particle weight of the subunit obtained from cytochrome oxidase by treating with 1 % SDS and with the minimum molecular weight calculated from heme *a* concentration of the highly purified preparation of cytochrome oxidase. Of course, it is necessary to investigate more precisely as to the form and weight of the particle observed on or released from the green membrane. But inferring from the form and approximate weight, the particle observed on the green membrane is a subunit of the unit particle of cytochrome oxidase (UPCO) and it is thought that two of the particle polymerize to form UPCO during the process of purification.

As described previously, main molecular state of cytochrome oxidase purified from the green membrane by ammonium sulfate fractionation in the presence of cholate was UPCO. When the solvent was replaced from cholate buffer to 0.25 % Tween 20-phosphate buffer, at first UPCO was the main molecular state of the fraction but polymerization of UPCO occurred gradually. The uniform preparation of molecular state of cytochrome oxidase, obtained by TAKEMORI *et al.* and ORII *et al.*, is thought to be a dimer of UPCO formed secondarily. And it is thought that the pretreatments, such as dialysis and Sephadex treatment, as used by TAKEMORI *et al.* and ORII *et al.*, respectively, of the purified preparation of cytochrome oxidase promote the formation of the dimer of UPCO. After removing the remaining UPCO and higher polymers etc. by centrifugation, the uniform preparation (dimers of UPCO) on ultracentrifugal analysis was obtained. The uniform preparation also polymerized gradually with lapse of time to form linear polymers and aggregates. With the polymerization the oxidase activity gradually decreased, as suggested by ORII and OKUNUKI (6).

The above results are diagrammatically summarized in Fig. 15. On

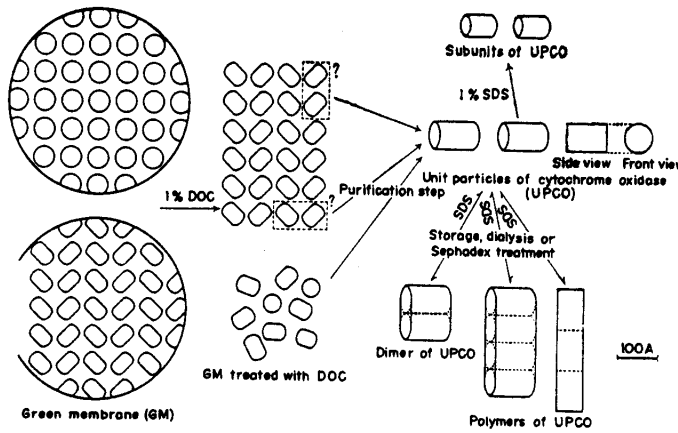

Fig. 15. Diagrammatic representation of the surface structure of the green membrane, DOC-treated green membrane and the molecular states of cytochrome oxidase.

On the surface of the green membrane, regular arrangement of small particles, measuring approximately 60 Å in average diameter, is observed. These particles are frequently arranged in a woven structure. When the green membrane is suspended in a 1 % deoxycholate solution, the membrane structure is degraded and converted to flat sheets. On the sheets the particles are mostly arranged in woven structure, sometimes the particles take a randon position and are released from the sheet. The unit particle of cytochrome oxidase are considered to be constructed from two of these particles. The smallest unit of active cytochrome oxidase (UPCO) has a cylindrical form (radius at the base, 70 Å in average ; height, 95 Å in average) in a configuration. Chain-like polymerization of the UPCO has occurred frequently. In the tube No. 6 (22.6 S position) of sucrose gradient ultracentrifugation of cyt. oxidase, dimers of UPCO are collected. In the tube No. 16 (5.7 S possition) of sucrose gradient ultracentrifugation of SDS-treated cytochrome oxidase, subunits of UPCO are collected. The subunit is considered to be a half of the unit particle of cytochrome oxidase.

the electron micrographs these particles were observed as an irregular form of particles which bore a resemblance to an ellipsoid or cylinder, but in the diagram these particles are dipicted as an elliptic cylinder or cylinder for convenience sake.

Concerning the electron microscopical studies on the molecular structure of the purified cytochrome oxidase, the authors have reported several times since 1966 (28—33). The studies were also conducted by MCCONNEL *et al.* (34), but their preparations of cytochrome oxidase were not a dispersed form but sheet-like aggregates similar to those illustrated in Fig. 10.

It is to be expected that studies on the correlation between fine structures and biochemical properties of the purified cytochrome oxidase and the cytochrome oxidase organized in a membrane can be promoted to a higher degree by using high resolution electron microscopy with biochemical methods as reported in this series of papers.

SUMMARY

For the purpose to reveal the correlation between molecular structure and biochemical functions of cytochrome oxidase the author studied purified cytochrome oxidase by using high resolution electron microscope and biochemical methods.

1. Cytochrome oxidase was purified from the cytochrome oxidase-rich submitochondrial membrane (green membrane), obtained from beef heart mitochondria, by three different methods; modification of the method of OKUNUKI *et al.*, method of FOWLER *et al.* and modification of the method of JACOBS *et al.* All the preparations showed a high specific activity under appropriate conditions and consisted mainly of small particles measuring approximately 80 to 90 Å in diameter.

2. The particle, measuring approximately 80 to 90 Å in diameter, took a cylindrical form measuring about 70 Å in diameter at the base and 95 Å in height in an appropriate condition. Many experimental results indicate that the particle is the smallest, fundamental unit of the active cytochrome oxidase. For this reason it was designated as the unit particle of cytochrome oxidase (abbreviated as UPCO).

3. The molecular weight of the unit particle, calculated from its volume and average density (1.24) of lipoproteins (3 : 7), was about 270, 000. The value was roughly twice the minimum molecular weight of 128, 000 calculated from the heme *a* content. Accordingly, it is considered that the unit particle contains two heme *a* molecule and two copper atoms.

4. It was suggested electron microscopically that the particle collected in the 22.6 S position by sucrose gradient ultracentrifugal analysis was a dimer of the unit particle of cytochrome oxidase and also that the particle collected in the 5.7 S position was a half of the unit particle of cytochrome oxidase.

5. It was also suggested that the particle observed on the green membrane was a subunit of cytochrome oxidase, containing one heme *a* and one copper atom, and the unit particle of cytochrome oxidase was constituted of two of the particles observed on the green membrane. Namely, the results indicate that the molecular state of cytochrome oxidase on the green membrane apparently differs from that of the purified cytochrome oxidase.

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ILLUSTRATIONS

Fig. 4. Cytochrome oxidase purified from beef heart mitochondria by the method of repeated ammonium sulfate fractionation in the presence of cholate-buffer solution and dissolved in 1 % cholate-buffer solution. PTA negative staining, × 200,000

Fig. 5. Cytochrome oxidase purified from beef heart mitochondria (BHM) by the same method as described in Fig. 4 and dissolved in 0.25 % Tween 20—0.05 M phosphate buffer, pH 7.4. PTA negative staining, × 200,000

Fig. 6. Monomers, dimers, polymers and subunits of cytochrome oxidase. PTA negative staining, ×600,000

a) An isolated unit particle of active cytochrome oxidase (UPCO) and a chain consisted of 6 UPCO. b) A chain consisted of 5 UPCO. c) Four isolated UPCO (side view) and a chain of UPCO. d) An isolated UPCO and a chain consisted of 4 UPCO. e) Three isolated UPCO (two of them, front view). f) Two isolated UPCO. One of them, front view and the other, side view. The side view of UPCO suggests that UPCO consists of two subunits. g) An isolated UPCO (front view) and a dimer of UPCO (front view). The picture shows that the dimer consists of two UPCO. h) Three dimers of UPCO. i) A dimer. j) Two dimers. k) Two dimers. These particles show that the dimer consists of two UPCO. l, m) Three and one subunit of UPCO, respectively.

Fig. 7. Rod shape particles of cytochrome oxidase which are possibly biuld up from UPCO by chain like-arrangement of the particles during storage in the medium of 0.25 % Tween 20-phosphate buffer, × 240,000

Fig. 8. Cytochrome oxidase collected in Tube No. 6 (22.6 S position) by sucrose gradient ultracentrifugation. These particles are likely to be dimers of the UPCO. PTA negative staining, × 400,000

Fig. 9. Subunits of cytochrome oxidase, which were obtained from cytochrome oxidase by treating with 1 % SDS and collected in the Tube No. 16 (5.7 S position) by sucrose gradient ultracentrifugation. PTA negative staining, × 394,000

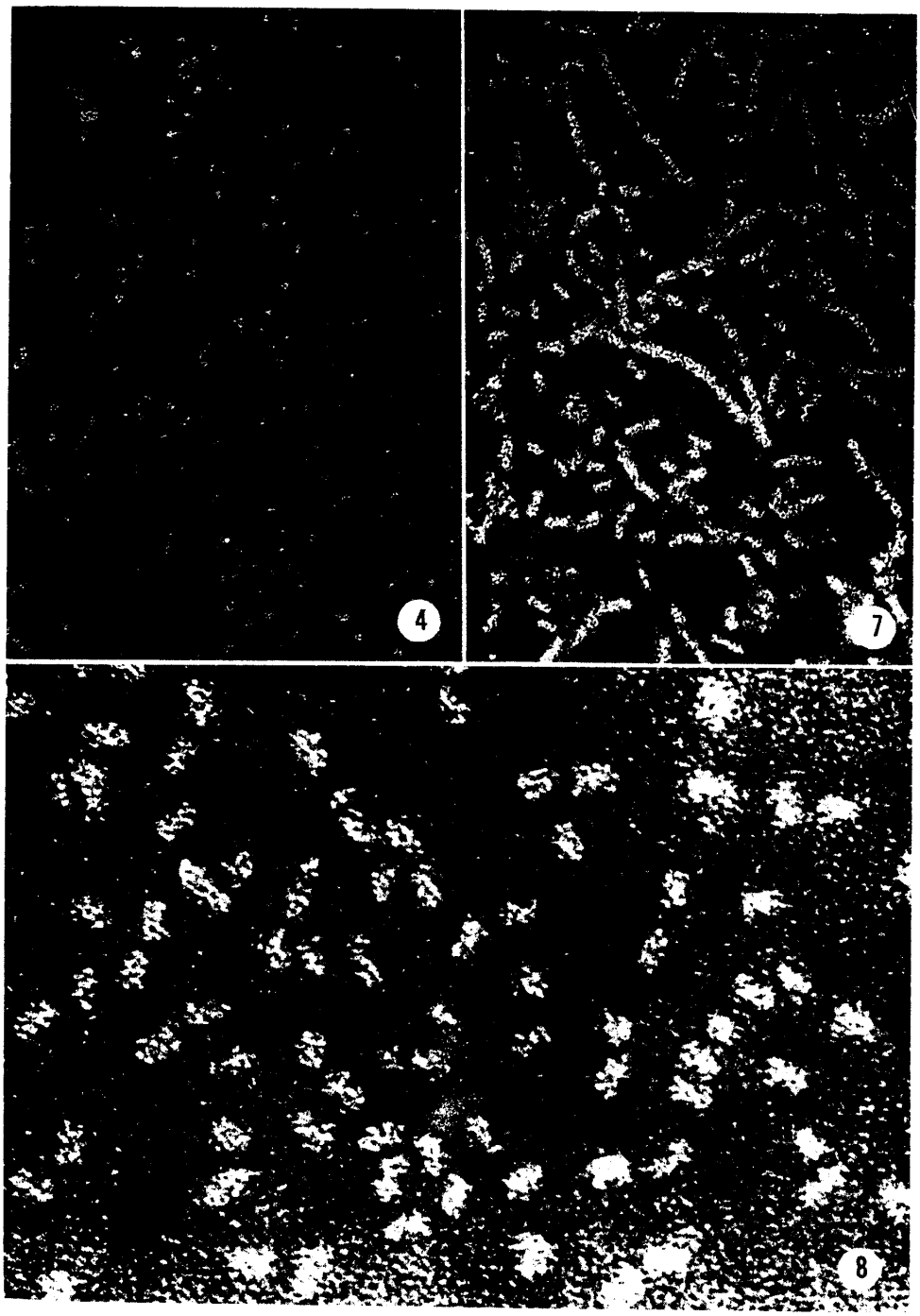
Fig. 10. Cytochrome oxidase purified from BHM by the method of ammonium sulfate fractionation in the presence of deoxycholate. In this preparation cytochrome oxidase is observed as sheet-like fragments. PTA negative staining, × 110,000

Fig. 11. Sheet-form cytochrome oxidase (as observed in Fig. 10) treated with 0.2 % sodium dodecyl sulfate. Rod shape particles similar to the particles in Fig. 7 are obesrved. PTA negative staining, × 226,000

Fig. 12. Partially purified cytochrome oxidase obtained from cytochrome oxidase-rich submitochondrial membrane (green membrane) by treating with potassium deoxycholate and potassium chloride. Isolated particles similar to UPCO and their aggregates are observed, × 200,000

Fig. 13. Cytochrome oxidase purified from green membranes by treating with Triton × 100 as described in the text. Isolated particles similar to UPCO and their aggregates are observed, × 200,000

Fig. 14. Green membrane suspended in 1 % potassium deoxycholate, pH 8.0. Sheets in which small particles are arranged in woven structure with center to center distance of about 70 Å and arranged at random, are observed. In some place the small particles are isolated from the sheets, ×200,000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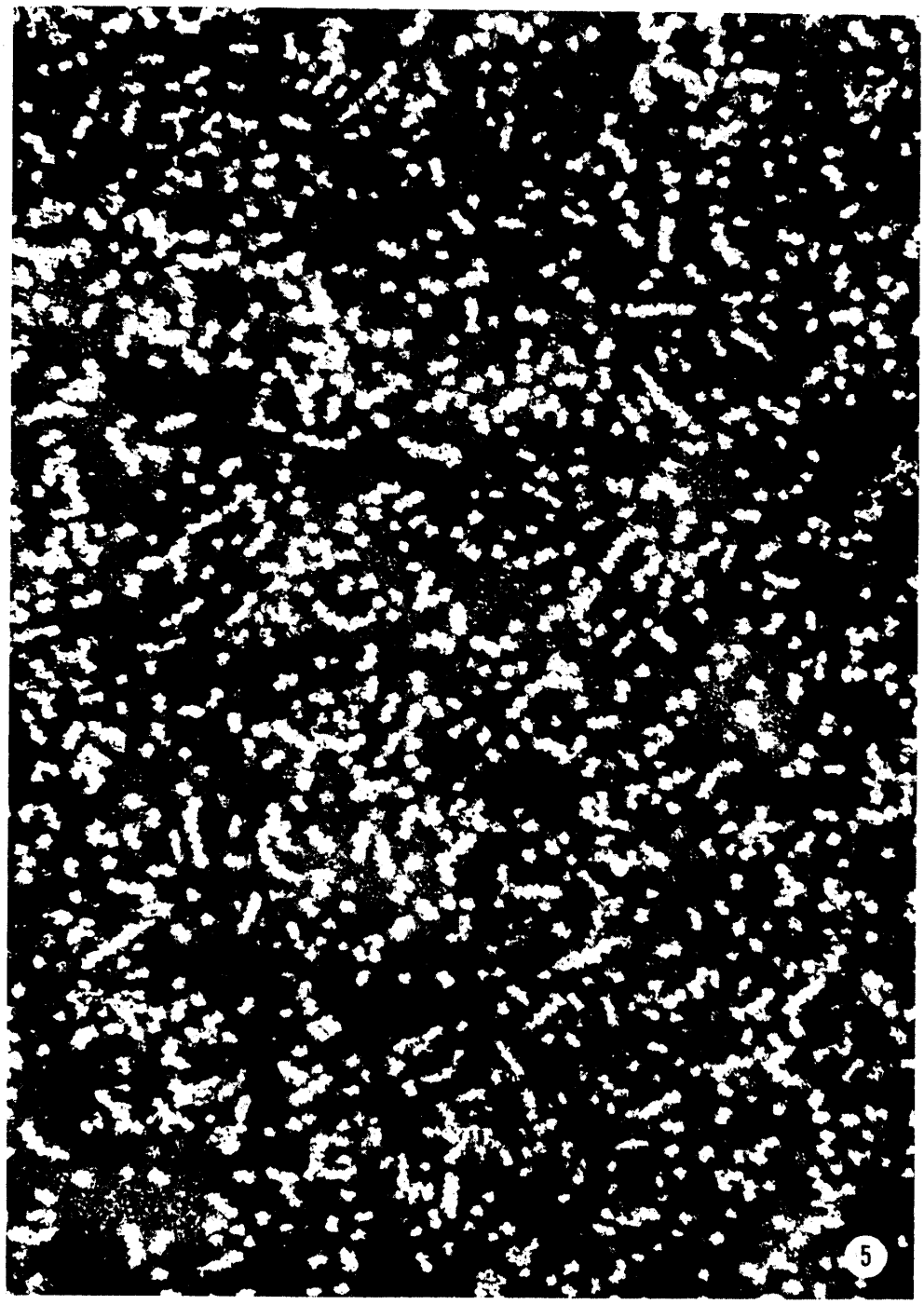
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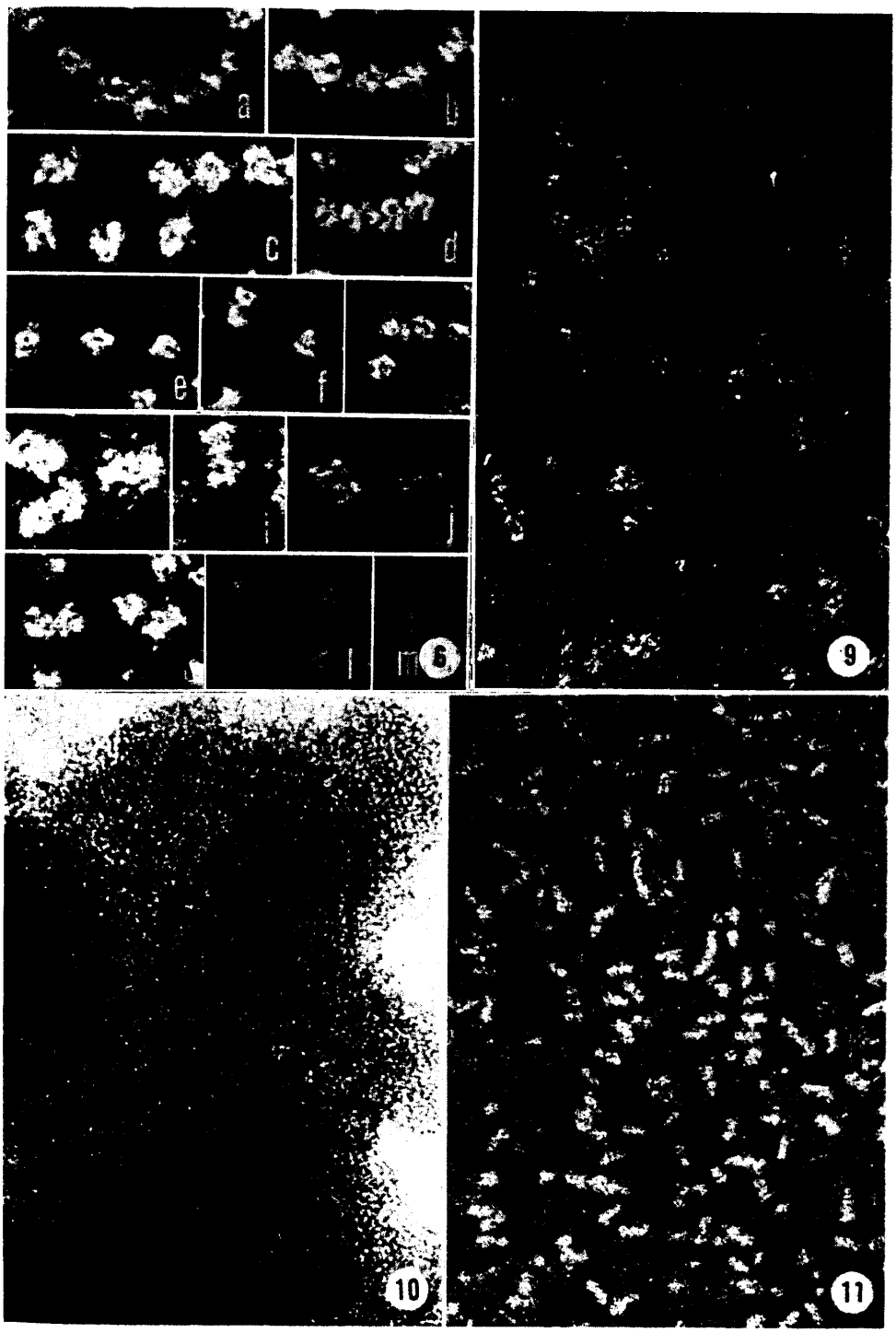
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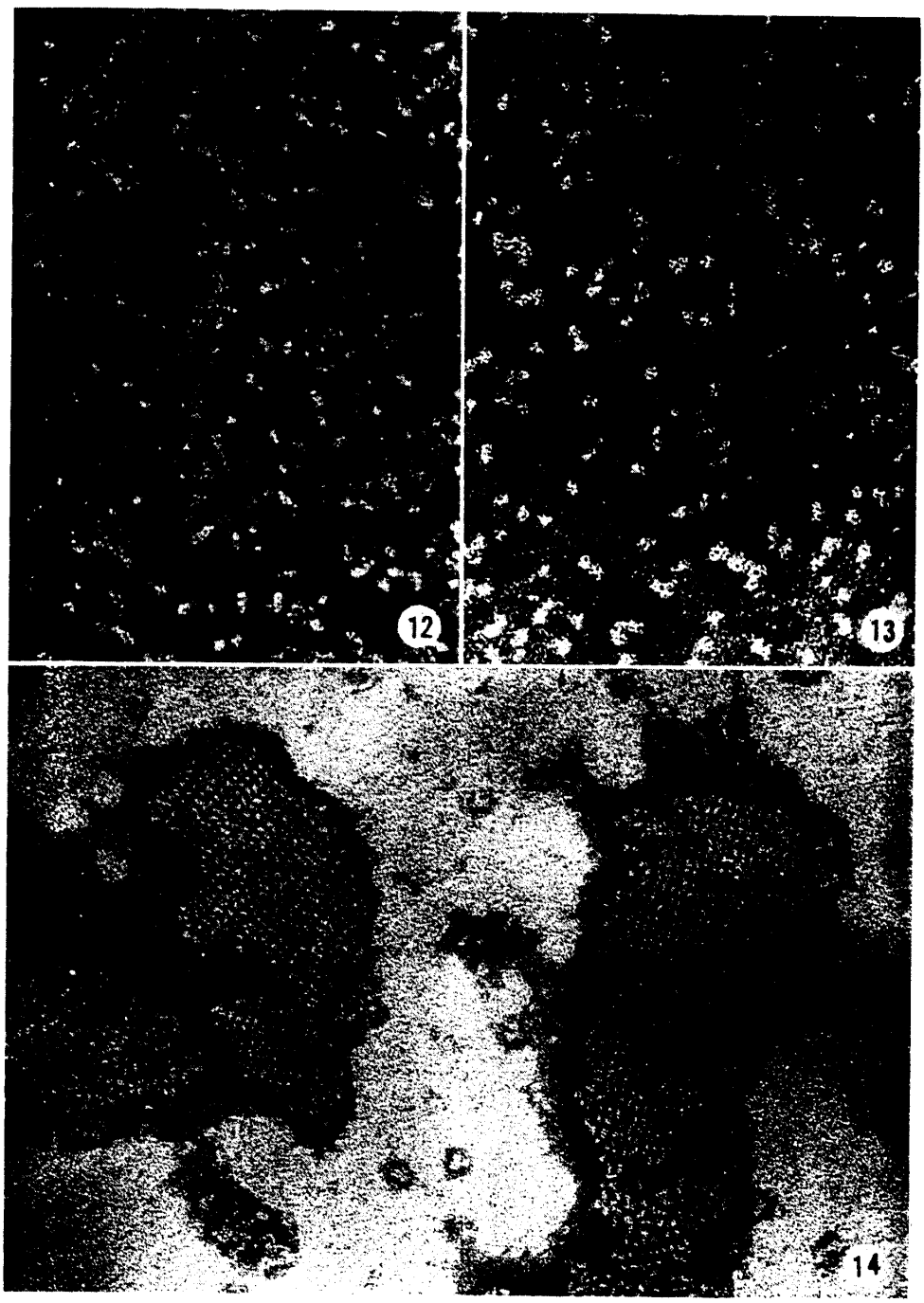
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