A Model Mosaic Membrane: Cytochrome Oxidase[1]

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Abstract

During purification of a lipid-free cytochrome oxidase, beef heart mitochondrial membranous structure was broken down through use of the nonionic detergent Triton, together with KCl. After removal of lipid, the purified cytochrome oxidase appears as 90Å diameter globules or as assemblies of rod-like structure with the same thickness. Upon addition of mitochondrial phospholipid, structural transformation of the enzyme occurs and enzyme activity is enhanced. Three phases of transformation in structure depending on the amount of phospholipid added are observed: 1) at 0-20 g Atom P of phospholipid/mole cytochrome oxidase there is transformation from swollen particles or rod-like elements (200Å) to sheets made up of 50Å globules; 2) at 20-65 g Atom P/mole, the 50Å globules are evenly dispersed to form mosaic membrane vesicles; 3) at higher than 65 g Atom P/mole, there is excess phospholipid around the membrane vesicle, and the subunits appear to be 30-50Å in diameter. During this transformation there is a regular increase in activity which attains maximum at 65 g Atom P/mole in oxidase and this activity remains unchanged even at higher phospholipid concentration up to 235 g Atom P/mole. We conclude that the oxidase protein globules form a mosaic membrane with phospholipid interspersed between the globules.

Introduction

Purified cytochrome oxidase has been shown to be able to form membranes. Deoxycholate will disperse the membrane structure and after removal of deoxycholate the solubilized cytochrome oxidase is organized to form membrane vesicles (8). In the study of the relationship between membrane formation and ionic strength, we observed that the membrane formation could be accomplished in the presence of high concentration of nonionic detergent (10). By employing nonionic detergents, Tritons, together with KCl, a lipid-free cytochrome oxidase has been prepared (2, 10) and it appears as 90Å globules. In the investigation of phospholipid function in the cytochrome oxidase reaction system (2) we found that this lipid-free preparation is rather inert to its substrate, cytochrome c. Maximal activity can not be obtained using even lipid-cytochrome c (5) as its substrate if phospholipid is absent on the enzyme side (2). Enzyme activity toward either cytochrome c or lipid-cytochrome c increases on addition of phospholipid micelles to the enzyme. In the present study we discuss the relationship between the enzyme activity and amount of phospholipid present as well as the structural transformation of the enzyme on addition of mitochondrial phospholipids.

Materials and Methods

Mitochondria were isolated by the method of Löw and Vallin (7) and were stored in concentrated suspension at —20° C before use. Enzyme and phospholipid were prepared from mitochondria which were less than

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two weeks old. Lipid-free cytochrome oxidase was prepared as described by Sun *et al.* (10 with some modification (2). One mM succinate was added before sonication and 0.02 M Tris pH 7.4 was used instead of phosphate buffer. This enzyme contained only 2-3 g Atom P/mole cytochrome oxidase or 2-3% phospholipid (w/w) and it has heme a concentration of 8-9 mμ moles/mg of protein as determined by its differential spectrum using a millimolar extinction coefficient ΔA at 605-630 mμ at 13.1 (12).

Phospholipid micelles and the lipid-free cytochrome oxidase were soluble and could not be centrifuged out at 108,000 x *g* for 1 hr. To study the amount of phospholipid bound to cytochrome oxidase during membrane formation, different amounts of phospholipid were added to 40 mg of lipid-free cytochrome oxidase and the mixture was diluted with 0.25 M sucrose, 0.02 M Tris, pH 7.4 to 3 mg of protein per ml. The mixture was sonicated at maximal output with a Branson's Sonifer for five 30 sec intervals and centrifuged at 108,000 x *g* for 30 min. The pellet was resuspended in sucrose-Tris buffer and centrifuged at the same speed. After three washes all free lipid and free cytochrome oxidase which were not in the membrane were washed out.

Protein was determined according to Yonetani (13). Molecular weight of cytochrome oxidase was based on the value of 72,000 (3). Phospholipid micelles were prepared by the sonication method of Fleischer and Fleischer (6). Cytochrome oxidase activities were assayed polarographically in the following mixture at pH 6.5: 16 mM potassium phosphate; 10 mM potassium citrate; 0.80 mM EDTA; 13 mM potassium ascorbate; 1.11 mM TMPD; 15 μM cytochrome c in a total volume of 1.8 ml using a Gilson oxygraph at 37° and the enzyme in the range of 5-20 μg.

Tritons and cytochrome c Type III were purchased from Sigma Chemical Company. Other chemicals were reagent grade.

Samples negatively stained with phosphotungstate, pH 6.8 were prepared for electron microscopy according to the procedure of Cunningham and Crane (4) and observed with a Philips EM 300.

Results

Membranes of mitochondrial electron transport particles (ETP) (Fig. 1) were split into two membranous fractions by using Triton X 114 and KCl as described by Prezbindowski *et al.* (9). Further purification of the green fraction results in a lipid-free cytochrome oxidase which appears as 90Å diameter globules or assemblies of rod-like structure with the same thickness (Fig. 2). This lipid-free enzyme has low activity which can be recovered upon addition of phospholipid. Figure 3 shows the relationship between the enzyme activity and the amount of phospholipid added. Three phases of the enzyme activity and enzyme structure related to the amount of phospholipid added can be observed. At low amount of phospholipid (0-20 g Atom P/mole cytochrome oxidase), the 90Å globules and rod-like elements began to swell to the size of

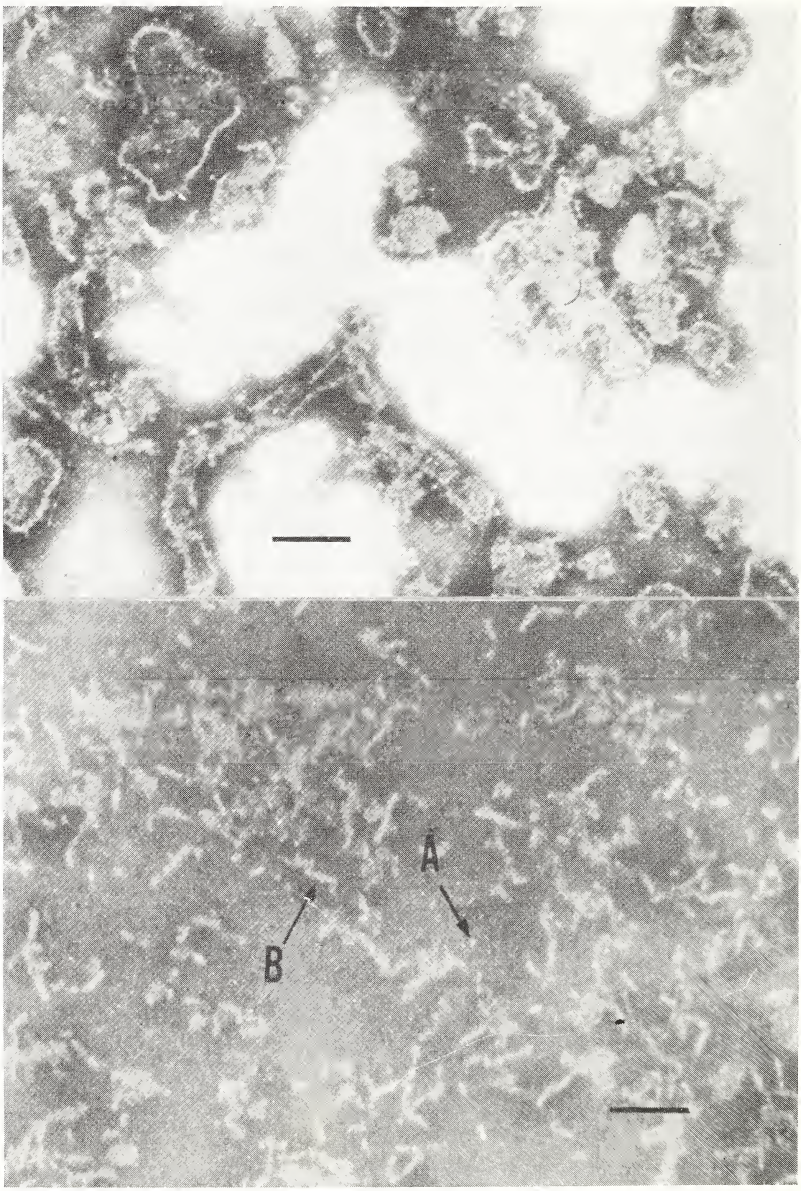

FIGURE 1. *Mitochondrial membrane fragments. Phosphotungstate. X 103,700. Marker 1000Å.*

FIGURE 2. *Purified lipid-free cytochrome oxidase showing 90Å globules and rod-like structure made of 90Å subunits. Phosphotungstate. X 103,700. Marker 1000Å. Arrow A indicates 90Å enzyme globules; arrow B, a rod-like element with 90Å subunits.*

200Å in diameter and gradually to transform to a small sheet of membrane made up to 50Å globules (Fig. 4). Addition of phospholipid rapidly changed the activity of the enzyme (Fig. 3). With addition of more phospholipid to the enzyme, (20-65 g Atom P/mole cytochrome oxidase), the lipoprotein globules became organized to form a larger sheet of membrane with the same size (50Å) globules visible in surface views (Fig. 5).

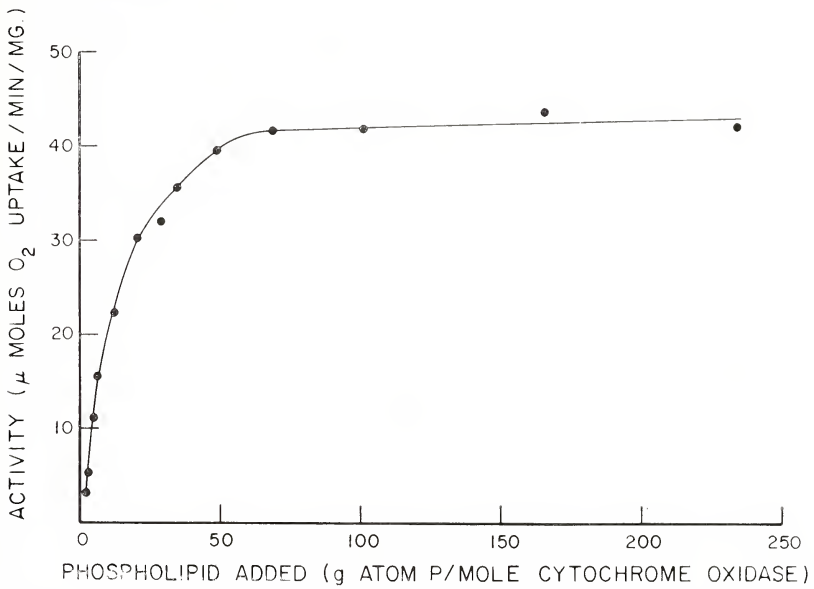

Figure 3. *Enzyme activity of a purified lipid-free cytochrome oxidase preparation and the amount of phospholipid added. Phospholipid was added and incubated at cold for 5 minutes before dilution for assay. This enzyme contained 1.5 mg of Triton x 100 per mg of protein.*

At phospholipid concentrations higher than 65 g Atom P/mole cytochrome oxidase, excess of phospholipid was observed around the membrane vesicles and the subunit was reduced to about 30-50Å diameter (Fig. 6). Activity at this stage remains at the maximum and addition of more phospholipid (up to 235 g Atom P/mole cytochrome oxidase) did not increase activity.

When the lipid-free enzyme was sonicated with increasing amounts of phospholipid micelles (up to 235 g Atom P/mole cytochrome oxidase) membrane obtained by centrifugation followed by several washes to remove unbound phospholipid and cytochrome oxidase showed regular increase in its phospholipid content up to a constant value of 65 g Atom P/mole cytochrome oxidase which corresponds to about 40% of lipid in the membrane (Fig. 8). Any excess phospholipid was washed out and in no experiment did the value of bound phospholipid in the washed membrane increase beyond this constant value. No phospholipid micelle struc-

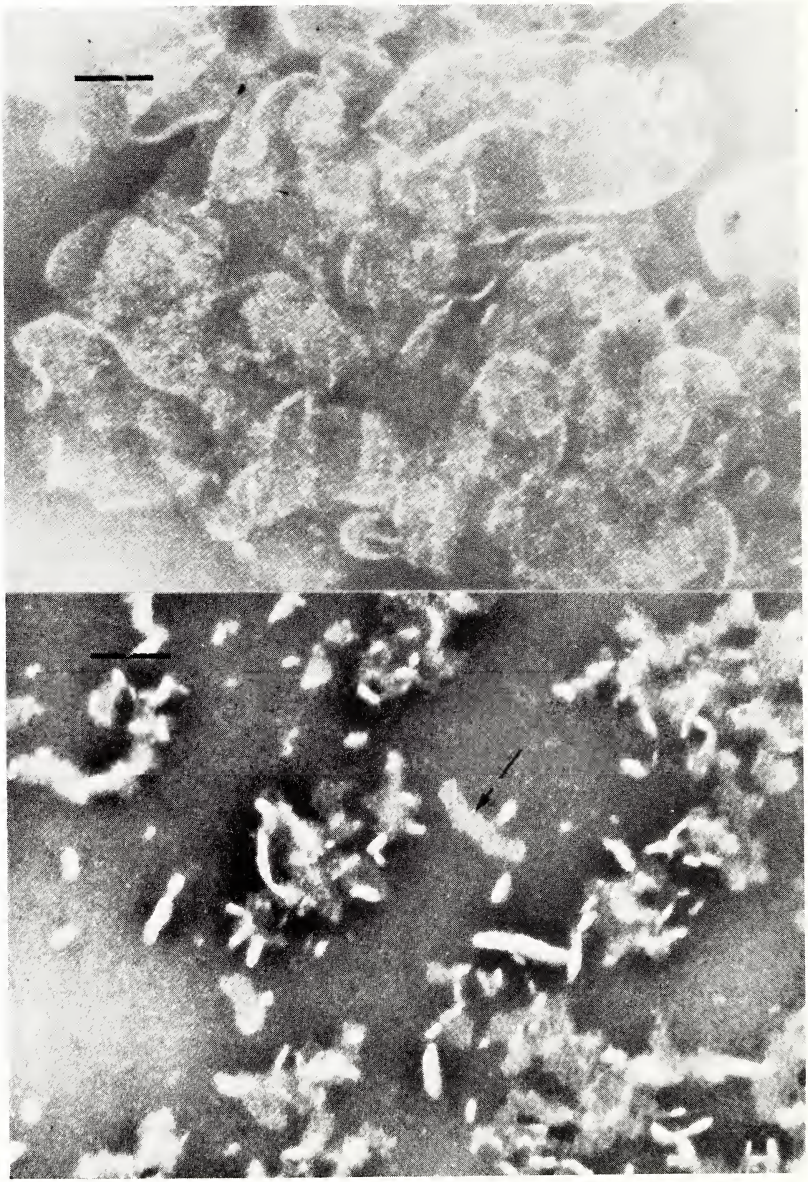

FIGURE 4. *Cytochrome oxidase with 10 g Atom P of phospholipid per mole of enzyme (or 10% phospholipid, w/w) showing swollen particles, rod-like elements and small membrane with 50Å subunits. Phosphotungstate. X 103,700. Marker 1000Å. Arrow points on a swollen rod-like element with 50Å subunits.*

FIGURE 5. *Cytochrome oxidase with 31 g Atom P of phospholipid per mole of enzyme (or 25% phospholipid, w/w) showing vesicle membrane with 50Å subunits phosphotungstate. X 103,700. Marker 1000Å.*

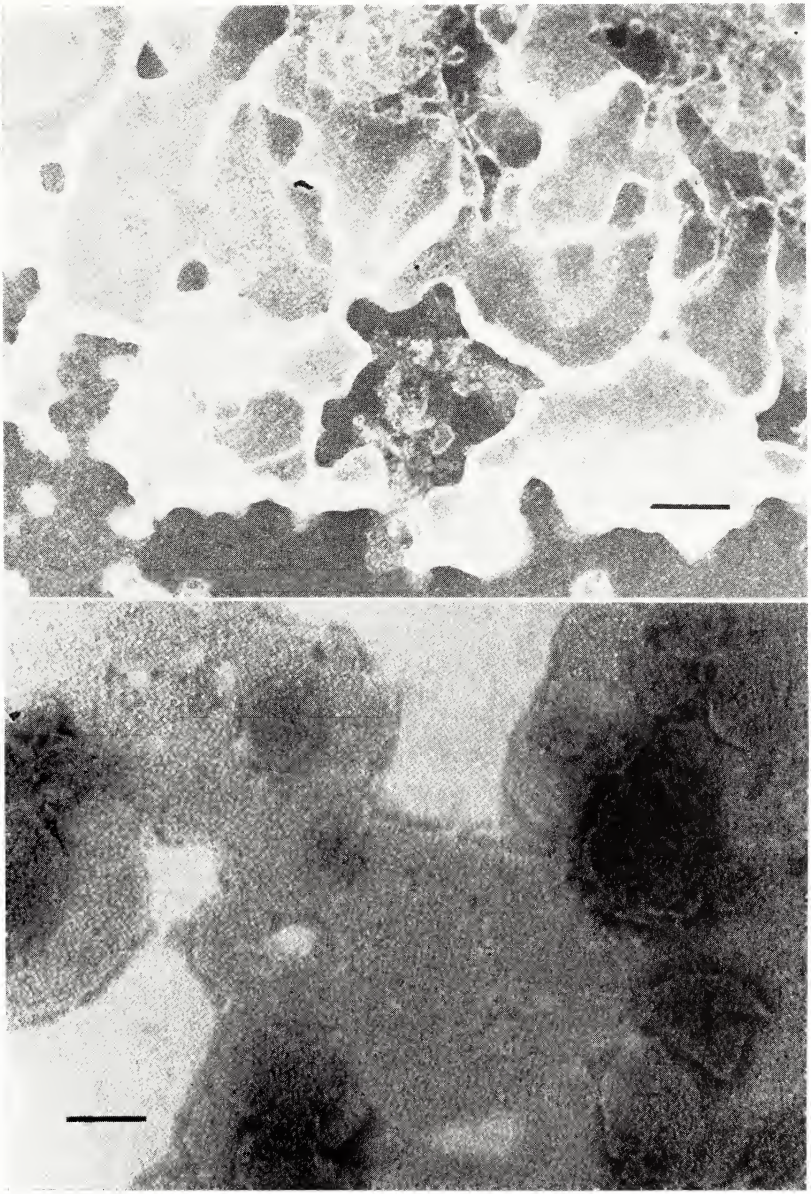

FIGURE 6. *Cytochrome oxidase with 235 g Atom P of phospholipid per mole of enzyme (70% phospholipid, w/w) showing excess of phospholipid micelles around membrane vesicles. Phosphotungstate. X 103,700. Marker 1000 Å.*

FIGURE 7. *Membraneous cytochrome oxidase after washing away the free phospholipid micelles. Excess of phospholipid (235 g Atom P of phospholipid per mole of cytochrome oxidase) was added to lipid-free cytochrome oxidase as described in the text and centrifuged at 108,000 x g for 30 minutes and 3 washes to remove unbound phospholipid. Phospholipid content decreased to 65 g Atom P of phospholipid per mole of cytochrome oxidase. No micelle is seen around membrane vesicles. Note 50 Å subunits. Phosphotungstate. X 103,700. Marker 1000 Å.*

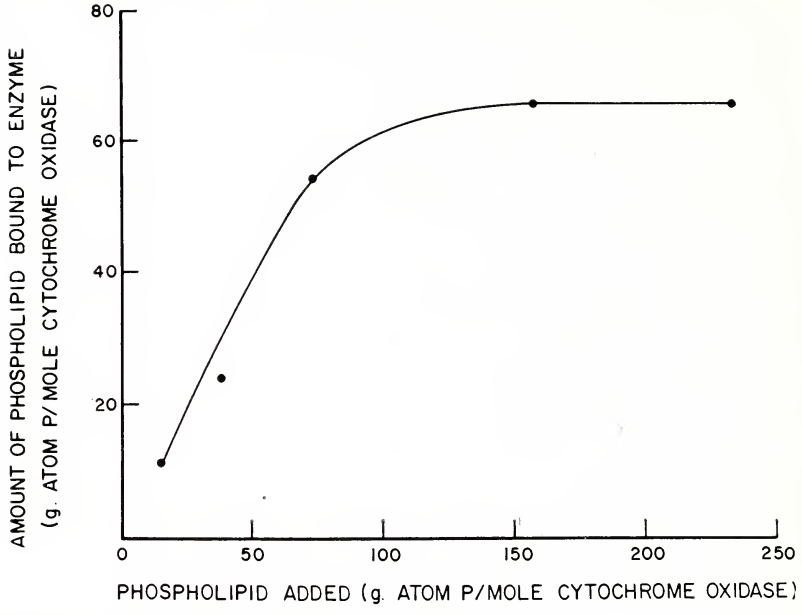

FIGURE 8. *Amount of phospholipid bound to cytochrome oxidase.*

ture was observed around the edge of the membrane vesicles after washing (Fig. 7). Subunits of the membrane consistently showed a diameter of 50Å and completely filled the sheet surfaces.

Enzyme with phospholipid added (vesicle membraneous form) was found to be much more stable to heat treatment than the lipid-free enzyme. Figure 9 shows the activity change of both lipid-free and lipid-added enzymes after incubation at different temperatures. After incubation at the temperature and time indicated, lipid-free enzyme was incubated with phospholipid at 4° C for 10 minutes before enzyme assay. Lipid added cytochrome oxidase has 90%, 81% and 19% of the original activity after 20 minutes incubation at 30° C, 38° C and 50° C respectively while the lipid-free enzyme had only 75%, 40% and 0% of original activity. The cytochrome oxidase of washed beef heart mitochondria was also found to be more stable to heat treatment (Fig. 10).

Discussion

The capability of cytochrome oxidase to form membrane was demonstrated earlier by McConnell *et al.* (8) and our laboratory (10). Membrane formation demonstrated in the present study requires the presence of both protein and phospholipid. Phospholipid content is also a critical factor for enzyme activity. Deficiency in lipid content causes the enzyme molecules to cluster together and may prevent access of substrate to the active site. As can be seen in Figures 2 and 4, on addition of phospholipid

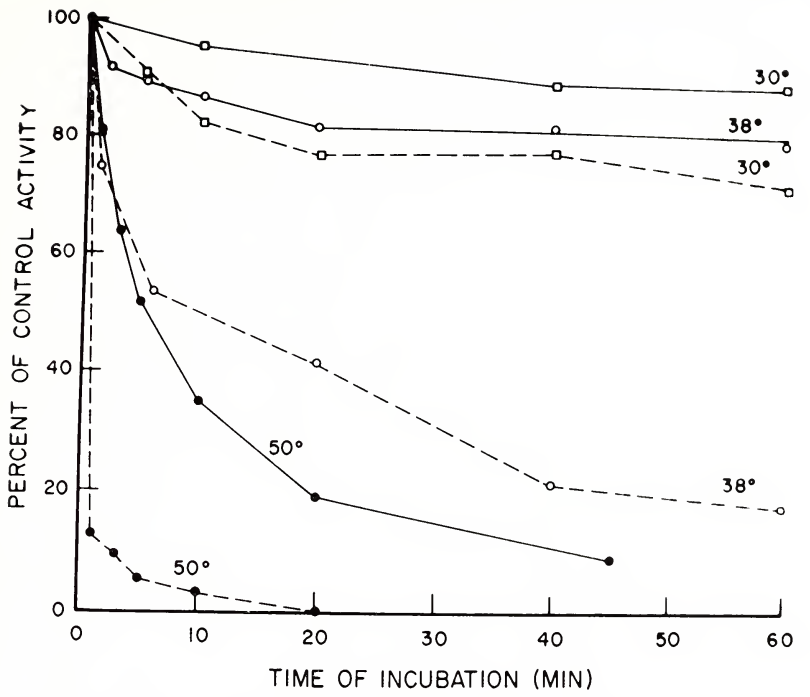

FIGURE 9. *Stability of lipid-free and lipid-added cytochrome oxidase activity. Enzyme was incubated at temperature indicated at protein concentration of 1 mg/ml. 120 g Atom P of phospholipid/mole enzyme was added before or after incubation. Solid lines are the lipid-added cytochrome oxidase activity; dotted lines the lipid-free cytochrome oxidase activity. Control activity as 100% was 41.2 μmoles O_2 uptake/min/mg.*

micelles the enzyme swells and 50Å lipoprotein globules are loosely packed into a sheet to form membrane vesicles. In this sheet form the substrate has ready access to active sites on the enzyme as shown by the high cytochrome oxidase activity.

Further addition of phospholipid micelles (20-65 g Atom P/mole enzyme) allows a more even dispersion of the enzyme in the form of mosaic membrane vesicles with 50Å globules evenly dispersed as shown in Figure 5. Thin sectioning of this membrane shows unit membrane structure which is 50-55Å thick. Once the phospholipid requirement for the membrane formation and maximal activity is reached, further addition of phospholipid does not further increase the activity. The negative staining data show excess phospholipid micelles around the edge of membrane vesicles. The globule size on the membrane is somewhat reduced to 30Å. This reduction of apparent globule size may indicate an excess of phospholipid among the lipoprotein globules.

Sonification facilitates the interaction of cytochrome oxidase protein and phospholipid to form tightly bound membrane. The unbound lipid is still in the state of micelles and can be easily removed by wash-

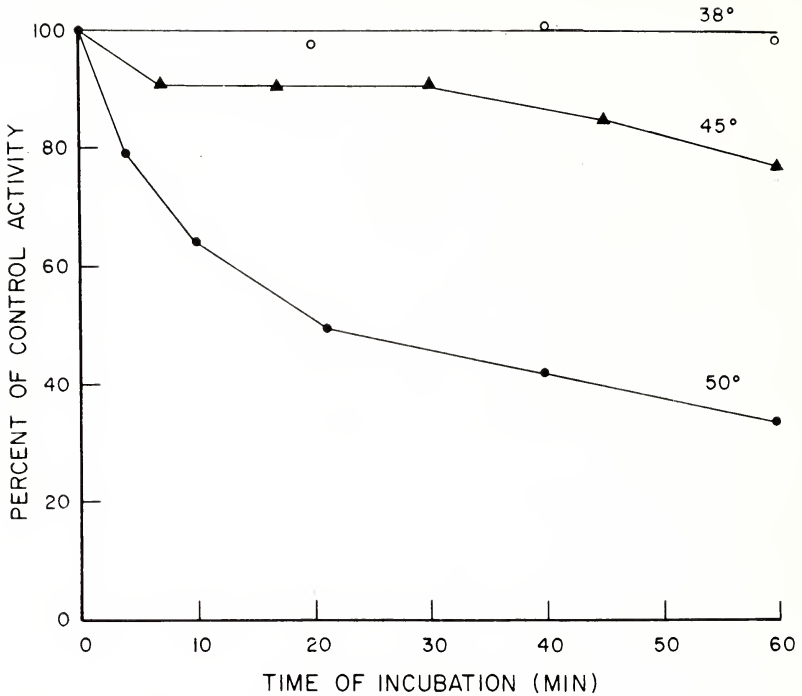

FIGURE 10. *Stability of cytochrome oxidase in a beef heart mitochondrial preparation. Mitochondria were adjusted to protein concentration of 4 mg/ml and incubated at indicated temperature. Control activity as 100% was 5.20 μmolcs 0_2 uptake/min/mg protein.*

ing. A consistent value of 60-70 g Atom phosphorus of phospholipid per mole of cytochrome oxidase was obtained after washing when an excess of phospholipid was added (Fig. 8). The same saturated value of phospholipid was obtained for the maximal activity of cytochrome oxidase (Fig. 3). The electron micrograph (Fig. 7) showed 50Å globules on the washed membrane surface and no phospholipid micelles were observed.

The 90Å particles characteristic of the lipid-free enzyme are equivalent to a molecular weight of 290,000 daltons if the protein density is assumed as 1.25. Molecular weight of monomer cytochrome oxidase is 72,000 (3). Thus, our lipid-free cytochrome oxidase may exist as a tetramer. Ball *et al.* (1) suggested that cytochrome oxidase might be a tetrapolymer consisting of four identical hemoprotein submolecules. Takemori *et al.* (11) also suggested that their $Emasol_{1130}$ solubilized cytochrome a as a pentamer based on sedimentation experiments. After cytochrome oxidase is reorganized into membrane the globule size appears to be 50-60Å which is equivalent to the molecular weight of 50,000-85,000. This value agrees with the molecular weight of 72,000 suggested by Criddle and Bock (3). Takemori *et al.* (11) suggested that cytochrome oxidase existed as a monomer with only one heme group

per molecule in mitochondrial particles, it tended to polymerize after extraction from mitochondria with bile salt and purification with ammonium sulfate. Similar polymerization probably also occurs in our preparation and depolymerization occurs again during reconstitution.

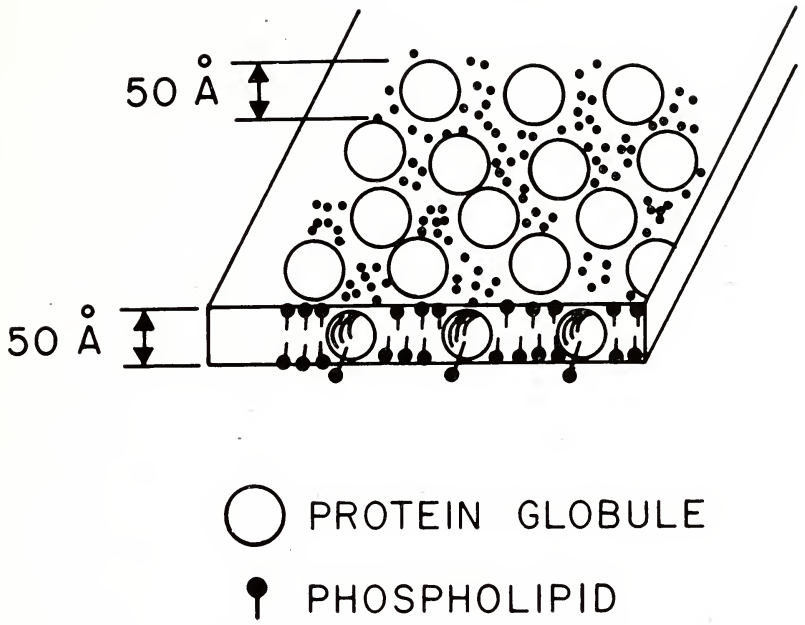

FIGURE 11. *A model mosaic membrane: cytochrome oxidase.*

In a study of the heat stability of the enzyme we found that the membraneous form of cytochrome oxidase (made by addition of phospholipid to lipid-free cytochrome oxidase) was more stable than the lipid-free enzyme. Figure 9 shows the cytochrome oxidase activity after incubation at a series of temperatures for enzyme to which phospholipid was added before or after incubation. The membraneous cytochrome oxidase has 90%, 81% and 19% of the control activity while the incubated lipid-free enzyme has only 75%, 40% and 0% of the control activity after incubation for 20 minutes at 30° C, 38° C and 50° C respectively. Stability of cytochrome oxidase activity in mitochondria is much greater than in the purified lipid-free state (Figs. 9 and 10).

Wallach and Gordon (14) proposed that in membrane formation the various subunits were assembled to form a protein lattice penetrated by cylinders of lipid. We propose that a model membrane made from interaction of purified membrane protein, cytochrome oxidase, and phospholipid may simulate in part the membrane structure in mitochondrial cristae as in Figure 11. Upon addition of phospholipid to lipid-free cytochrome oxidase a complex forms between these two components, and structural transformation occurs parallel to and in as pronounced a

fashion as the enhancement of enzyme activity. Protein molecules, as membrane subunits, are assembled to form mosaic structure with phospholipid. After formation of protein-lipid complex, the enzyme is much more insensitive to heat treatment than the lipid-free enzyme. The membrane of cytochrome oxidase may represent a model for some biological membranes in vivo. Further comparison between this membrane model and the mitochondrial cristae membrane, with respect to sensitivity to enzyme attack (e.g., phospholipases and proteases) as well as comparison of molecular conformations will be necessary before we can tell how closely the model membrane reflects the structure in cristae.

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