BBA 45675

PHYSICAL STATE OF CYTOCHROME OXIDASE

RELATIONSHIP BETWEEN MEMBRANE FORMATION AND IONIC STRENGTH

F. F. SUN, K. S. PREZBINDOWSKI, F. L. CRANE AND E. E. JACOBS

Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907, and Department of Biophysics, Stanford University, Stanford, Calif. (U.S.A.)

(Received December 18th, 1967)
(Revised manuscript received February 26th, 1968)

SUMMARY

Preparations of lipid-containing and lipid-depleted cytochrome oxidase from beef heart mitochondria through use of Triton non-ionic detergents is described. The lipid-containing oxidase is shown to form either a membranous or dispersed state. The dispersed state requires the presence of salt whereas the membranous condition is obtained by reducing the ionic strength. The membranous form shows low activity in comparison to the dispersed form. Typical unit membrane structure can be shown in sections of the membranous oxidase or in reaggregated membranous oxidase even though the lipid content is as low as eight percent. Both the dispersed and membranous oxidase show 50-Å globules by negative staining whereas the lipid-free oxidase shows strings of 20 Å diameter.

INTRODUCTION

McCONNELL *et al.*[1] have recently demonstrated that purified cytochrome oxidase subunits were able to reorganize themselves into membrane-like materials. This process is dependent on the removal of solubilizing agent and the presence of phospholipids. GREEN *et al.*[2] extended this observation to many other lipoprotein membranes and used it as a basis to explain such phenomena as the reconstitution of the electron transport chain and the requirement of lipid for certain membrane-bound enzymes. According to this hypothesis, lipoproteins of the natural membrane can be dispersed by bile salts into their repeating units. Upon diluting, the repeating units interact with one another forming membrane sheets which are morphologically and enzymatically indistinguishable from the original membrane. However, in the absence of phospholipids, the repeating units lose their membrane-forming ability and exist only in a state of bulk phase in which the enzymic activity cannot be fully demonstrated.

In the investigation of lipid function in the cytochrome oxidase reaction, we have found that a soluble, lipid-containing, purified cytochrome oxidase prepared by non-ionic detergent from beef heart mitochondria tends to reaggregate into vesicular membranes. In contrast to the result of McCONNELL *et al.*[1], we observed that the

membrane formation can be accomplished in the presence of high concentration of non-ionic detergent provided the ionic strength in the solution is low enough. Thus, this phenomenon can be explained as ionic interaction of the lipoprotein subunits. The use of non-ionic detergents gives us the advantage that we can alter the concentrations of detergents and salts separately, so that we can give broader scope to a study of the physical state of the enzyme.

Enzymic analyses indicate that the oxidase in membranous state is rather inert. Only partial activity can be demonstrated when the enzyme exists in this state. Once trapped in a membrane, the enzyme subunits can no longer interact with micellar phospholipids. It remains to be determined whether the active dispersed state or the rather inactive membranous state is of greater significance in interpretation of cytochrome oxidase in a physiological system.

MATERIALS AND METHODS

Triton X-114 and Triton X-100 were supplied by Rohm and Haas Company, Philadelphia, Pa. Cytochrome *c* type III was purchased from Sigma Chemical Company. All other reagents used were reagent grade. Glass-distilled water was used throughout this investigation.

Protein was analyzed by the modified biuret method according to YONETONI[3]. Phospholipids were separated by thin-layer chromatography followed by phosphorus analysis with the method of BARTLETT[4]. Cytochrome oxidase activities were assayed spectrophotometrically by the method of SMITH[5] at pH 6.0. The concentration of heme *a* was determined by its differential spectrum using extinction coefficient ΔA at 605–630 mμ as 13.1 (ref. 6). Spectra were recorded on a Unicam SP-500 spectrophotometer. Triton X-100 concentration was measured by its ultraviolet absorption at 276 mμ. The extinction coefficient was taken as $E^{1\%}_{276\,m\mu} = 10$.

Enzyme preparation

1. *Pure membrane cytochrome oxidase.* Beef heart mitochondria were prepared according to the method of LOW AND VALLIN[7]. The frozen suspension was thawed and diluted with an equal volume of 0.25 M sucrose buffered with 0.01 M potassium phosphate (pH 7.4) (referred to hereafter as sucrose phosphate buffer). The diluted material was subjected to sonication (Branson sonifier; maximum output) for 3 min and centrifuged at 30000 $\times$ *g* for 30 min. The turbid supernatant and dark brown residue were discarded and the yellowish middle layer of mitochondria was collected. The washed mitochondria were suspended in sucrose phosphate buffer and protein concentration was adjusted to 30 mg/ml. Enough 10% aqueous Triton X-114 was added to make 0.6 mg/mg of protein; KCl was added to give an 0.2 M final concentration. The mixture was allowed to stand at 0° for 20 min and centrifuged at 78000 $\times$ *g* for 1 h. The clear supernatant and a tightly packed brownish residue were discarded and the middle, fluffy, cytochrome-rich fraction was collected. This fraction was washed twice with sucrose phosphate buffer and suspended in the same medium with protein concentration adjusted at 30 mg/ml.

Subsequent fractionation steps were similar to the procedure described by SUN AND JACOBS[8] for mitochondria. The washed fluffy fraction was treated with 10% aqueous Trition X-100 to achieve 1 mg/mg protein and solid KCl to 1 M. The mixture

was allowed to stand 20 min and centrifuged at 105000 × *g* for 30 min. The red supernatant containing almost all cytochrome *b* and c_1 was poured out and the green residue was collected and homogenized in sucrose phosphate buffer.

The same treatment was repeated. The protein concentration was kept at 20 mg/ml and Triton X-100 at 1 mg/mg protein *plus* 1 M KCl were added. The insoluble enzyme was collected by centrifugation. The second extraction removed some residual cytochrome *b* and c_1 and also reduced the phospholipid content. This fraction was referred to as membranous cytochrome oxidase because of its membranous appearance under electron microscopic examination.

2. *Pure dispersed cytochrome oxidase.* This preparation consists of a soluble enzyme made from the membrane cytochrome oxidase. The membranous enzyme was suspended in sucrose phosphate buffer with protein concentration adjusted to 30–35 mg/ml. Exactly 1.5 mg of Triton X-100 per each mg of protein were added. Solid KCl was then added to reach 1 M. The mixture was allowed to stand at 0° for 1–2 h and centrifuged at 105000 × *g* for 30 min. The dark green supernatant was saved and kept at —15°.

3. *Reaggregated cytochrome oxidase.* This consists of an insoluble preparation made by dialyzing the pure dispersed enzyme against 1000 vol. of 1% Triton X-100 buffered with 0.01 M neutral potassium phosphate for 2 h. The enzyme was collected by centrifuging at 105000 × *g* for 30 min.

4. *Lipid-free cytochrome oxidase.* This preparation was made almost free from lipids by using an extremely high concentration of Triton X-114. Beef heart mitochondria were washed and sonicated as described in the membranous enzyme section. The washed mitochondria were treated with 2.5 mg Triton X-114 (from a 10% aqueous solution) per each mg of protein *plus* KCl to the final concentration of 0.2 M. The mixture was centrifuged at 78000 × *g* for 1 h and the residue was collected.

The protein concentration of the green residue fraction was again adjusted to 30 mg/ml and sufficient 10% aqueous Triton X-100 was added for 1.2 mg per each mg of protein. Solid KCl was then stirred in to 1 M. After the salt was completely dissolved the mixture was centrifuged at 105000 × *g* for 30 min. The sedimented brownish residue was discarded and the dark green supernatant liquid was dialyzed for exactly 2 h against 100 vol. of 1% Triton X-100 containing 0.01 M neutral phosphate. More yellowish protein precipitated during this period and was removed by centrifugation at 105000 × *g* for 30 min. Prolonged dialysis at this stage should be avoided since it leads to the aggregation of cytochrome oxidase.

The clear dialysate was applied to a column of diethylaminoethyl-cellulose (DEAE-cellulose) column in the chloride form. The size of column depends on the volume of the dialysate. A 20 mm × 80 mm column was ordinarily used for approx. 50 ml of dialysate. The ion-exchange cellulose was equilibrated with 0.01 M neutral phosphate before packing into the column. The enzyme was then loaded and followed by washing with 10 vol. of 1% Triton X-100 containing 0.01 M neutral phosphate. The green band was eluted with 0.2 M neutral phosphate containing 1% Triton X-100 in minimal volume. The enzyme can be kept at —15° for 1 week without losing activity.

5. *Deoxycholate cytochrome oxidase.* This is a soluble cytochrome oxidase prepared by the deoxycholate method of FOWLER, RICHARDSON AND HATEFI[9].

The purity of each enzyme preparation was ascertained by measuring its heme *a*

content and band pattern in disc electrophoresis[10]. Pure cytochrome oxidase gives only two major bands in acrylamide gel (F. F. SUN AND F. L. CRANE, unpublished study). Any other bands were considered as impurities.

Micellar phospholipid preparation

Micellar phospholipid sol was prepared by the sonication method of FLEISCHER AND FLEISCHER[11].

Electron microscopy

Sectioned samples for electron microscopy were prepared according to the method of MOLLENHAUER[12] using an OsO_4 fixation period of 2 h. Sections were post-stained with 1% barium permanganate or $KMnO_4$. Samples negatively stained with phosphotungstate (pH 6.8 with KOH) were prepared according to the procedure described by CUNNINGHAM AND CRANE[13]. Samples were observed and photographed using the Philips EM 200.

RESULTS

Four different cytochrome oxidase preparations have been obtained through the use of Triton. The lipid-containing enzyme can exist in two states, depending on the salt concentration. The lipid-free enzyme can only exist in a soluble state. The chemical properties of the four preparations are listed in Table I.

TABLE I

PROPERTIES OF VARIOUS FORMS OF CYTOCHROME OXIDASE

Heme *a* contents are expressed in mμmoles/mg protein. Activities are expressed in μmoles cytochrome *c* oxidized per min per mg.

Preparation	*Heme a content*	*Phospholipid content* (%)	*Triton content* (%)	*Activity without phospholipid*	*Activity with phospholipid*
Mitochondria	0.97	26	—	3.49	5.87
Membranous oxidase	8.0	18	1.7	7.37	7.17
Dispersed oxidase	8.8	18	3.4	56.00	73.00
Aggregated oxidase	8.1	8	—	3.00	9.21
Resolubilized oxidase	8.1	8	—	27.36	64.00
Lipid-free oxidase	9.0	2	—	5.5	65.00
Reconstituted oxidase	8.9	21	—	46.80	52.00

Fig. 1 shows the electron micrograph of a negatively stained sample of the original membrane preparation. Since this fraction has not gone through a solubilization stage, it is likely that this preparation still bears some relation to the structure of the native enzyme. This fraction consists predominately of vesicular membranes with orderly arrays of 50-Å particles spreading over their surface. In certain areas, random agglomerates were also observed. The thin section of this fraction shows exclusively membranous appearance (Fig. 2). Each membrane is 55 Å thick with clearly visible unit membrane structure.

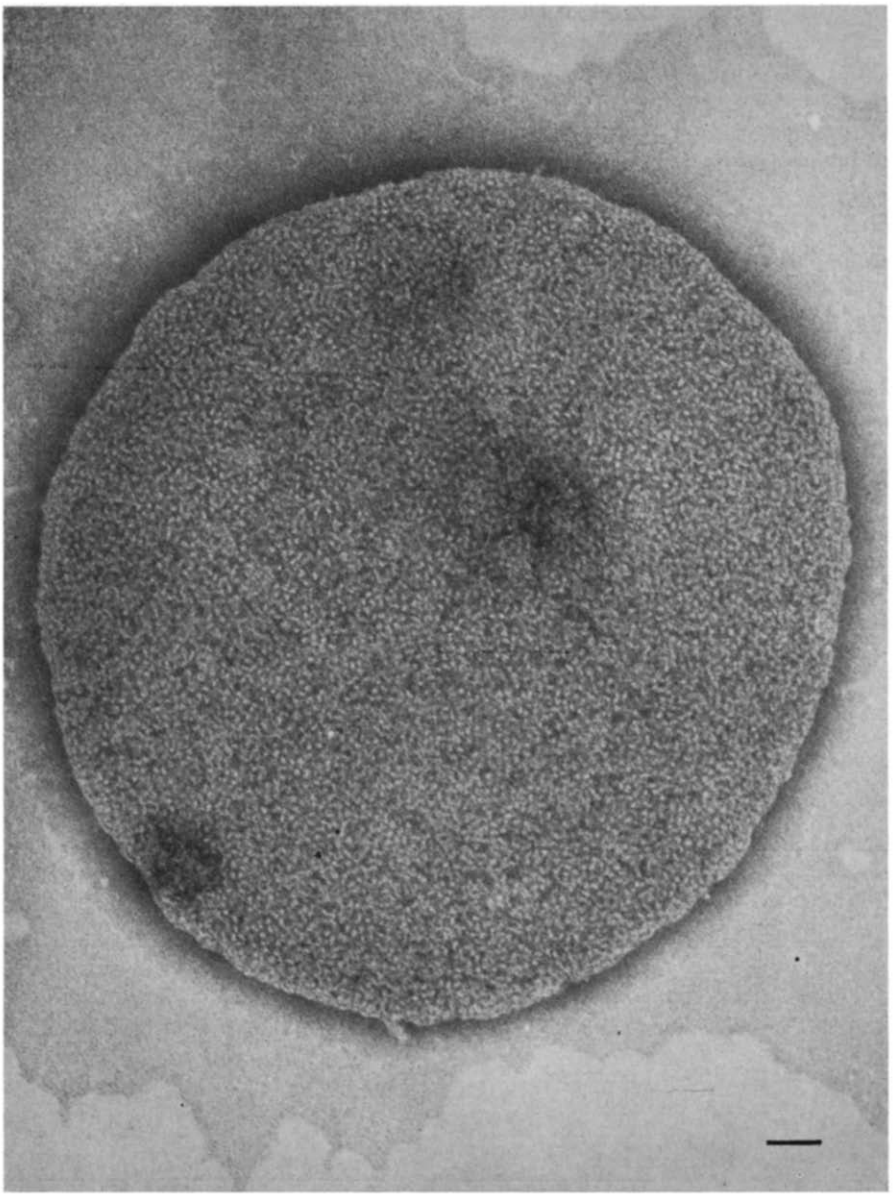

Fig. 1. Membranous cytochrome oxidase, negatively stained with 1% phosphotungstate. 50-Å, electron-translucent particles are spread over the surface of the vesicular membranes. Marker indicates 500 Å.

This fraction was not active even though the heme *a* content approaches a standard cytochrome oxidase preparation. Addition of phospholipid micelles had no stimulative effect. The lack of activity cannot be explained on the basis of the lack of phospholipids since this preparation contains approx. 20% of its weight as phospholipids. Determination of Triton showed that this preparation contained less Triton

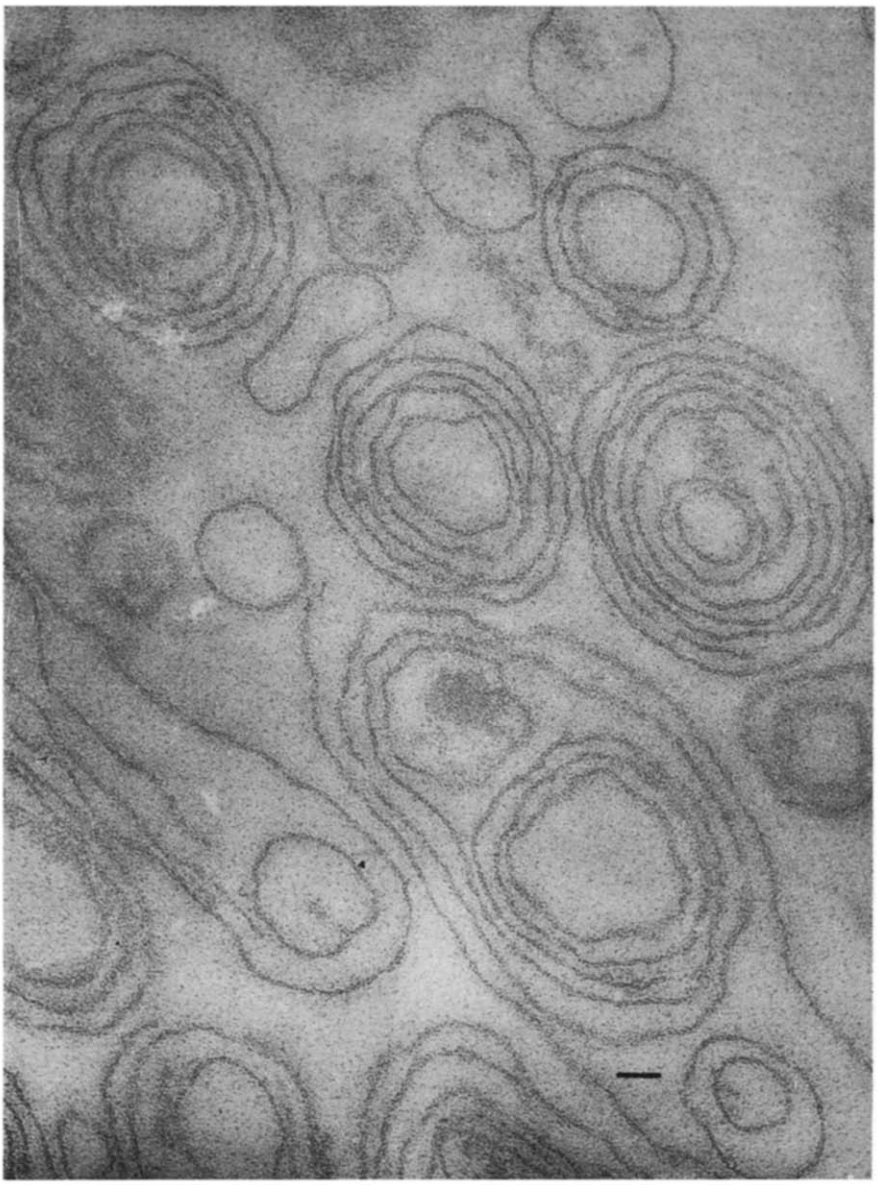

Fig. 2. Thin section of membranous cytochrome oxidase, fixed in OsO_4 and poststained with $KMnO_4$. Note the "unit membrane" structure which is approx. 55 Å thick. Marker represents 500 Å.

than a more active preparation which indicated the low activity can not be caused by Triton inhibition. We could only bring back the activity by completely dispersing the enzyme with high ionic strength in the presence of more Triton X-100.

Fig. 3 shows the dispersed cytochrome oxidase. The activity of this preparation was very high even in the absence of added phospholipids. The enzyme appears as

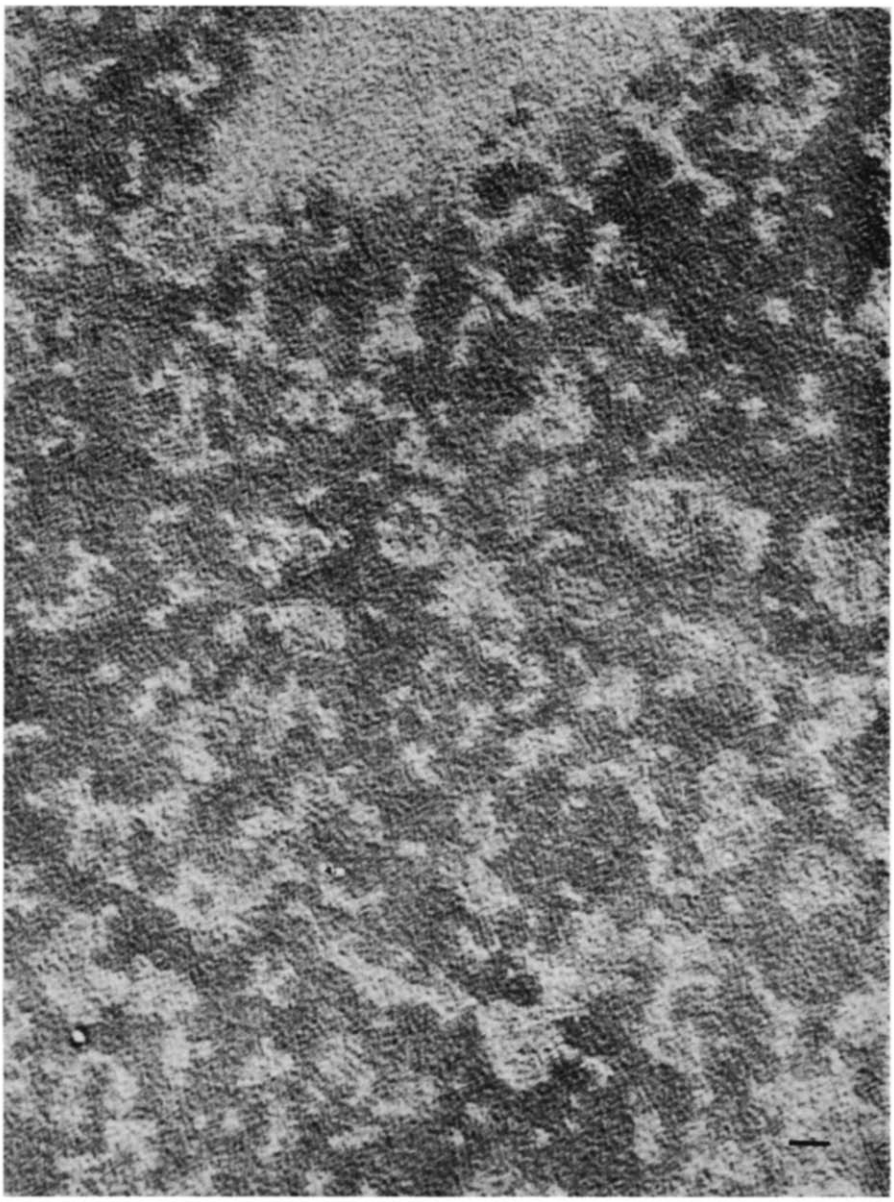

Fig. 3. Dispersed cytochrome oxidase, phosphotungstate stained. Marker indicates 200 Å.

small particles evenly dispersed on the grid. Addition of micellar phospholipids stimulated the activity about 30%. The physical state of the preparation was not very stable. Incubation at 37° or dialyzing against 1% Triton X-100 caused the enzyme to aggregate rapidly.

Fig. 4 shows the aggregated enzyme after a short period of dialysis. The membrane nature is apparent. However, the aggregated enzyme appears more broken

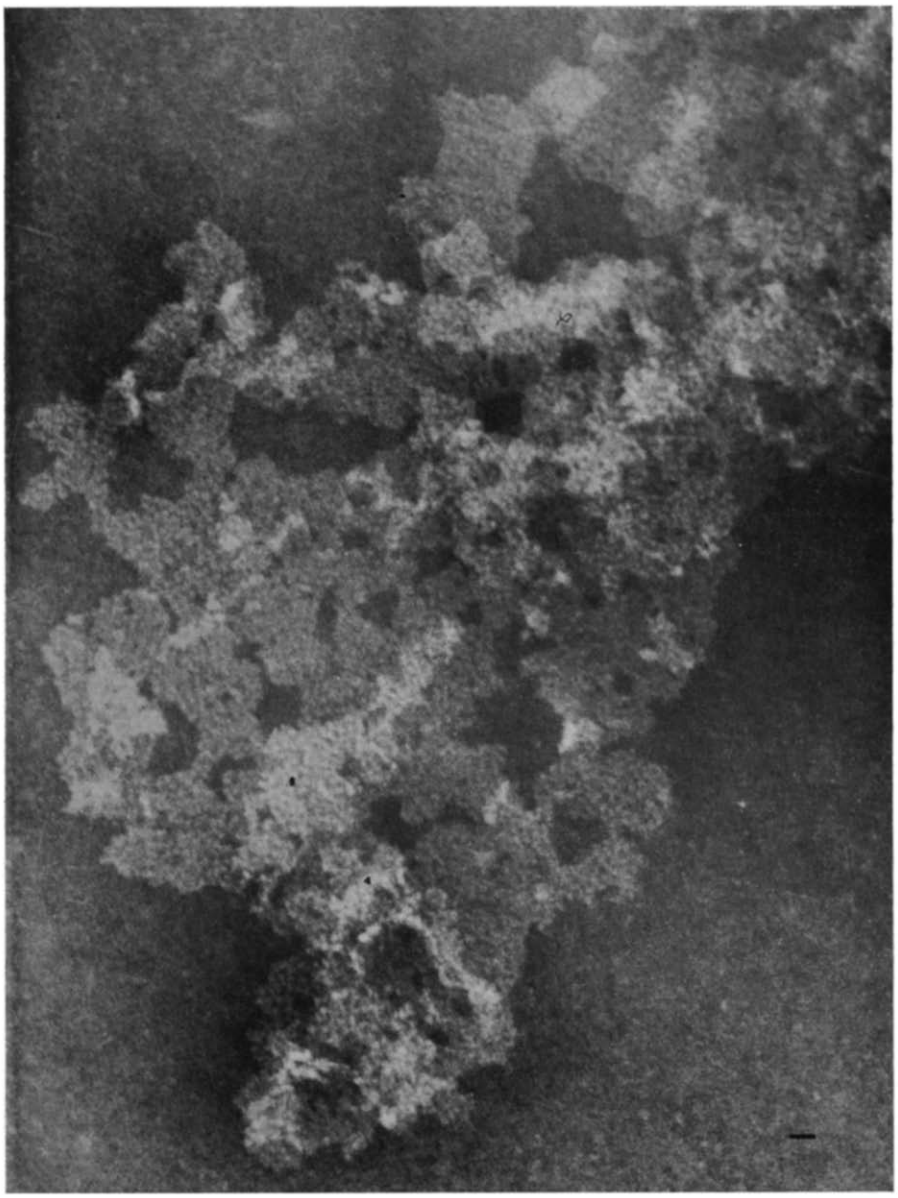

Fig. 4. Aggregated cytochrome oxidase, stained with phosphotungstate. This preparation does not exhibit the regular vesicular nature of the original membranous cytochrome oxidase. Marker indicates 200 Å.

up than the original form, lacking the orderly arrays of subunits. Thin section of this fraction (Fig. 5) shows clear unit membrane structure which is indistinguishable from the original membranous preparation. The enzyme resembles the membranous preparation in its low activity and in being insensitive to stimulation by micellar phospholipids.

Fig. 5. Thin section of aggregated cytochrome oxidase, showing thickness approximately equal to that of the membranous preparation (55 Å) and also giving the "unit membrane" appearance. Marker is 500 Å.

It was of interest to determine whether the membrane formation phenomenon observed in this case was due to the removal of inorganic salts. Three portions of the concentrated lipid-containing soluble oxidase were dialyzed separately against cold 3% Triton X-100, 1 M KCl and the mixture of both for 2 h. Only the first tube (dialyzed against plain 3% Triton X-100) showed heavy precipitates. This result

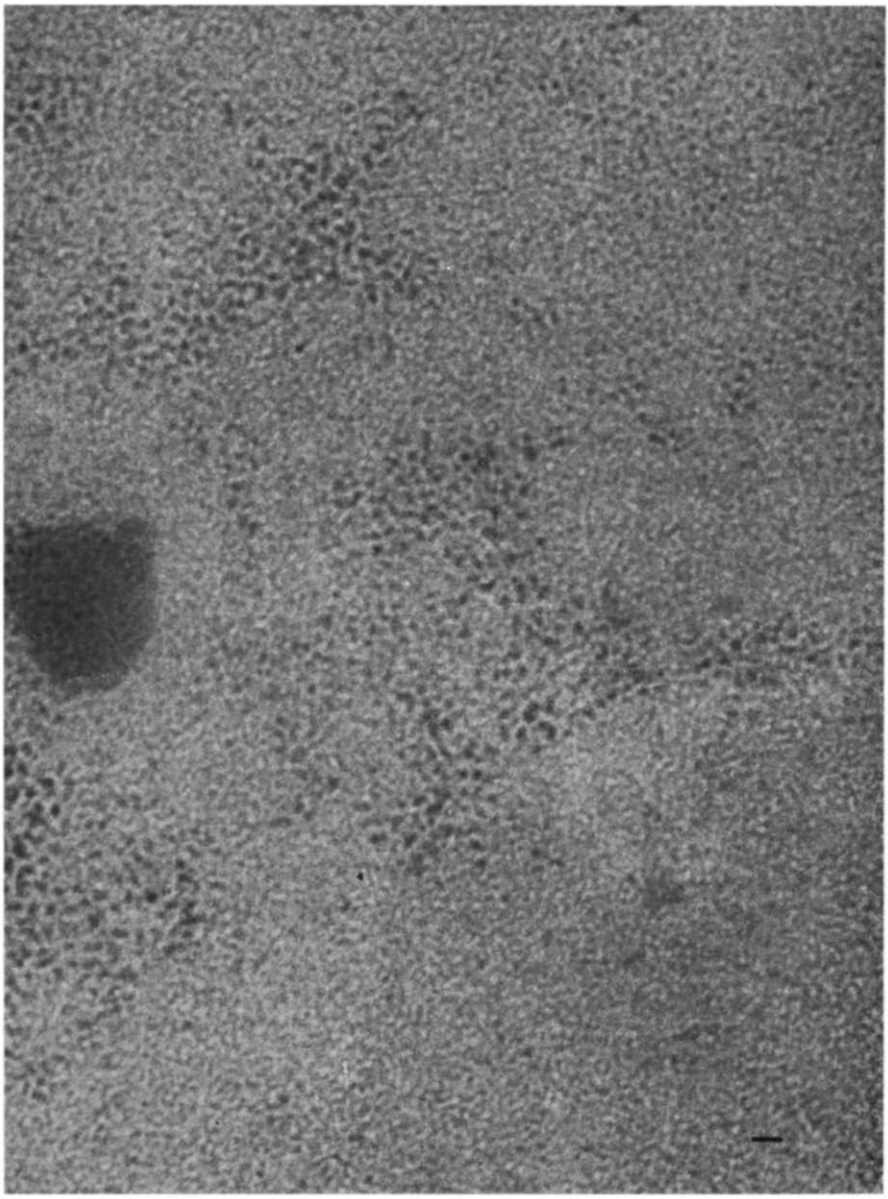

Fig. 6. Lipid-free cytochrome oxidase. Note the non-membranous form which occurs in the absence of phospholipids. Phosphotungstate stained. Marker represents 100 Å.

clearly indicated that the membrane formation was caused by the removal of salt only. The second tube (1 M KCl) had been extensively dialyzed for 2 days. Only a small amount of precipitate formed and this was found to be predominately contaminating structural protein (low in heme).

It should be mentioned that extensive dilution of the concentrated lipid-containing soluble oxidase with Tris buffer failed to induce membrane formation. This

was contrary to the observations of McCONNELL *et al.*[1]. However, we found the deoxycholate cytochrome oxidase preparation of FOWLER, RICHARDSON AND HATEFI[9] did organize into membrane after extensive dilution. The difference may be caused by the difference in detergent used, *i.e.*, the difference in charge properties of the detergent–phospholipid–protein complex.

The activity of the aggregated cytochrome oxidase can be readily increased by resolubilization. The resolubilized enzyme had lower activity in the absence of micellar phospholipids, but can be stimulated to the same level as the original soluble preparation by the addition of lipids. The loss in activity after the aggregation solubilization cycle was evidently due to the loss of lipids.

Dialysis of the resolubilized enzyme against 1% Triton X-100 produced no precipitate. However, the ability to form membrane can be readily restored by the addition of micellar phospholipids. This result confirmed the observation of McCONNELL *et al.*[1] that the membrane formation process is lipid dependent.

Lipid-free cytochrome oxidase formed no membrane in the absence of phospholipids (Fig. 6). However, the enzyme had strong affinity for phospholipids. Once mixed together, the mixture turned turbid almost immediately and the precipitate could be easily sedimented. Electron microscopic examination of the precipitate showed small pieces of vesicular membranes with subunits on their surface. The membrane appears quite similar to the original membranous oxidase preparation but occurs in relatively smaller vesicular units. The enzyme activity depends on the initial phospholipid concentration in the reaction medium. At least 15 μg phospholipid phosphorus were needed per each mg of enzyme protein to obtain a fully active enzyme. Table II shows that the reconstituted enzyme complex was inactive unless saturated with phospholipids. The membrane formation itself does not require as much lipid. The concentration of enzyme protein in the reaction medium was also important. Concentrated solutions of both enzyme and lipids always facilitated the reconstitution.

TABLE II

EFFECT OF PHOSPHOLIPID ADDITION ON ACTIVITY OF THE LIPID-FREE OXIDASE

Concentrated micellar phospholipids sol (10–15 μatoms P per ml) was mixed with concentrated cytochrome oxidase (8–10 mg/ml) in the proportion indicated. The mixture was incubated for 5 min at room temperature and diluted appropriately for assay.

Preparation	*Phospholipid added (weight ratio of protein + lipids)*	*Phospholipid recovered (% of total weight)*	*Activity (μmoles/min per mg protein)*
Lipid-free oxidase	—	2	3.0
Lipid-free oxidase	1:0.38	13.8	2.3
Lipid-free oxidase	1:1	54	77.6

The reaction between lipid-free cytochrome oxidase and micellar phospholipids was found to be temperature dependent. Turbidometric measurement of the protein lipid mixture under various temperature is shown in Table III. It is clear that the formation of membrane was rather sluggish at 0° but almost instantaneous at 20°. The product of this experiment has been checked carefully on the electron microscope. The membranous nature of the reconstituted enzyme was apparent.

TABLE III

EFFECT OF TEMPERATURE ON RATE OF AGGREGATION OF RECONSTITUTED OXIDASE

Equal weights of lipid-free cytochrome oxidase and micellar phospholipids were mixed in cuvettes at the temperature indicated. Turbidities were followed by measuring absorbance change at 520 mμ in a Unicam P-500 spectrophotometer equipped with a constant temperature block.

Temp.	*Initial rate of increase in turbidity (absorbance/min)*
0°	0.28
10°	2.40
20°	Instantaneously forms dense turbidity

DISCUSSION

The capability of membrane formation by mitochondrial proteins was first demonstrated in RACKER's laboratory with coupling factor F_4, and followed by extensive studies in GREEN's laboratory with a number of submitochondrial lipoproteins. The general conditions required for membrane formation were (1) the presence of phospholipids, (2) the absence of dispersing agents such as bile salts. The first requirement has been fully confirmed in this investigation. The second requirement, however, should be better defined as either a decrease of ionic strength in the medium or a decrease in bile salts.

We have clearly shown that purified cytochrome oxidase can exist in two physical states, namely membranous and dispersed states. The phospholipid content and the ionic strength of the medium were the major factors that determine the state in which a given preparation may exist.

From an enzymatic standpoint the membrane state is generally inactive unless saturated with phospholipids before its formation. Once formed, the enzyme particles are in a "trapped" state and lose their ability to interact with micellar phospholipids or other enzyme complexes of the electron transport chain. On the other hand, the enzyme in the dispersed state exists as individual free particles, which not only have maximal catalytic capacity but also retain full ability for interaction with phospholipid micelles or other electron transport enzyme particles.

Numerous investigations in the past have indicated that particulate cytochrome oxidase with phospholipid content comparable to the original mitochondria was always less active than solubilized preparation. SMITH AND CAMERINO[14] reported that the rate of cytochrome oxidase reaction catalyzed by the particulate Keilin–Hartree preparation was highly variable and always less than the maximal turnover rate possible. Large consistent activities could be obtained only after the particulate enzyme was solubilized by deoxycholate. On the other hand, OKUNUKI[15] has indicated that the oxidase activity in his preparation rises 2- to 3-fold under appropriate conditions in which the cytochrome *a* is depolymerized. In separate studies, a highly active deoxycholate cytochrome oxidase preparation of FOWLER, RICHARDSON AND HATEFI[9] lost part of its activity after extensive dilution. The only active membranous oxidase preparation was our reconstituted phospholipid-rich preparation which contained up to 50% of its weight as phospholipids. It is rather doubtful that functional

cytochrome oxidase *in situ* is so enriched. The high lipid content may only be secondary binding after the enzyme is saturated with phospholipids.

The low activity in membranous cytochrome oxidase can be satisfactorily explained in terms of its phospholipid content. Normal purified cytochrome oxidase contains approx. 20% phospholipids which is not enough to saturate all enzyme molecules. Even if the enzyme is in the dispersed state, exogenous added micellar phospholipid is still needed to achieve the maximal activity. Recent experiments showed that at least 15 μg/mg enzyme protein (27% by weight) was required for full activity (F. F. SUN AND F. L. CRANE, unpublished study). The deficiency in lipids may cause the enzyme molecules to squeeze against each other in the limited space of a membrane and results in the distortion of active sites. On the other hand, if the dispersed enzyme is allowed to form membranes in the presence of excess phospholipids, each enzyme molecule will have enough space to be aligned in proper spatial arrangement and the resulting enzyme will be active. Since the phospholipid requirement is quite high for this enzyme (60 molecules per one heme *a*), it is unlikely that the lipids serve as a coenzyme in the catalytic function.

It is clear that formation of the membranes described here requires both phospholipid and oxidase protein. The membranes which we observe differ from simple smectic phase bilayers of phospholipid by their spatial arrangement and the presence of the 50-Å globules throughout. The original membranous oxidase with 18% lipid is mostly in the vesicular form shown by the sections and concentric vesicles are quite common. On the other hand, the reaggregated oxidase with 8% lipid is not vesicular but shows sheets both in section and by surface view. The ratio of lipid to protein therefore can influence the overall shape of the membrane.

The relation between protein and lipid molecules in the membrane is more difficult to determine. We postulate that the 50-Å globules are lipoprotein elements made up of cytochrome oxidase and phospholipid. These combine together, probably with additional phospholipid, to make up the membrane. The alternative explanation of membrane formation would be that a bilayer of phospholipid is formed to which the 50-Å globules are attached on the surface. Our view that the globules make up an integral part of the membrane is based on the following considerations: (a) The surface of the vesicles are smooth under negative staining and do not show 50-Å projections. There must be some roughening of the surface in order to obtain negative staining unless affinity of the stain for certain regions and not others is to be envoked as the basis for seeing the white spots. (b) The edges of the reaggregated sheets with 8% lipid do show globules on the edges. (c) The globules are 50 Å in diameter and the membranes are 50 Å thick in section. A layer of globules packed on the surface of a phospholipid bilayer would be 90 Å thick. (d) When small amounts of phospholipid are added to the lipid-free oxidase only small clusters of globules or rows of globules appear. At 4% phospholipid about 50% of the material is in globular form whereas the rest is still in the dispersed 20-Å string form. No membranes are found in fixed material at this stage even though the equivalent amount of free phospholipid shows typical smectic micelles as layers of membranes. (e) Smectic phase phospholipid micelles are found interspersed among the granular oxidase membranes when the phospholipid exceeds 50%. The two types of membrane are clearly distinct and show no evidence of fusion. (f) The calculated surface area of a phospholipid bilayer is hardly sufficient to contain all of the cytochrome oxidase molecules in the membrane

with 8% lipid. In this membrane there are 13.7 molecules of phospholipid per molecule of cytochrome oxidase (heme *a*). If each phospholipid molecule provides a surface area of approx. 100 Å^2 (ref. 16) then 685 Å^2 of phospholipid bilayer are available to support the 1880 Å^2 subtended by the 50-Å spheres of cytochrome oxidase if the oxidase is on only one side of the bilayer. If the oxidase is on both sides of the bilayer then 1370 Å^2 are available for each 1880 Å^2 of oxidase. Since the 8% phospholipid membrane appears mostly as a single layer of globules in Fig. 4 it appears that the phospholipid available is not enough to form a continuous bilayer under the oxidase. This membrane was separated by centrifugation and no mass of free oxidase globules has been observed which would allow concentration of the phospholipid under only part of the oxidase molecules. (g) Pure phospholipid micelles exposed to the same conditions of salt and Triton as the oxidase membrane remain as membranous lamellae and do not disperse.

From these observations we propose that the oxidase globules are immersed in the lipid and contribute to the membrane structure. The lipid can still be considered as the continuous phase of the membrane surrounding the globules. The greater stability of vesicles of 20% lipid membranes as compared to the fragility of 8% lipid membranes may be attributed to more continuous lipid phase between the globules.

The induction of membrane formation by decrease of ionic strength has clearly demonstrated that a significant part of the binding force in this membrane is polar bonding. Hydrophobic force may play only a supporting role. Whether this observation can be extended to other mitochondrial protein remains to be studied.

The physiological significance of membrane formation phenomenon is still unknown. The artificially formed membrane does retain many biochemical and morphological features resembling those of the native membrane. The present evidence, however, still cannot allow us to rule out completely the possibility of artifact in the structures or enzymic functions observed.

ACKNOWLEDGEMENTS

Supported under a grant AM 04663 from the National Institute of Arthritis and Metabolic Diseases. F.L.C. is supported by a career grant K6,21,839 from the National Institute for General Medical Science. We wish to thank Mrs. MILDRED WETZEL for mitochondria preparation and Miss JUDY HALL for assistance with photography.

REFERENCES

1 D. G. MCCONNELL, A. TZAGOLOFF, D. H. MACLENNAN AND D. E. GREEN, *J. Biol. Chem.*, 241 (1966) 2373.
2 D. E. GREEN, D. W. ALLMANN, E. BACHMANN, H. BAUM, K. KOPACZYK, E. F. KORMAN, S. LIPTON, D. H. MACLENNAN, D. G. MCCONNELL, J. F. PERDUE, J. S. RIESKE AND A. TZAGOLOFF, *Arch. Biochem. Biophys.*, 119 (1967) 312.
3 T. YONETANI, *J. Biol. Chem.*, 236 (1961) 1680.
4 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
5 L. SMITH, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 732.
6 W. H. VANNESTE, *Biochim. Biophys. Acta*, 113 (1966) 175.
7 H. LOW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
8 F. F. SUN AND E. E. JACOBS, *Biochim. Biophys. Acta*, 143 (1967) 639.
9 L. R. FOWLER, S. H. RICHARDSON AND Y. HATEFI, *Biochim. Biophys. Acta*, 64 (1962) 170.

10 K. TAKAYEMA, D. H. MACLENNAN, A. TZAGOLOFF AND C. STONER, *Arch. Biochem. Biophys.*, 114 (1966) 223.
11 S. FLEISCHER AND B. FLEISCHER, in R. W. ESTABROOK AND M. Z. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 407.
12 H. H. MOLLENHAUER, *Stain Technol.*, 39 (1964) 111.
13 W. P. CUNNINGHAM AND F. L. CRANE, *Plant Physiol.*, 40 (1965) 1041.
14 L. SMITH AND P. W. CAMERINO, *Biochemistry*, 2 (1963) 1432.
15 K. OKUNUKI, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Vol. 14, Elsevier, New York, 1966, p. 232.
16 L. L. M. VAN DEENEN, *Ann. N.Y. Acad. Sci.*, 137 (1966) 717.