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Proteolipids. VI. The Dual Role of Phospholipid in Cytochrome Oxidase Reaction*

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Abstract

After being deprived of solubilizing agent, the lipid-free cytochrome oxidase requires Triton X100 and additional phospholipid to obtain maximal activity. High levels of Triton X100 affect the interaction of phospholipid and cytochrome oxidase, thus decreasing the activity. In the terminal segment of the electron transport system, phospholipid serves not only to enhance the interaction between cytochrome *c* and cytochrome *a*, but also to maintain favorable molecular arrangements of reacting groups in both hemoproteins. The relationship between the enzyme activity and phospholipid content as well as the ultrastructure of the enzyme is discussed.

Introduction

It has been postulated that cytochrome *c* serves as the mobile electron carrier which shuttles electrons between complex III and complex IV in the mitochondrial electron transport system. A lipid cytochrome *c* complex was suggested[1] to be the functional species working between these two complexes. This concept was greatly enhanced by our recent discovery that lipid cytochrome *c* was oxidized much faster than soluble cytochrome *c* by a lipid-free cytochrome oxidase preparation.[2] This result seems to indicate that the role of phospholipid in this segment was to promote the interaction between cytochrome *c* and cytochrome *a*. Since it would make no difference which components the lipid would attach to, as long as one component is lipid bound, the reaction would be carried out at its maximal rate.

However, electron microscopic studies from this[3] and other laboratories,[4,5] plus biochemical evidence, have revealed that the above conclusion is oversimplified. Both the structure and the catalytic capacity of the two hemoproteins, cytochromes *c* and *a*, have been shown to be phospholipid dependent. Lipid cytochrome *c* can exist in two different molecular states, the inactive aggregated state and the active micellar state.[2] The catalytic ability is determined not only by the presence of phospholipid but also by the orientation of lipid on the protein molecule.

The same rule also applies to the cytochrome oxidase. It has been shown[3,6] that the enzyme can exist in several molecular states, depending on the phospholipid content and the environment, such as the ionic strength and the presence of detergents. Maximal

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activity of a particular enzyme preparation could not be obtained unless both hemoproteins were in favorable molecular states for interaction. For example, one can use lipid cytochrome *c* in the micellar state to interact with lipid-free cytochrome oxidase in the completely dispersed state to obtain enhanced activity. On the other hand, a similar level of activity can be obtained by reaction of soluble cytochrome *c* with a completely dispersed phospholipid containing oxidase. Absence of phospholipid results in inefficient interaction and low activity which is probably due to the unfavorable steric arrangement of the two cytochromes.

In continuing our research on the effects of phospholipid on the molecular states of cytochrome oxidase, we have found that when the lipid-free cytochrome oxidase preparation was deprived of detergents, aggregation occurred and the enzyme lost partially its ability to interact with lipid cytochrome *c*. Addition of detergents readily redispersed the aggregates and restored the activity with lipid cytochrome *c*. When phospholipid was present in both hemoproteins, cytochrome oxidase reached higher activity than when phospholipid was only on one of the hemoproteins. Thus, it seems that the phospholipid bound to lipid cytochrome *c* is not enough to fulfil the entire requirement for maximal reaction capacity. Additional phospholipid must be supplied in order to maintain proper structural configuration in the cytochrome oxidase itself. This communication defines the role of phospholipid in the terminal step of electron transport system.

Experimental Procedure

Reagents and procedures for the isolation of lipid-free cytochrome oxidase and micellar mixed mitochondrial phospholipid have been described in previous publications,[2,3] except that 0·02 M Tris-HCl, pH 7·4, was susbtituted for phosphate buffer in the preparation of lipid-free soluble cytochrome oxidase. The final preparation contained 8–9 mμmoles of heme *a*/mg of protein, 2–3 g-atom P/mole of cytochrome *a* or 2–3% phospholipid (w/w), and 0·5–1·0 mg of Triton X100/mg of protein.

The detergent-free, lipid-free cytochrome oxidase was prepared by treatment of the Triton preparation with diethyl ether. Soluble lipid-free Triton containing enzyme was diluted to 2–4 mg/ml and mixed with 20 volumes of diethyl ether, and shaken for 1 min. The green aqueous layer was pipetted out. Ether treatment of the enzyme was repeated six times or until no Triton was detected by the assay indicated below. After lyophilization to remove residual ether, the enzyme was sonicated in 0·02 M Tris, pH 7·4, for four 30 sec intervals. Triton was not detectable in this preparation.

Phospholipid micelles and cytochrome *c* were centrifuged separately at 108,000 $\times$ *g* for 1 h to remove any aggregates before lipid cytochrome *c* preparation. Lipid cytochrome *c* was prepared by mixing concentrated soluble cytochrome *c* (>0·5 mM) and phospholipid micelles (>500 μg P/ml). Phospholipid micelles and cytochrome *c* were mixed in a molar ratio of 31 P:1 cytochrome *c* and stirred at 4° for 10 min before diluting and centrifugation at 108,000 $\times$ *g* for 30 min. The pellet was collected and resuspended in 0·02 M Tris, pH 7·4, and recentrifuged for 30 min. A small drop of α-tocopherol and 0·5 M ascorbate, pH 7·0, was added to the suspension of the collected pellets to make a final concentration of ascorbate 2 mM. The mixture was sonicated at maximal output for five 30 sec intervals with a Branson's Sonifer and centrifuged to remove any pre-

cipitation with a clinical centrifuge for 1 min. Cytochrome *c* concentration was adjusted to 0·5 mM for assay.

Analytical procedures for cytochromes, phospholipid, and protein were identical to those described elsewhere.[2,3] Molecular weight of cytochrome oxidase was based on the value of 72,000.[7] Triton X100 was determined spectrophotometrically by the formation of a blue complex with ammonium cobaltothiocyanate. The reagent was prepared by dissolving 7·5 g of cobalt nitrate hexahydrate and 50 g of ammonium thiocyanate in water to make the final volume to 500 ml. Triton-containing enzyme was extracted with 20 volumes of acetone at room temperature three times with centrifugation at 3000 × *g*. The acetone extract was evaporated to dryness and redissolved in water. Two milliliters of the reagent was pipetted into a cuvette and 1 ml of Triton X100 containing sample was added to make a final volume of 3 ml. Absorbance was measured at 630 μm against the diluted reagent without Triton.

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 m MTMPD (*N,N,N',N'*-tetra-methyl-*p*-phenylenediamine dihydrochloride); 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.

Negative staining with 2% phosphotungstic acid was carried out as described by Cunningham and Crane[8] and preparations were observed with a Philips EM 300.

Results

We have reported previously that the lipid-free cytochrome oxidase rapidly oxidized lipid cytochrome *c* complex using ascorbate-TMPD as electron donor.[2] These experiments, however, suffered from some variations in the maximal velocities obtained from one batch of enzyme preparation to another. Lately, we have observed that a few batches of freshly prepared cytochrome oxidase did not obtain their maximal activities toward lipid cytochrome *c*. Analysis of the lipid phosphorus of the preparations disclosed that the variation was caused by differences in phospholipid content. This was also confirmed with a detergent-free cytochrome oxidase preparation. The final detergent concentration and phospholipid content in the enzyme have effect on its activity.

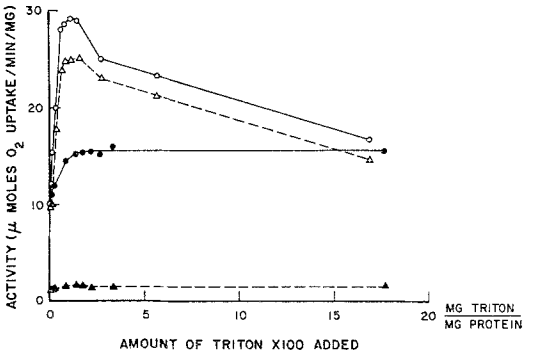

Figure 1. Effect of Triton X100 on cytochrome oxidase activity. Key: –▲–▲–, soluble cytochrome *c* as substrate assayed with lipid-free cytochrome oxidase; —●—●—, lipid cytochrome *c* as substrate with lipid-free cytochrome oxidase; –△–△–, soluble cytochrome *c* as substrate with lipid-added cytochrome oxidase; —○—○—, lipid cytochrome *c* with lipid-added cytochrome oxidase. This enzyme was a detergent-free, lipid-free preparation. Different amounts of Triton X100 were added to the enzyme. The lipid-added cytochrome oxidase was made as follows: 50 μg phospholipid phosphorus/mg of protein (or 116 g-atom P/mole of cytochrome *a*) added to enzyme and incubated 5 min at 4° before being diluted for assay.

Figure 1 shows the relationship between the oxidase enzyme activity and the concentration of Triton X100 in the final enzyme. Triton was completely removed and then different amounts of Triton X100 were added to the enzyme to study the

effect of Triton in the assay system with either soluble cytochrome *c* or lipid cytochrome *c* as substrate. Phospholipid was initially associated either with enzyme and/or cytochrome *c*. It took approximately 1·5 mg of Triton X100/mg of protein to reach the maximal rate for all cases. Soluble cytochrome *c* showed little, if any, response in the lipid-free cytochrome oxidase to the increased Triton X100 levels. Activity decreased at a Triton X100 concentration of more than 2 mg/mg of protein when phospholipid was added to cytochrome oxidase with either soluble cytochrome *c* or lipid cytochrome *c* as substrate, but not when no phospholipid was initially added to the oxidase. This indicates that Triton X100 does affect the interaction between phospholipid and enzyme. This was also shown in a study of the effect of Triton X100 on cytochrome oxidase activity of a beef heart mitochondria preparation (Fig. 2). At higher levels of Triton X100 the cytochrome oxidase activity of the mitochondria was only half of that without Triton added, and highest activity was obtained at Triton level of 0·25 mg/mg of protein. Figure 3 shows the enzyme activity of various amounts of lipid-added cytochrome oxidase assayed with soluble cytochrome *c* and lipid cytochrome *c* at two different cytochrome *c* concentrations. Both Figs. 1 and 3 show that high activities were always obtained when phospholipid was present in both cytochrome *c* and cytochrome oxidase. At Triton X100 concentration of 1·5 mg/mg of protein 29·0 μmoles O_2 uptake/min/mg of protein was obtained when phospholipid was present in both cytochrome *c* and cytochrome oxidase, while 25 and 15 μmoles O_2/min/mg were obtained when phospholipid was only present in cytochrome oxidase or cytochrome *c*, respectively (Fig. 1). The same conclusion can also be obtained from Fig. 3. At two concentrations of cytochrome *c* tested the lipid-added cytochrome oxidase had higher activity toward lipid cytochrome *c* than soluble cytochrome *c* at the same amount of phospholipid added to cytochrome oxidase.

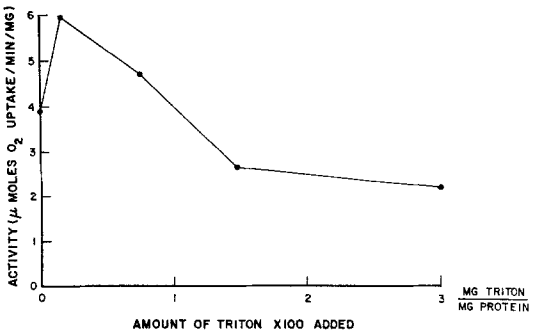

Figure 2. Effect of Triton X100 on cytochrome oxidase activity of beef heart mitochondria. Beef heart mitochondria preparation was adjusted to protein concentration of 4 mg/ml. Indicated Triton X100 was added to mitochondria and incubated 5 min at 4° before being diluted for assay.

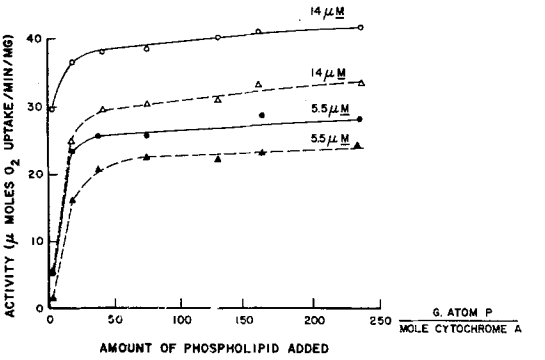

Figure 3. Effect of phospholipid on cytochrome oxidase activity with lipid cytochrome *c* and soluble cytochrome *c* as substrate. Phospholipid was incubated with lipid-free cytochrome oxidase and assayed with either lipid cytochrome *c* or soluble cytochrome *c* at two different concentrations, 14 μM and 5·5 μM. It shows higher enzyme activity always obtained with phospholipid on both hemoproteins. Solid lines: assayed with lipid cytochrome *c*; dotted lines: soluble cytochrome *c*.

Tables I and II show the effect of detergents on the lipid-depleted and lipid-added cytochrome oxidase activity. In the absence of phospholipids in the system (soluble cytochrome *c* as substrate, Table I) maximal activity could not be obtained even when

Triton X100, deoxycholate, or Emasol$_{1130}$ was added to the oxidase. However, the requirement for phospholipids were present in cytochrome *c* (lipid cytochrome *c* as substrate, Table II). The rate reached a maximum before any phospholipid was added in the presence of detergents.

TABLE I. Effect of detergents on the reactivity of the cytochrome oxidase preparation (I)

Amount of PLP added to the enzyme (g-atom P/mole cytochrome *a*)	Activity with addition of			
	Nil	Triton X100	DOC	Emasol$_{1130}$
0	1·0	7·5	3·8	3·9
8·6	24·5	21·5	18·7	28·3
17·2	38·3	27·0	24·2	34·0
31·3	40·3	41·0	34·8	35·2
85·8	43·0	45·0	39·7	38·9
137·0	42·6	39·5	39·5	40·8
257·0	42·6	41·5	33·7	43·5

All activities are expressed in μmoles of oxygen uptake/min/mg of enzyme protein. 0·15 ml aliquot of each detergent (10%, w/v) was added to 1 ml of lipid free cytochrome oxidase preparation which contained 0·70 mg of Triton X100/mg of protein (final protein concentration 5·60 mg/ml). Various amounts of phospholipids (PLP) were added and the mixtures were allowed to stand at 4° for 10 min before diluted to 100 μg/ml for assay. Emasol$_{1130}$ (polyoxyethylenesorbitane monolaurate) is a nonionic detergent from the Kao Soap Company, Tokyo. DOC stands for deoxycholate.
Substrate: Soluble cytochrome *c* at the concentration of 15 μM in the final assay mixture.

TABLE II. Effect of detergents on the reactivity of the cytochrome oxidase preparation (II)

Amount of PLP added to the enzyme (g-atom P/mole cytochrome *a*)	Activity with addition of			
	Nil	Triton X100	DOC	Emasol$_{1130}$
0	6·9	60·0	46·5	42·8
8·6	60·5	61·3	48·3	45·0
17·2	61·1	61·5	47·4	43·8
31·3	61·8	61·3	50·8	41·0
85·8	58·5	60·0	43·0	41·3
137·0	57·7	59·2	43·5	41·0
257·0	56·4	58·5	42·5	41·0

All expressions are identical to those listed in the legend of Table 1.
Substrate: Lipid cytochrome *c* at the concentration of 15 μM in the final assay mixture.

The above observations were further reinforced by the results of electron microscopic studies. The lipid-free cytochrome oxidase appeared as 90 Å globules or assemblies made of globules (Fig. 4). After removal of Triton the enzyme aggregated as shown in Fig. 5. With addition of phospholipids to the lipid-free cytochrome oxidase preparation

a massive formation of membrane vesicles was observed (Fig. 6). Smaller membrane vesicles were also formed in the absence of detergent (Fig. 7). The dimension of globules in the membrane was 50–55 Å.

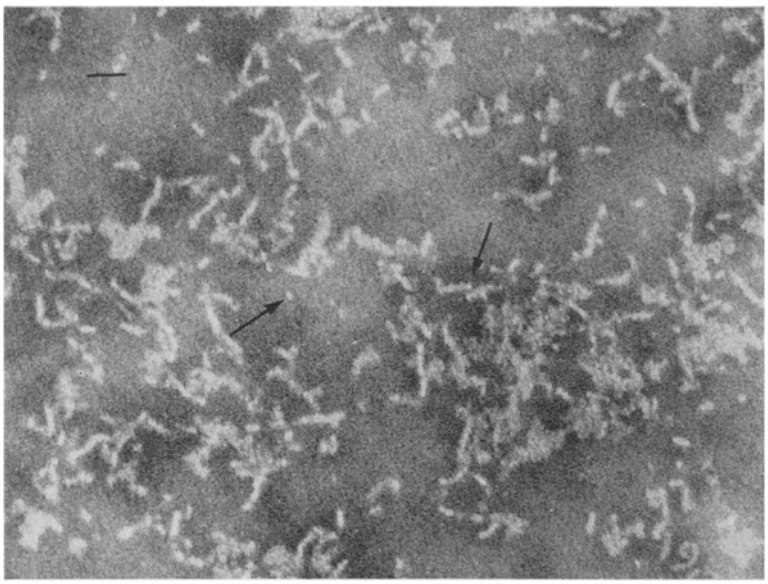

Figure 4. Lipid-free cytochrome oxidase. The preparation contained 1·5 mg of Triton X100/mg of protein. Arrows at 90 Å globules. Negatively stained. ×103,000. Marker 500 Å.

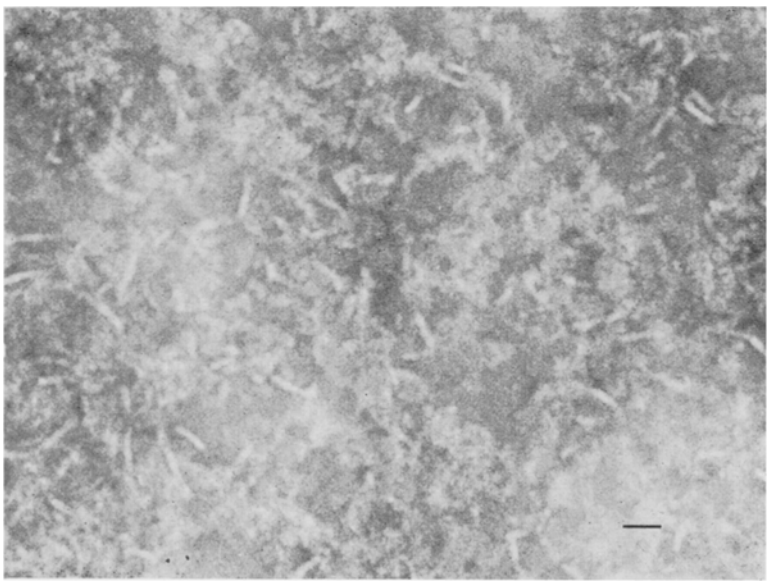

Figure 5. Detergent-free, lipid-free cytochrome oxidase. Negative staining with 2% PTA. ×103,000. Marker 500 Å.

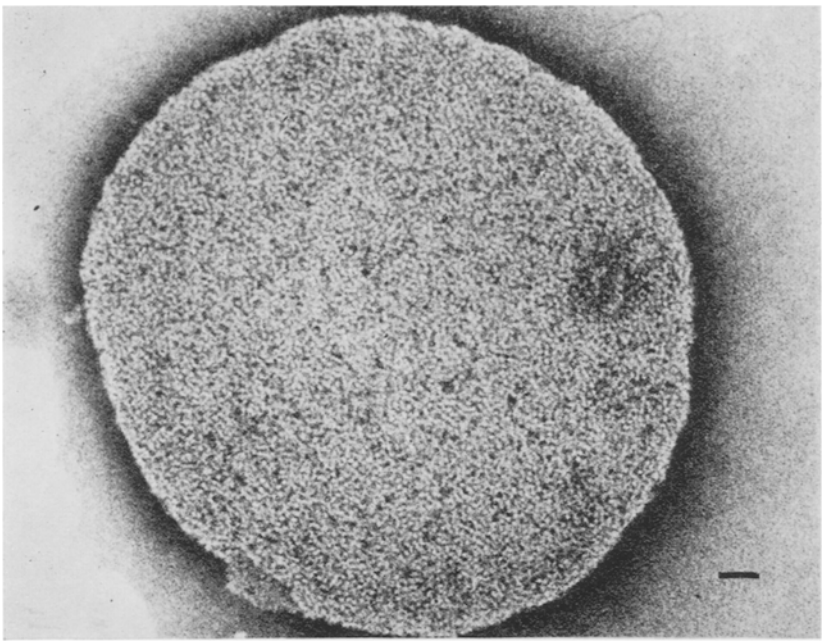

Figure 6. Membranous cytochrome oxidase I. 28 g-atom P of phospholipid/mole of cytochrome *a* or 23% of phospholipid (w/w) were added to a lipid-free cytochrome oxidase preparation which contained 1·5 mg of Triton X100/mg of protein. Note 50 Å globules on membrane vesicles. ×103,000. Marker 500 Å.

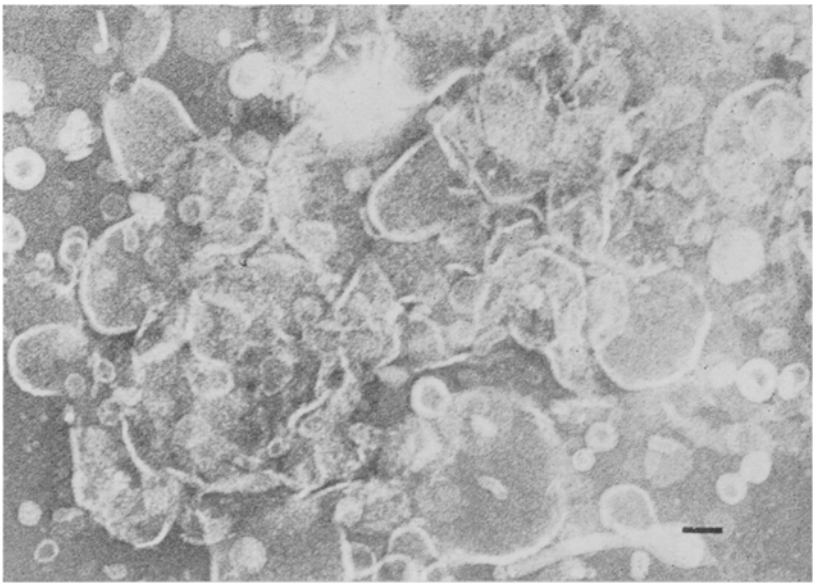

Figure 7. Membranous cytochrome oxidase II. 65 g-atom P of phospholipid/mole of cytochrome *a* or 41% of phospholipid (w/w) were added to a detergent free cytochrome oxidase preparation and incubated at room temperature (23°) for 1 h. ×103,000. Marker 500 Å.

Discussion

It has been shown that some chemical parameters relate closely with the molecular state of cytochrome oxidase. For example, treatment with sodium dodecyl sulfate (SDS) and thioglycollate has been shown to be able to disperse the active enzyme into a state bearing only one heme, one iron, and one copper per molecular unit in solution.[7,9] On the other hand, Takemore *et al.*[10] reported that in the case of cytochrome *a* solubilized with 0·5% solution of the ionic detergent sodium cholate the protein behaved as a polydispersed aggregated system encomposing a distribution of protein aggregates of different molecular weight. These and other properties have been elaborated quite extensively by Okunuki and co-workers.[11,12] They found that only four sulfhydryl groups among seven were titratable in their polymeric aggregate of cytochrome *a*, but all seven SH groups were readily detectable when the enzyme was dispersed by 0·5% SDS. Furthermore, all intrinsic copper in the SDS-treated enzyme formed chelation compound with sodium bathocuprone-disulfonate, whereas only the extraneous copper would chelate if the enzyme existed in the aggregated state. This evidence clearly indicates that essential components such as the copper atom and SH groups are buried deeply in the aggregated enzyme so they would not be available for their catalytic role. On the other hand, it is conceivable that SDS, which they used as a dispersing agent, mimics the role of phospholipids in native cytochrome oxidase to keep the essential reacting groups in a functional orientation.

It appears that the role of phospholipids in cytochrome oxidase is to maintain a suitable orientation in both cytochrome *c* and cytochrome *a*, and facilitate the interaction of both cytochromes. The enzyme can exist in different physical states, depending on the treatment.[3,6] In the absence of both lipid and detergent the enzyme is in a state of aggregation. It forms 90 Å globules or assembles to rod-like structure with thickness of 90 Å at the presence of Triton X100. The lipid-depleted preparations are inactive to its substrate, cytochrome *c*, because of its molecular polymerization.[6] Addition of phospholipids causes the enzyme to rearrange into a membranous form which is found to have high activity and to maintain a suitable configuration to expose all the necessary prosthetic groups for interaction. The size of enzyme globules is changed from 90 Å to 50–55 Å when phospholipids are added and membrane is formed. The 50–55 Å globule is equivalent to a monomer of cytochrome *a*.[6] Oxidase with low phospholipid content (8·6 g-atom P/mole cytochrome *a*) is active only when lipid cytochrome *c* is used as substrate. Also, addition of detergents to phospholipid-free enzyme readily brings the activity to maximum when lipid cytochrome *c* is used as substrate but not when soluble cytochrome *c* is used.

It can be seen from Fig. 1 that addition of phospholipid gives a five-fold increase of enzyme activity to a detergent-free, lipid-free cytochrome oxidase preparation. Maximal enzyme activity is reached when Triton X100 is added. This indicates that both Triton X100 and phospholipid are needed to obtain maximal enzyme activity.

Lipid cytochrome *c* was shown to be oxidized much faster than soluble cytochrome *c* by a lipid-free cytochrome oxidase.[2] However, it is apparent in this study that in addition to phospholipid associated with cytochrome *c* a dispersing agent is required to reach maximum oxidase activity. This dispersing agent may either be a detergent or phospholipid. Better activity is attained with lipid cytochrome *c* than with soluble cytochrome *c*

even when detergents are used as dispersing agent. The oxidase can be active either when spread into a membranous layer with phospholipid or when dispersed by a detergent without membrane formation.

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