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Phospholipids in the Cytochrome Oxidase Reaction

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Abstract

Phospholipids and Emasol activate cytochrome oxidase by increasing its affinity for its substrate, cytochrome *c*. Cardiolipin was most effective in activating cytochrome oxidase among phospholipids tested. Prior formation of a cytochrome *c*-cytochrome oxidase complex changes the effect of phospholipids. In addition to their structural role in the last segment of the electron transport system, phospholipids can protect the enzyme from heat treatment and mercurial inhibition. They facilitate the interaction between cytochrome oxidase and cytochrome *c*, as well as the cytochrome *c* analogue, protamine.

Introduction

Cytochrome oxidase is an insoluble particulate in inner membrane of mitochondria and it has been suggested that the insolubility of the enzyme might be due to its association with the lipids of mitochondria [1]. Chuang *et al.* [2] reported that their detergent-free, lipid-depleted cytochrome oxidase which contains only 3% phospholipids (w/w), is still insoluble. Its insolubility may be due to the properties of the protein itself [3]. Bile salts have been used to solubilize the enzyme [4, 5] and most cytochrome oxidase preparations have a high lipid content, expecially phospholipids [6, 7]. We have reported that 73% of the residual phospholipids in our lipid-depleted cytochrome oxidase is

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cardiolipin but neither lecithin nor phosphatidyl ethanolamine was found [8]. Triton X114 membranous and reconstituted cytochrome oxidase preparations [2] and deoxycholate-cholate preparation of Fowler *et al.* [9] have a high content of cardiolipin [8] while Marinetti *et al.* [5] and Fleischer *et al.* [6] showed different lipid compositions in their enzyme preparations.

Earlier workers [10] claimed that phospholipids could simply be replaced by detergents, such as Emasol or Tween. Though Emasol can activate the lipid-depleted cytochrome oxidase to a similar level as phospholipids can, it does not form membrane structure with cytochrome oxidase protein [2]. In addition to our previous discussion on the structural role of phospholipids, the present communication deals with the functional role of phospholipids in the cytochrome oxidase reaction.

Materials and Methods

Beef heart mitochondria were isolated according to the method of Löw and Vallin [11] and were stored in concentrated suspension at -20° before use. Lipid-depleted cytochrome oxidase was prepared by the Triton procedure [12]. The enzyme preparations contain 1.3-1.8 μg P/mg protein and have a heme *a* content of 8-9 mμ moles/mg protein* as determined by their differential spectra, reduced *minus* oxidized, using a millimolar extinction coefficient ΔA at 605 nm and 630 nm as 13.1 [13]. The preparation has two major bands on polyacrylamide gel electrophoresis [15]. Mixed and purifed beef heart mitochondria phospholipid sols were prepared as described earlier [2]. Phosphorus was determined by the method of Chen *et al.* [16]. Protein concentration was determined by the method of double strength biuret [17]. Molecular weight of cytochrome oxidase was based on the value of 72,000 [18]. Cytochrome oxidase and TCHQ (tetrachlorohydroquinone) oxidase [18] activities were assayed polarographically with a Gilson KM oxygraph at 37°.

Cytochrome *c* (Type III), *p*-chloromercuribenzoic acid (PCMB), DEAE cellulose, sodium dodecyl sulfate Triton and protamine sulfate were obtained from Sigma, tetrachlorohydroquinone from Eastman. Emasol-1130 (polyoxyethylene sorbitan monolaurate) was supplied by Kao-Atlas, Tokyo. All other reagents were of reagent grade.

* Heme *a* content would be 10-11 mμ moles/mg protein if 16.5 mM^{-1} cm^{-1} was used as the absorbance index for $A_{605\ nm}$ (reduced) *minus* $A_{630\ nm}$ (reduced) [14]. A difference of a factor of 1.25 exists between two methods of calculation.

Results

Phospholipids, Emasol and Cytochrome Oxidase Activity

The enzyme activity of cytochrome oxidase has been shown to be lipid dependent. After removal of the lipids from cytochrome oxidase the enzyme activity was very low (3-5 μmoles O_2/min/mg protein). Upon addition of phospholipids or certain detergents, the enzyme activity rapidly increased (Figure 1). Lineweaver-Burk plots of the data gave straight lines (Figure 2). Half maximal activity, obtained by extrapolating the straight line to the abscissa, was at a phospholipid concentration of 10.0 g Atom P/mole or 12.6 mole of Emasol/mole of cytochrome oxidase. The pattern of the effect of phospholipids and Emasol on the enzyme were very similar, namely, both decrease the Km of the enzyme (Figures 3 and 4). The presence of phospholipids or Emasol decreased the Km for cytochrome oxidase from 10×10^{-6} M at the lipid-depleted state to 2.3×10^{-6} M respectively. These data clearly indicate that both phospholipids and Emasol facilitate the interaction between the substrate, cytochrome *c*, and the enzyme, cytochrome oxidase.

Because of the preferential binding of cardiolipin to cytochrome oxidase over other lipids [8], differences were expected in the effect of purified phospholipids on cytochrome oxidase. Figure 5 shows the effect of purified phospholipids on the enzyme activity. Cardiolipin activated the enzyme much more than did lecithin and phosphatidyl ethanolamine. This was also demonstrated in assays at different concentration of cytochrome *c* with fixed amounts of phospholipids

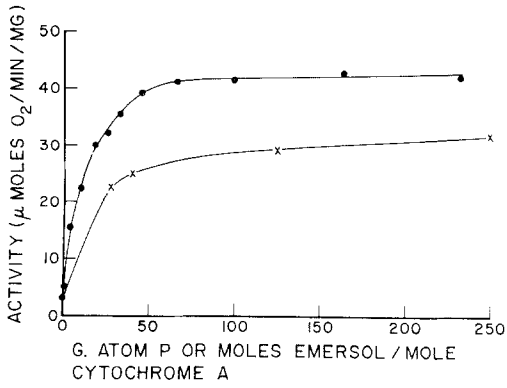

Figure 1. Activation of cytochrome oxidase by phospholipids and Emasol. Lipid-depleted cytochrome oxidase was incubated with mixed total mitochondrial phospholipids or Emasol-1130 for 10 min at 4° before being diluted for assay. xEmasol, ●PLP.

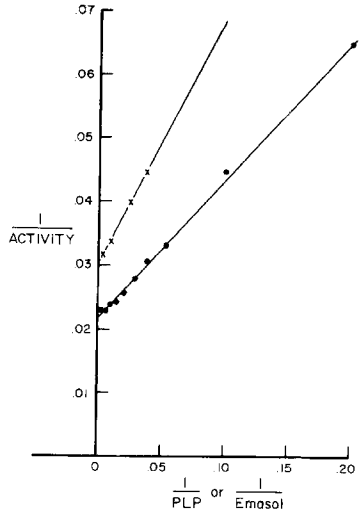

Figure 2. Double reciprocal plot of enzyme activity and concentrations of phospholipids or Emasol. 1/activity is expressed as (μmoles O_2/min/mg protein)$^{-6}$; 1/PLP, (g Atom P of phospholipids/mole cytochrome oxidase)$^{-1}$; 1/Emasol, (moles Emasol-1130/mole cytochrome oxidase)$^{-1}$. xEmasol, ●PLP.

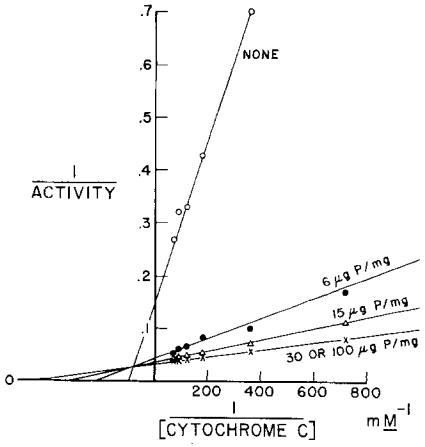

Figure 3. Effect of phospholipids on activity of cytochrome oxidase. Various concentrations of mixed total mitochondrial phospholipids were incubated with cytochrome oxidase for 10 min at 4° before being diluted for assay.

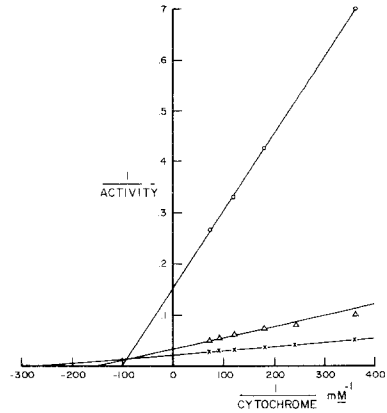

Figure 4. Effect of Emasol on activity of cytochrome oxidase. ○ none, △ 0.2 mg and x 5 mg Emasol per mg protein.

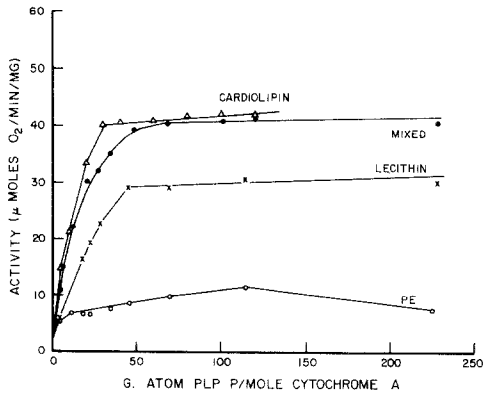

Figure 5. Effect of purified mitochondrial phospholipids on cytochrome oxidase activity. Mixed, mixed total mitochondrial phospholipids; PE, phosphatidyl ethanolamine.

(Figure 6). Phosphatidyl ethanolamine did not change the Km of the enzyme (Km for both the lipid-depleted enzyme and phosphatidyl ethanolamine-treated enzyme was 10.0×10^{-6} M) while lecithin and cardiolipin decreased the Km to 3.6×10^{-6} M and 2.1×10^{-6} M, respectively.

Phospholipids and Heat Stability of Cytochrome Oxidase

Cytochrome oxidase to which phospholipids were added was found to be much more stable to heat treatment than the lipid-depleted enzyme.

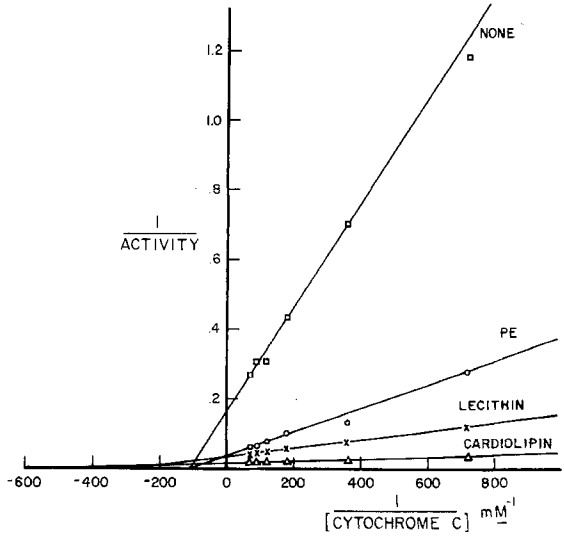

Figure 6. Effect of purified mitochondrial phospholipids at different concentrations of cytochrome *c*.

Figure 7 shows the activity of lipid-containing and lipid-depleted cytochrome oxidase after exposure to various temperatures. After exposure to heat for the time indicated, phospholipids were added to the lipid-depleted enzyme for assay. Lipid-containing cytochrome oxidase has 90%, 81% and 19% of the original activity after 20 min incubation at 30°, 38° and 50°, respectively, while the lipid-depleted enzyme had only 75%, 40% and 0% of the original activity, respectively. Heat stability of the purified cytochrome oxidase in the presence of phospholipids was similar to cytochrome oxidase in beef heart mitochondria preparations (Figure 8). Although Emasol had a similar activation effect on the lipid-depleted enzyme, it could not protect the enzyme activity as well as phospholipids could (Figures 7 and 9). Emasol-containing enzyme had 70%, 38% and 0% of the original activity after 20 min incubation at 30°, 38° and 50°, respectively, which is the same as the lipid-depleted cytochrome oxidase without any addition.

Phospholipids and Inhibition of Cytochrome Oxidase Activity by Mercurials

PCMB (*p*-chloromercuribenzoate) only slightly inhibited the lipid-depleted cytochrome oxidase (about 10%). The mercurial, however, inhibited the enzyme to a greater extent in the presence of SDS (sodium

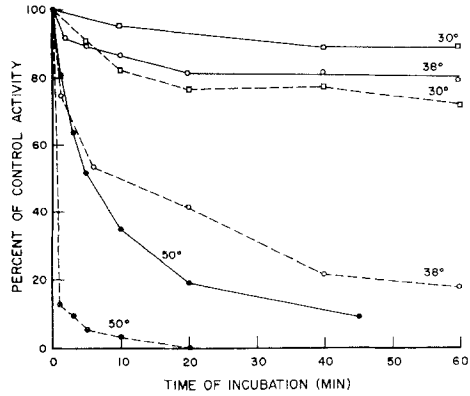

Figure 7. Heat stability of lipid-containing and lipid-depleted cytochrome oxidase. Enzyme was incubated at temperature indicated at protein concentration of 1 mg/ml. 50 μg phospholipid phosphorus/mg enzyme protein was added before or after incubation. Solid lines are the lipid-containing cytochrome oxidase; dotted lines, lipid-depleted enzyme with phospholipids added after incubation. Control activity as 100% was 41.2 μmoles O_2 /min/mg protein.

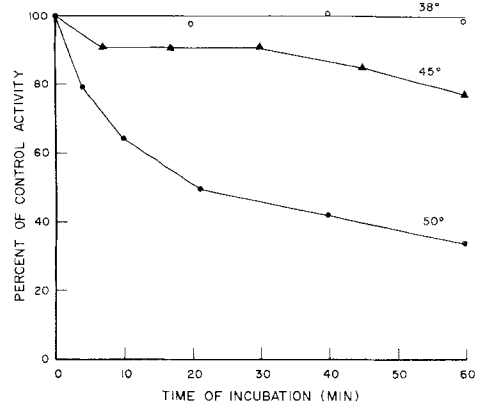

Figure 8. Heat stability of cytochrome oxidase in beef heart mitochondria. Beef heart mitochondria were adjusted to protein concentration of 4 mg/ml and incubated at indicated temperature. Control activity as 100% was 5.2 μmoles O_2 /min/mg protein.

dodecyl sulfate). Figure 10 shows the SDS treated lipid-depleted cytochrome oxidase activity after incubation with PCMB. It inhibited 90% of the enzyme activity after 60 min incubation.

When PCMB was incubated with SDS treated enzyme in the absence of phospholipids, it inhibited 16%, 79% and 95% of the enzyme activity at PCMB concentrations of 15, 30 and 37×10^{-6} M, respectively

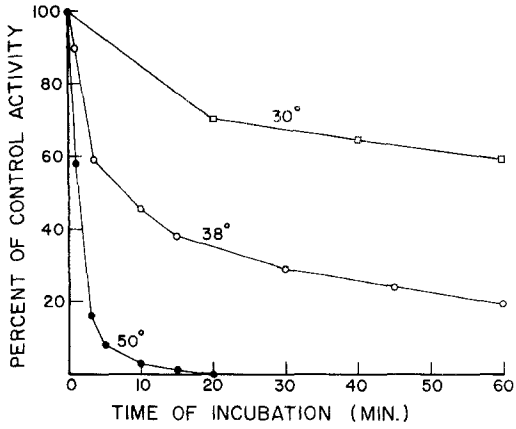

Figure 9. Heat stability of Emasol-treated lipid-depleted cytochrome oxidase. 30 mg Emasol-1130/mg enzyme protein was added before incubation. Control activity as 100% was 43.2 μmoles O_2/min/mg protein.

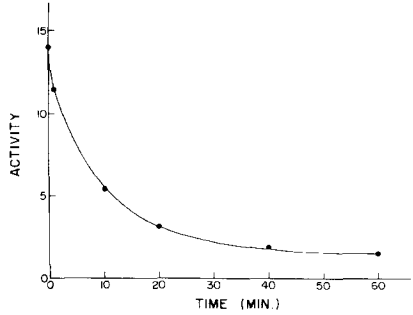

Figure 10. Inhibition of cytochrome oxidase activity by mercurial. Lipid-depleted cytochrome oxidase at concentration of 1 mg/ml in the presence of 0.05% sodium dodecyl sulfate was incubated with 3.7×10^{-5} M *p*-chloromercuribenzoate at 23°.

(Table I). The same concentrations of PCMB only inhibited 0%, 4% and 59% of the activity of the phospholipid-containing enzyme, respectively. Preincubation of dithiothreitol decreased inhibition from 59% to 16% when phospholipids were added before PCMB incubation and 95% to 69% when phospholipids were added after incubation.

Phospholipids and Inhibition of Cytochrome Oxidase Activity by Protamine

Basic proteins, such as protamine, salmine, ribonuclease, lysozyme and histone have been found to be potent inhibitors of cytochrome oxidase

TABLE 1. Inhibition of cytochrome oxidase activity by *p*-chloromercuribenzoate

PCMB concentration (10^{-6} M)	PLP added before incubation	PLP added after incubation
0	33.7 (100%)	29.1 (100%)
15	34.0 (100.9%)	24.5 (84%)
30	20.5 (60.8%)	6.1 (20.9%)
37	13.9 (41.2%)	1.5 (5.1%)
37 (13 mM dithiothreitol added prior to PCMB)	28.4 (84.3%)	9.1 (31.3%)

Activity is expressed as μmoles O_2 /min/mg protein. Parentheses are percentage of control activity.

Lipid-depleted cytochrome oxidase at concentration of 1 mg of protein/ml in 0.05% sodium dodecyl sulfate incubating with PCMB at 23° for 1 h. PLP was added before or after PCMB incubation.

reactions due to the similarity of their net charge with cytochrome *c*, the substrate for the enzyme [20, 21]. Figure 11 shows the inhibition of lipid-depleted and lipid-containing cytochrome oxidase by protamine. The lipid-containing enzyme was completely inhibited at a protamine concentration of 50×10^{-6} M while the lipid-depleted enzyme still had 30% of its original activity at the same concentration of protamine. Analysis of the effect of protamine on cytochrome oxidase by Lineweaver-Burk plot shows that both lipid-depleted and lipid-containing enzyme are competitively inhibited by protamine (Figs 12 and 13). The Km for the lipid-containing enzyme changed to much higher value in the presence of protamine than did the Km for the lipid-depleted

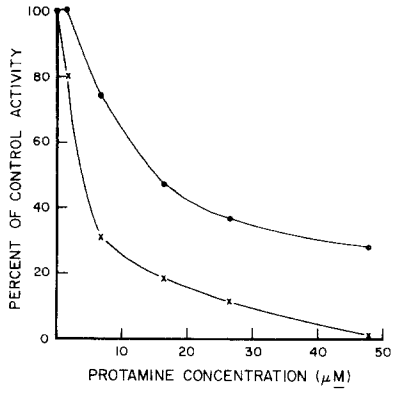

Figure 11. Inhibition of cytochrome oxidase activity by protamine. Lipid-depleted and lipid-containing (50 μg P/mg protein) cytochrome oxidase was assayed in the presence of protamine. Upper curve, lipid-depleted cytochrome oxidase; lower curve, lipid-containing enzyme.

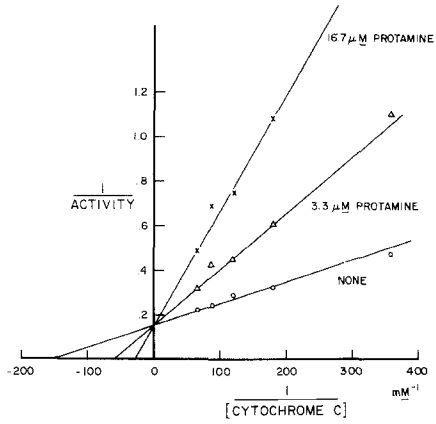

Figure 12. Effect of protamine on lipid-depleted cytochrome oxidase activity.

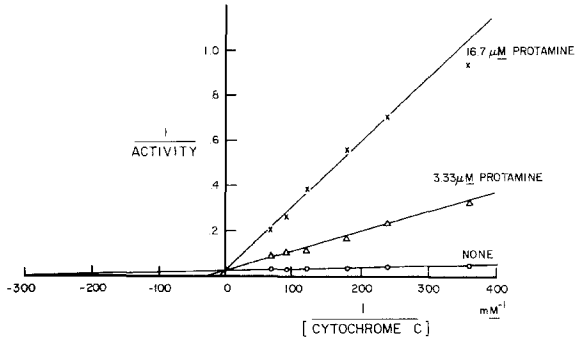

Figure 13. Effect of protamine on lipid-containing cytochrome oxidase activity.

cytochrome oxidase. In the absence of protamine, the Km for the lipid-containing enzyme was 3.3×10^{-6} M and changed to 33×10^{-6} M and 125×10^{-6} M at protamine concentrations of 3.3×10^{-6} M and 16.7×10^{-6} M, respectively (Figure 13) while that of the lipid-depleted cytochrome oxidase changed from 6.7×10^{-6} M to 16×10^{-6} M and 34×10^{-6} M, respectively (Figure 12).

Effect of Phospholipids on Cytochrome c-Cytochrome Oxidase Complex Activity

Cytochrome oxidase has been reported to form a complex with cytochrome *c* [22]. An experiment was designed to demonstrate involvement of phospholipids in the complex and its activity. Figure 14

shows the effect of the sequence of addition of cytochrome *c*, cytochrome oxidase and phospholipids or Emasol on the activity. When phospholipids or Emasol was added before cytochrome oxidase-cytochrome *c* complex was formed (Figure 14B and D) the enzyme had much higher activity than when the enzyme was added before phospholipids or Emasol (Figure 14A and C). If phospholipids or Emasol

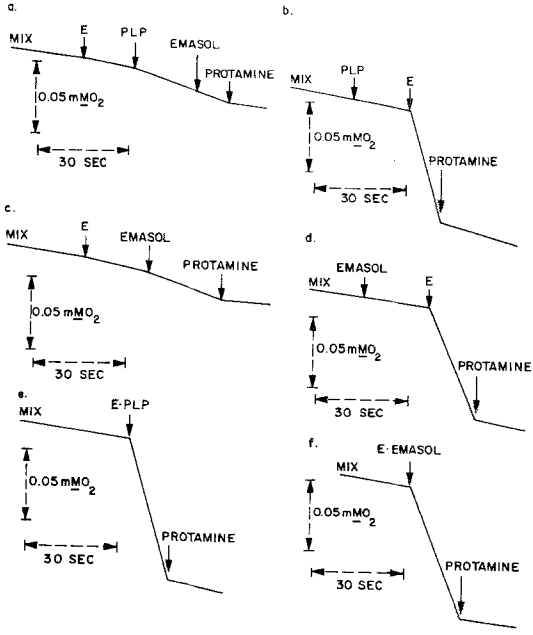

Figure 14. Effect of cytochrome *c*, cytochrome oxidase, and phospholipids or Emasol addition sequence on cytochrome oxidase activity. Mix, the mixture of ascorbate, N,N,N′,N′-tetramethyl-*p*-phenylene-diamine dihydrochloride, cytochrome *c* and assay buffer; E, lipid-depleted cytochrome oxidase, 15 μg of protein; PLP, 25 μg phospholipid phosphorus; Emasol, 0.25 mg; protamine, 0.48 mg in 1.8 ml assay medium.

was incubated with cytochrome oxidase before the assay (Figure 14E and F), it also exhibited much higher activity than when phospholipids were added during the assay. In all cases, protamine inhibited enzyme activity (Table II).

Phospholipids and Emasol also exhibited a similar effect on TCHQ (tetrachlorohydroquinone) oxidase activity of the lipid-depleted preparation (Fig 15 and Table 3). Once the protamine-cytochrome oxidase complex was formed, neither phospholipids nor Emasol could activate enzyme activity to a large extent.

TABLE 2. Effect of cytochrome *c*, cytochrome oxidase, and phospholipids or emasol addition sequence on cytochrome oxidase activity

Sequence of addition	Activity (μmoles O_2/min/mg)
Mix + E	3.8
Mix + E + PLP	11.0
Mix + PLP + E	46.0
Mix + PLP + E + protamine	1.0
Mix + E + Emasol	9.8
Mix + E + Emasol + protamine	0.3
Mix + Emasol + E	29.5
Mix + Emasol + E + protamine	0.8
Mix + (E + PLP)	40.4
Mix + (E + PLP) + protamine	0.4
Mix + (E + Emasol)	30.0
Mix + (E + Emasol) + protamine	0

Mix, the mixture of ascorbate, TMPD, cytochrome *c* and assay buffer; E, the lipid-depleted cytochrome oxidase; PLP, 25 μg P; Emasol-1130, 0.25 mg; protamine, 0.48 mg.

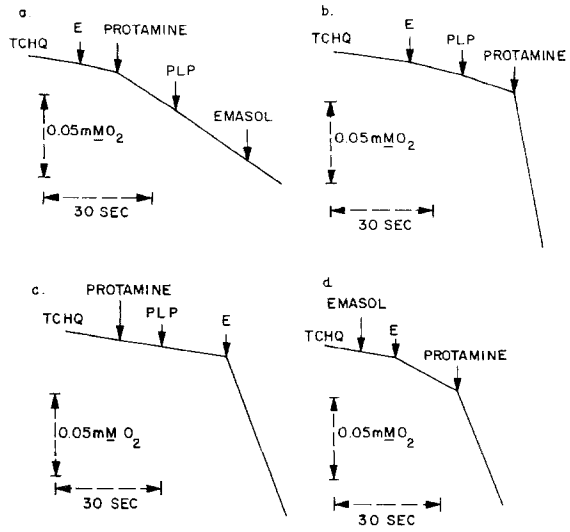

Figure 15. Effect of protamine, cytochrome oxidase, and phospholipids or Emasol addition sequence on TCHQ oxidase activity. TCHQ, tetrachlorohydroquinone 5 μmoles; E, lipid-depleted cytochrome oxidase, 0.15 mg of protein; PLP, 25 μg P; protamine, 48 mg (or 3×10^{-7} M); Emasol, 0.25 mg in 1.8 ml assay medium.

TABLE 3. Effect of protamine, cytochrome oxidase, and phospholipids or Emasol addition sequence on TCHQ oxidase activity

Sequence of addition	Activity (μmoles O_2/min/mg)
TCHQ + E	0.4
TCHQ + E + PLP	0.4
TCHQ + E + PLP + protamine	3.8
TCHQ + E	0.3
TCHQ + E + protamine	1.1
TCHQ + E + protamine + PLP	1.1
TCHQ + Emasol+ E + protamine	2.6
TCHQ + Emasol+ protamine + E	4.3

TCHQ, tetrachlorohydroquinone; E, lipid-depleted cytochrome oxidase, 0.15 mg; PLP, phospholipid 27 μg; protamine, 48 mg; Emasol-1130, 0.25 mg in 1.8 ml assay media.

Discussion

Upon addition of phospholipids to the lipid-depleted cytochrome oxidase the enzyme activity rapidly increases. This is due to the dispersing effect of the phospholipids which spread the enzyme into membranous layers [2, 22] and possibly exposing the active site to the substrate by changing the conformation of the protein [2, 12]. It has been postulated that [10] phospholipid is not a specific activator of cytochrome oxidase because it could simply be replaced by detergents, such as Emasol and Tween. Although Emasol can activate the lipid-depleted cytochrome oxidase to the same level as phospholipids can, we have never been able to show detergents can form membrane structure with cytochrome oxidase protein [2]. Both Emasol and phospholipids decrease the Km for cytochrome *c* to a similar degree and kinetically they exhibit the same pattern of effects on cytochrome oxidase, but Emasol does not play a structural role as phospholipids do. None of the detergents (Emasol, Tween, deoxycholate, cholate, sodium dodecyl sulfate or Triton), lysophosphatides, or fatty acids form membrane with cytochrome oxidase.

Further evaluation on the unique role of phospholipids in cytochrome oxidase was made to test whether phospholipids could protect the enzyme from heat treatment or chemical effect. Study of the effect of heat on enzyme activity shows that lipid can protect the enzyme from heat. The lipid-containing cytochrome oxidase has 90%, 81% and 19% of the control activity while the lipid-depleted enzyme has only 75%, 40% and 0% of the control activity after incubation for 20 min at 30°, 38°

and 50°, respectively. In the same heat treatment experiment, the Emasol-containing cytochrome oxidase loses activity to the same extent as the lipid-depleted cytochrome oxidase. Phospholipid-containing cytochrome oxidase exhibits the same stability to heat treatment as the cytochrome oxidase in intact mitochondria.

Cytochrome oxidase can form a complex with cytochrome *c* [22]. Cytochrome oxidase requires cytochrome *c* not only as an electron donor but also as a component of the enzymatically active complex [22, 24]. Attempts to study the involvement of phospholipids in the formation of cytochrome *c*-cytochrome oxidase complex of King's type [22] showed that cytochrome oxidase could form a complex with cytochrome *c* whether in the presence or absence of phospholipids (T. F. Chuang and F. L. Crane, unpublished observation). Since phospholipids are not necessary for the cytochrome *c*-cytochrome oxidase complex formation, the significance of phospholipids or other cytochrome oxidase activating agents, such as Emasol and Tween, in the cytochrome *c*-cytochrome oxidase complex is doubtful. This was evaluated by demonstrating the effect of the sequence of addition of cytochrome *c*, cytochrome oxidase and phospholipids or Emasol in the assay medium on the activity. When phospholipids or Emasol are present during the complex formation between cytochrome *c* and cytochrome oxidase, the enzyme activity is 3 to 4 times higher than when phospholipids or Emasol are added after the complex has been formed. It therefore appears that once the cytochrome *c*-cytochrome oxidase complex is formed, phospholipids are somehow blocked from orienting the two cytochromes so that maximal enzymatic activity cannot be obtained. It should be pointed out that the preformed complex can still take a membranous form when phospholipids are added.

As expected, phospholipids behave in the same way in TCHQ oxidase activity when phospholipids are added after enzyme-protamine complex has been formed, as they do in the cytochrome oxidase reaction. This could be explained by the fact that protamine-oxidase complex of the TCHQ oxidase system is analogous to the cytochrome *c*-cytochrome oxidase complex in the cytochrome oxidase system.

Study of protamine inhibition on cytochrome oxidase further supports the concept of the role of phospholipids in this segment of the electron transport chain. Protamine competitively inhibits both lipid-depleted and lipid-containing enzyme. It alters the Km for the lipid-depleting cytochrome oxidase to a lesser extent than that of the lipid-containing enzyme. In other words, it inhibits more enzyme activity in the presence of phospholipids than in the absence of them. These data in accordance with our earlier report [12, 25] suggest that phospholipids function to maintain a suitable orientation of cytochrome oxidase and facilitate the interaction of the enzyme with cytochrome *c*. Phospholipids also faciliate the interaction between the enzyme and

protamine, a substrate analogue, and potentiate its competitive inhibition.

Zahler and Fleischer [26] used lipid depleted mitochondrial membranes to measure the effect of phospholipid on the kinetics of cytochrome oxidase. Our results differ from theirs in that the Km decreased rather than increased on addition of phospholipids. In both systems the V_{max} increased on addition of phospholipid. This difference would be consistent with their postulate that detergents modify the enzyme. On the other hand the presence of other proteins in the membrane may modify the response to phospholipid and prevent aggregation of the lipid-free oxidase. Lack of aggregation could explain their lack of detergent activation.

Acknowledgements

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