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Structural Role of Phospholipids in Ubiquinol-Cytochrome *c* Reductase†

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ABSTRACT: The role of phospholipids in ubiquionol-cytochrome *c* reductase has been studied by the following methods: (1) removal and restoration of phospholipids, (2) circular dichroism measurements, and (3) phospholipase A_2 treatment. Over 90% of the phospholipids in the cytochrome b–c_1 III complex (a highly purified ubiquinol-cytochrome *c* reductase) can be removed by repeated precipitation with ammonium sulfate in the presence of 0.5% sodium cholate. The delipidated enzyme complex is inactive. Full restoration of enzymatic activity can only be achieved with a freshly prepared delipidated enzyme complex, made in the presence of 20% glycerol. As the age of the delipidated enzyme increased, the amount of activity restored decreased and the incubation time required to reach maximal activity increased. Removal of phospholipids from the cytochrome b–c_1 III complex resulted in an immediate decrease of ~15% in molar ellipticities in both the far-UV and the Soret regions. A further decrease in ellipticities was observed upon incubation of the delipidated enzyme at 0 °C in 50 mM phosphate buffer, pH 7.4. Replenishing phospholipids to the delipidated enzyme complex restored enzymatic activity and the molar ellipticity in both regions. The absolute requirement for phospholipids in the cytochrome b–c_1 III complex was also demonstrated by treatment of the enzyme with purified phospholipase A_2. The inactivation of the cytochrome b–c_1 III complex by phospholipase A_2 was not prevented by the presence of excess exogenous ubiquinone but was prevented by the presence of ethylenediaminetetracetic acid (EDTA). The enzymatic activity of the phospholipase A_2 treated complex is fully restorable upon the addition of EDTA and phospholipids.

The phospholipid (PL)[1] requirement in all four mitochondrial electron transfer complexes has been well documented (Ragan & Racker, 1973; McPhail & Cunningham, 1975; Hatefi et al., 1962; Baum et al., 1967; Yu et al., 1979a). The active complexes, as isolated, contain ~20% phospholipids. However, the degree of dependence of enzymatic activity on PL varies. Some regain activity when PL's are replaced by nonionic detergents (Yu et al., 1975), whereas others absolutely require

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[1] Abbreviations used: PL, phospholipid; Q, ubiquinone; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

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PL's (Regan & Racker, 1973; Yu et al., 1978). For example, in succinate-cytochrome *c* reductase, PL's are absolutely required for enzymatic activity (Yu et al., 1978). No detergents or fatty acids can restore activity to the delipidated enzyme. On the other hand, the requirement for PL in cytochrome *c* oxidase is not absolute, as several nonionic detergents such as Tween 80 or Emasol 1130 can partially restore enzymatic activity in the delipidated oxidase (Yu et al., 1975). The strict requirement for PL in the electron-transfer complex is evidenced by changes in the spectral properties and stability of the PL-depleted preparations. For instance, succinate-cytochrome *c* and ubiquinol-cytochrome *c* reductases show significant spectral changes (Yu et al., 1979b) and become very labile after removal of PL. Cytochrome *c* oxidase, however, shows very little difference in stability and spectral properties between the delipidated and intact preparations (Yu et al., 1975). When PL's were removed from succinate-cytochrome *c* reductase, some of the redox components in the complex underwent a redox state change while others did not. Cytochrome c_1, for example, became reduced in the absence of exogenous substrate (Yu et al., 1978) upon the removal of PL from succinate-cytochrome *c* reductase or ubiquinol-cytochrome *c* reductase, indicating that some unknown component, which is in the reduced state in the presence of PL, has become oxidized and has transferred an electron to cytochrome c_1 in the absence of PL. The midpoint potential of such an unknown component is PL dependent, because the midpoint potential of cytochrome c_1 has been shown to be constant in the presence or absence of PL (Chiang & King, 1979).

Since the enzymatic activity of the isolated succinate dehydrogenase (Davis & Hatefi, 1971; Ackrell et al., 1977; Yu & Yu, 1980) is independent of PL and succinate dehydrogenase in the delipidated succinate-cytochrome *c* reductase has the same properties as observed in the intact complex (Yu et al., 1973), the action site of PL in succinate-cytochrome *c* reductase is, therefore, located in the cytochrome b–c_1 region. The availability of the highly purified and soluble ubiquinone-cytochrome *c* reductase (cytochrome b–c_1 III complex) (Yu & Yu, 1980) has facilitated conformational study of this complex through circular dichroism spectral measurement. In this report we wish to present the effects of PL on the structure of the cytochrome b–c_1 III complex and its enzymatic activity. Part of this report has been presented previously (Yu & Yu, 1979).

Experimental Procedures

Horse cytochrome *c*, type III, phospholipase A_2 from bee venom, crystalline bovine albumin, and sodium cholate were procured from Sigma Chemical Co. Ammonium sulfate was obtained from Schwarz/Mann, and asolectin was obtained from Associated Concentrates Inc. Q_2 was synthesized according to the method of Shunk et al. (1958) with modifications. Enzyme preparation and assays of beef heart succinate-cytochrome *c* reductase (Yu et al., 1974) and ubiquinol-cytochrome *c* reductase (the cytochrome b–c_1 III complex) (Yu & Yu, 1980) were carried out according to the reported procedures.

The delipidated cytochrome b–c_1 III complex was prepared essentially according to the method described for the preparation of the PL-depleted succinate-cytochrome *c* reductase (Yu et al., 1978). A purified cytochrome b–c_1 III complex as prepared (Yu & Yu, 1980) was diluted in 50 mM sodium–potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate to a protein concentration of ~10 mg/mL. The solution was incubated at 0 °C for 20 min before being subjected to ammonium sulfate precipitation. Neutralized, saturated ammonium sulfate solution was used to bring the solution to 45% saturation, and the precipitate formed was collected by centrifugation at 27000*g* for 20 min. This precipitate was dissolved in 50 mM sodium–potassium phosphate, pH 7.4, containing 0.5% sodium cholate and 20% glycerol to a protein concentration of 10 mg/mL. The solution was once again brought to 45% ammonium sulfate saturation with neutralized, saturated ammonium sulfate solution. The precipitate thus obtained was dissolved in the same phosphate–cholate–gylcerol buffer and ammonium sulfate precipitation was repeated 3 times. The resulting precipitate was finally dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 0.67 M sucrose to a protein concentration of 20 mg/mL. Some delipidated preparations were finally dissolved in 50 mM sodium–potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate.

Samples of the intact and delipidated cytochrome b–c_1 III complex used for the circular dichroism measurement were diluted in 10 mM Tris-HCl buffer, pH 7.8, containing 0.1% sodium cholate and 50 mM ammonium sulfate to ensure complete solubility. Protein concentration was determined by the biuret method in the presence of H_2O_2 (Yonetani, 1961) using bovine serum albumin as the standard.

The reincorporation of Q and PL was done by first mixing the delipidated cytochrome b–c_1 III complex, at a protein concentration of ~10 mg/mL, with Q (10 nmol/mg of protein) and then with an asolectin micelle solution (0.3 mg/mg of protein). The mixture was then diluted with 50 mM phosphate buffer, pH 7.4, to a protein concentration of ~1 mg/mL and incubated at 0 °C, and samples were withdrawn for assay at given times until the maximal activity was reached. The Q_2 was dissolved in 95% ethanol to a concentration of 1–2 mg/mL. The asolectin micelle solution was prepared in water at 10 mg/mL by sonification.

Cytochrome *b* (Berden & Slater, 1970), cytochrome c_1 (Yu et al., 1972), nonheme iron (Brumby & Massey, 1967), phospholipids (Ames & Dubin, 1960) and Q (Redfearn, 1967) were determined according to reported procedures.

All visible spectral measurements and assays were conducted in a Cary spectrophotometer, Model 14. The circular dichroism spectra were determined in a Cary spectrophotometer, Model 60, with CD attachment, Model 6002, calibrated with *d*-10-camphorsulfonic acid. Molar ellipticity in the Soret region was expressed on the basis of the concentration of cytochrome c_1, whereas that in the far-UV region was calculated by amino acid residues, using an average amino acid residue weight of 115. $NaDodSO_4$ polyacrylamide gel electrophoresis was performed according to Weber & Osborn (1969).

Results

Removal of Phospholipids from Cytochrome b–c_1 III Complex. Over 90% of the enzymatic activity and the PL in the cytochrome b–c_1 III complex are removed by the method of cholate–ammonium sulfate precipitation. Maximal removal of phospholipids and Q requires five precipitation cycles. Figure 1 shows the enzymatic activity and PL remaining after each cycle of ammonium sulfate precipitation in the presence of 0.5% sodium cholate. The presence of 20% glycerol is necessary from the second cycle of ammonium sulfate precipitation on, in order to avoid irreversibly inactivating the delipidated cytochrome b–c_1 III complex. When prepared in the absence of glycerol, the delipidated cytochrome b–c_1 III complex shows very little activity after reincorporation of Q and PL, although the chemical compositions of delipidated preparations made in the presence and absence of glycerol are

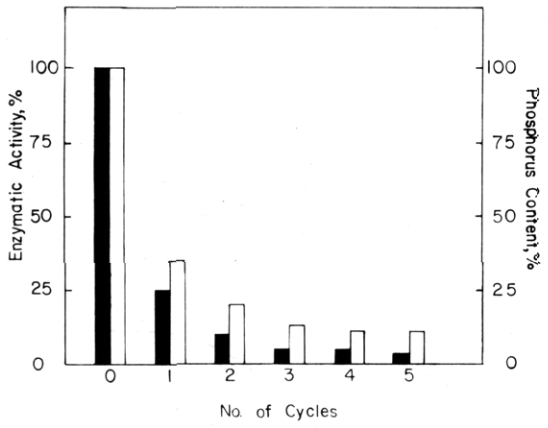

FIGURE 1: Correlation of enzymatic activity and phospholipid content of cytochrome b–c_1 III complex during ammonium sulfate precipitation in the presence of 0.5% sodium cholate. 100% activity equals 140 μmol of cytochrome c reduced min^{-1} (mg of ptotein)$^{-1}$ at 23 °C, and 100% phospholipids indicates 0.25 μmol of phosphorus (mg of protein)$^{-1}$. Activity (■); phosphorus content (□).

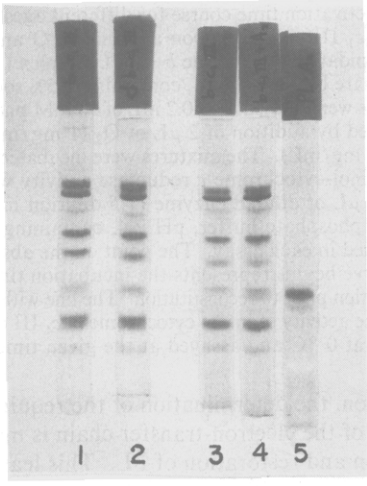

FIGURE 2: Sodium dodecylsulfate–polyacrylamide gel electrophoretic patterns of the intact, delipidated, and phospholipase A_2 treated cytochrome b–c_1 III complex. The trace detergents used in the preparation of the complex were removed by diluting the protein to 0.2 mg/mL in 50 mM sodium–potassium phosphate buffer, pH 7.4, and centrifuging at 45000 rpm for 2 h in a Beckman centrifuge, Model L, rotor 50. The samples were then digested with 1% $NaDodSO_4$ and 1% β-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.0, at 55 °C for 2 h. The amounts of proteins used in the gel column were, from left to right, 12, 12, 10 and 10 μg for the intact (1), delipidated (2), intact (3), and phospholipase A_2 treated (4) cytochrome b–c_1 III complex, respectively, and 3 μg for phospholipase A_2 (5).

identical. Table I compares the PL and other essential components in the intact and delipidated cytochrome b–c_1 III complex. With the exceptions of PL and Q, the delipidated complex has an essential component content identical with that of the intact complex. The repeated ammonium sulfate precipitation in the presence of 0.5% sodium cholate does not alter the polypeptide composition of the complex. Figure 2 (gels 1 and 2) shows the identical $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of intact and delipidated cytochrome b–c_1 III complex preparations.

Circular Dichroism Spectra of Cytochrome b–c_1 III Complex and Its Delipidated Preparation. The cytochrome b–c_1 III complex and its delipidated preparation are soluble in aqueous solution without addition of detergent. Perhaps the bound detergent used during preparation is sufficient to disperse the protein. When the enzyme complex was in a relatively high protein concentration (20 mg/mL), a nearly transparent solution was obtained. At low protein concentrations (1 mg/mL) in the absence of added detergent, the preparation became slightly turbid. This turbility disappeared when the solution was made in dilute detergent. To ensure an absolutely transparent sample for circular dichrosim measurement, we routinely diluted the sample in 10 mM

Table I: Chemical Composition of the Cytochrome b–c_1 III Complex and Its Delipidated Preparation

components	intact cytochrome b–c_1 III complex (nmol/mg)	delipidated cytochrome b–c_1 III complex (nmol/mg)
cytochrome b	10.3	10.3
cytochrome c_1	5.9	5.9
nonheme iron	6.9	6.9
phospholipids[a]	250	25.3
ubiquinone	3	0.4

[a] Phospholipids are expressed in nanomoles of phosphorus per milligram of protein.

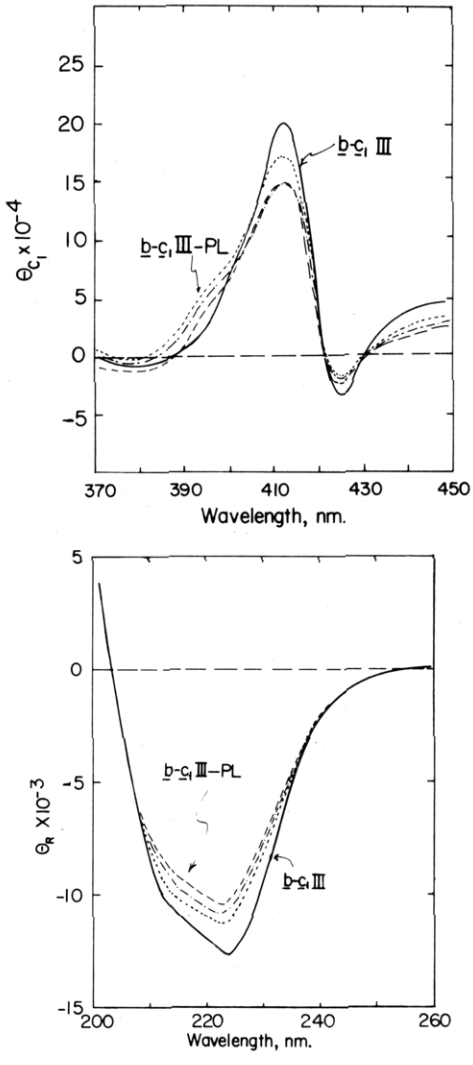

FIGURE 3: Circular dichroism spectra of the cytochrome b–c_1 III complex and its delipidated preparation in the Soret (A) and far-UV (B) regions. Both the intact and delipidated cytochrome b–c_1 III complex preparations were diluted to 1 mg/mL with 10 mM Tris-HCl buffer, pH 7.8, containing 0.1% sodium cholate and 50 mM ammonium sulfate. 1 cm and 0.1 cm light path cuvettes were used in the Soret and UV regions, respectively. The solid line (—) represents intact cytochrome b–c_1 III complex. The dotted line (- - -), dashed line (–·–), and broken line (– – –) represent freshly made, 1-h-old and 3-h-old delipidated samples, respectively.

Tris-HCl buffer, pH 7.8, containing 0.1% sodium cholate (or deoxycholate) and 50 mM ammonium sulfate. Figure 3A shows the Soret circular dichroism spectra of cytochrome b–c_1 III complex and its delipidated preparations at different ages. For simplicity, only the oxidized form was measured. The cytochrome b–c_1 III complex has a Soret circular dichroism extremum at 413 nm with a molar ellipticity of 2×10^5. The molar concentration was calculated by the cytochrome c_1 concentration, assuming that 1 mol of cytochrome b–c_1 III complex contains 1 mol of cytochrome c_1. Upon removal of PL, a significant decrease in ellipticity, ~16%, was observed. When the delipidated preparation was incubated at 0 °C, a further decrease (25%) in ellipticity was observed, especially in the first hour of incubation. The decrease in the 413-nm extremum resulting from the removal of PL was coupled with an increase in ellipticity at 394 nm, but the increased ellipticity at 394 nm gradually diminished during the incubation of the delipidated enzyme.

It is known that the ellipticity in the far-UV region, is correlated to the peptide bond configuration (α helix and β structure) of proteins (Greenfield & Fasman, 1969). Therefore, it is of interest to see whether PL's have any effect on the circular dichroism spectra of this region. As indicated in Figure 3B, the intact cytochrome b–c_1 III complex shows the typical double extrema of negative ellipticity in the far-UV region, with a molar ellipticity of 1.3×10^4 at 233 nm. When PL's were removed from the preparation, a significant decrease in molar ellipticity was observed. This indicates that removal of PL's from the cytochrome b–c_1 III complex causes some changes in polypeptide structure.

It has been shown in Table I that the delipidated cytochrome b–c_1 III complex is also deficient in ubiquinone. Restoring Q to the PL- and Q-depleted cytochrome b–c_1 III complex, however, produces no significant effect on the circular dichroism spectrum of either the Soret or far-UV region. Q alone was found to be ineffective in restoring enzymatic activity to the delipidated complex.

Stabilization Effect of Phospholipids in Cytochrome b–c_1 III Complex. Like other electron-transport membrane protein complexes, the cytochrome b–c_1 III complex as isolated is very stable. However, the enzyme stability decreases when PL's are removed from the complex. Only freshly prepared delipidated cytochrome b–c_1 III complex is capable of regaining the full original activity upon reincorporation of Q and PL. As the age of the delipidated cytochrome b–c_1 III complex increases, the amount of activity restored upon addition of Q and PL decreases. After a 6-h incubation at 0 °C the delipidated cytochrome b–c_1 III complex lost 50% of its ability to regain unbiquinol-cytochrome c reductase activity upon addition of PL. Figure 4 shows the reactivation time course for the delipidated enzyme of different ages. When the freshly prepared delipidated cytochrome b–c_1 III complex was reactivated by addition of Q and PL, the enzymatic activity was restored quickly. Maximal activity (the activity of intact complex) was restored to the enzyme incubated up to 2 h at 0 °C upon mixing with Q and PL. When the delipidated complex became older, the activity that could be restored decreased, and the activation time required to reach that partial activity increased greatly. These results indicate that the change in structure proceeds continuously after the PL's are removed and it is that change, due to prolonged incubation of the delipidated complex, that is irreversible.

Inactivation of the Cytochrome b–c_1 III Complex by Phospholipase A_2. Although the absolute requirement for PL in the cytochrome b–c_1 III complex is demonstrated in the previous section, the determination of the requirement of PL in this region of the electron-transfer chain is mainly derived from depletion and restoration of PL. This leaves the possibility that the inactivation may have been due partly to a conformational change induced by the repeated ammonium sulfate–cholate precipitations and that the replenished PL acted only to return the conformation to the original active form, rather than having a direct catalytic or structural role. To account for this possibility, we have taken another approach to demonstrate the absolute requirement for PL in this region of the electron-transport chain. This approach is to digest the PL with purified phospholipase A_2 and follow the enzymatic activity as the digestion proceeds. The result is shown in Figure 5. Under the tested conditions, a 50% inactivation of ubiquinol-cytochrome c reductase activity required only 3 min. While the kinetics of the inactivation have not been analyzed in detail, inactivation due to the presence of proteolytic enzymes in the phospholipase A_2 has been ruled out as the phospholipase A_2 treated complex (gel 4, Figure 2) shows a protein subunit pattern in the $NaDodSO_4$–polyacrylamide gel electrophoretic column identical with untreated cytochrome b–c_1 III complex (gel 3, Figure 2). Therefore, the inactivation observed could not have been due to the destruction of protein in the cytochrome b–c_1 III complex. The purity of phospholipase A_2 is illustrated in the gel 5 of Figure 2.

It is known that the activity of phospholipase A_2 is dependent on calcium ions (Roberts et al., 1977). Removal of calcium ions from the system by EDTA inactivated the phospholipase A_2 activity. Table II shows the reactivation of phospholipase A_2 treated cytochrome b–c_1 III complex by EDTA and PL. A full restoration of activity was observed when the phospholipase A_2 treated complex was mixed with

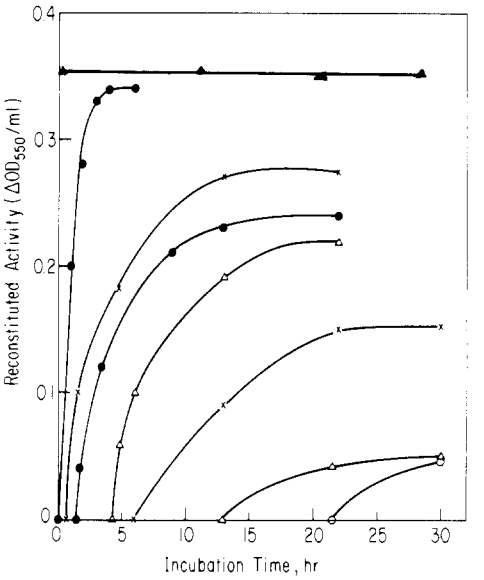

FIGURE 4: Reactivation time course for different ages of delipidated cytochrome b–c_1 III complex upon addition of Q and PL. 20-μL aliquots of delipidated cytochrome b–c_1 III complex (10 mg/mL, in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate) at the given ages were diluted with 0.2 mL of 50 mM phosphate buffer, pH 7.4, followed by addition of 2 μL of Q_2 (1 mg/mL) and 40 μL of asolectin (10 mg/mL). The mixtures were incubated at 0 °C. The restored ubiquinol-cytochrome c reductase activity was assayed at given times. 1 μL of diluted enzyme (1:5 dilution in 50 mM sodium–potassium phosphate buffer, pH 7.4, containing 0.1% sodium cholate) was used in each assay. The point on the abscissa at which the activity curve begins represents the incubation time of the delipidated preparation prior to reconstitution. The line with solid triangles (▲) indicates the activity of intact cytochrome b–c_1 III complex which was incubated at 0 °C and assayed at the given times.

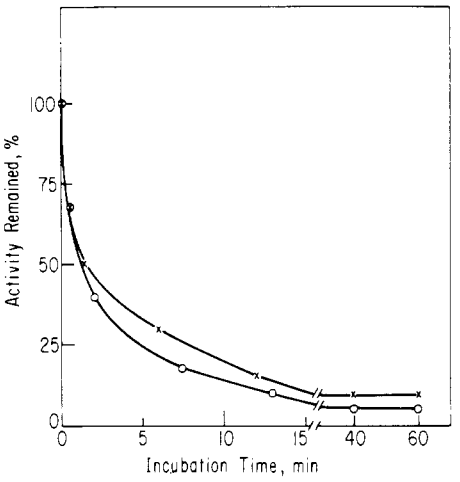

FIGURE 5: Inactivation of cytochrome b–c_1 III complex by phospholipase A_2 in the presence and absence of excess exogenous Q. 1 mL of cytochrome b–c_1 III complex (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate in the presence (×) and absence (○) of excess Q_2 (0.6 mM) was incubated with 5 μL of phospholipase A_2 (2 mg/mL, in 50% glycerol), at 23 °C. Aliquots of the incubated enzyme were withdrawn and assayed for ubiquinol-cytochrome c reductase activity at a fixed Q_2H_2 concentration (75 μM).

Table II: Reversible Inactivation of the Cytochrome b–c_1 III Complex by Phospholipase A_2

no.	treatments[a]	activity[b] (%)
1	cytochrome b–c_1 III complex at 0 °C	100
2	cytochrome b–c_1 III complex at 23 °C	98
3	cytochrome b–c_1 III complex + A_2	2
4	(cytochrome b–c_1 III complex + EDTA) + A_2	98
5	(cytochrome b–c_1 III complex + A_2) + EDTA + PL	101

[a] The cytochrome b–c_1 III complex was diluted in 50 mM phosphate buffer, pH 7.4, containing 2.5% glycerol and 0.1% sodium cholate to a protein concentration of 1 mg/mL. 0.25-mL aliquots of the diluted protein were incubated at 0 °C (1) and at room temperature in the absence (2) or in the presence (3) of phospholipase A_2 for 1 h. 10 μL of phospholipase A_2 (2 mg/mL, in 50 mM phosphate buffer, pH 7.4, containing 50% glycerol) was used. In (4), the cytochrome b–c_1 III complex was mixed with 10 μL of 0.1 M EDTA, pH 6.6, prior to the incubation with phospholipase A_2 at room temperature. In (5), the same treatment as in (3) was used, except that at the end of incubation, 10 μL of EDTA solution was added and the solution was then mixed with 25 μL of phospholipids (10 mg of asolectin/mL). [b] Activity was followed by reduction of cytochrome c using Q_2H_2 (75 μM) as substrate. 100% activity equals 140 μmol of cytochrome c reduced min^{-1} (mg of protein)$^{-1}$ at 23 °C.

3.5 mM EDTA and 1 mg of asolectin/mg of protein. If EDTA was added prior to the addition of phospholipase A_2, no inactivation of the cytochrome b–c_1 III complex was observed upon incubation. These results suggest that the role of PL is not simply that of providing a fluid environment but is indeed required to maintain the structural integrity of the complex.

Effect of Ubiquinone on the Action of Phospholipase A_2. Phospholipase A_2 inactivated ubiquinol-cytochrome c reductase activity by hydrolyzing PL, as demonstrated in Figure 5. However, a recent brief report (Shimomura et al., 1979) states the phospholipase action on mitochondrial electron-transfer complexes can be prevented by addition of excess Q to the system. Since the report was based on results obtained from crude complexes and submitochondrial particles, it is of interest to see if a similar result can be obtained in the cytochrome b–c_1 III complex. Figure 5 compares the inactivation of the cytochrome b–c_1 III complex by phospholipase A_2 in the presence and absence of excess Q. The final concentration of Q in the system was 600 μM. In contrast to the reported result, we found that the presence or absence of excess Q in the system made no difference to inactivation by phospholipase A_2.

Discussion

Repeated ammonium sulfate precipitation in the presence of cholate has been shown to be effective in the removal of PL from electron-transfer complexes (Yu et al., 1973; Ragan & Racker, 1973; Yu et al., 1975). The degree of PL removed, however, varies. Phospholipids in cytochrome c oxidase can be almost completely removed, and the resulting PL-depleted enzyme preparation is stable and can be fully reactivated upon addition of PL. Complex I (Regan & Racker, 1973), on the other hand, can be depleted of no more than 50% of its PL without destroying the enzyme. As described in the previous section, more than 90% of the PL in the cytochrome b–c_1 III complex can be removed by the same procedure, but the activity of the delipidated preparation can be restored only when glycerol (20%) is included during the ammonium sulfate precipitation in the presence of cholate. Even under these conditions, the delipidated enzyme is only stable for a short period of time. The mechanism of the protective effect of glycerol is not clear at present.

Since the activity of the freshly made delipidated cytochrome b–c_1 III complex can be fully restored, it is reasonable to assume that the process of removal of PL did not itself cause any harmful damage but changed the cytochrome b–c_1 III complex from a stable to a more labile state, which then became denatured upon prolonged incubation. In other words, the structural or conformational change resulting from the removal of PL is reversible, at least in a functional sense. It is the change resulting from prolonged incubation of the delipidated enzyme that produced the irreversibly inactivated form. The change from the delipidated form to the inactivated form probably occurs in more than one step, since freshly made delipidated cytochrome b–c_1 III complex regains its original activity upon reincorporation of PL after a brief incubation (<3 h) at 0 °C, whereas an aged delipidated preparation required a much longer incubation time before a partial activity could be detected. If no further change in the protein structure occurred after removal of PL from the complex, one would have expected to see a similar reactivation process in the freshly prepared and aged delipidated preparations, even though the maximal extent of activity restoration would have decreased resulting from denaturation upon incubation.

The effect of PL on the circular dichroism spectra of the cytochrome b–c_1 III complex indicated that a strong interaction between protein and PL exists. There are at least seven different molecular weight polypeptides present in this enzyme complex, and whether the change in structure results from change of a single polypeptide or a collective change of structure is not known. As also indicated in Figure 3, a further change in structure was observed upon incubation of the delipidated preparation at 0 °C. This result parallels those obtained in the Soret region. Since the Soret circular dichroism spectra reflect mainly the heme environment of cytochromes b and c_1, and, to a minor extent, the nonheme iron–sulfur protein, it is perhaps safe to suggest that perturbation of the protein surrounding heme b and heme c_1, as well as perturbation somewhere within the secondary structure of the cytochrome b–c_1 III complex has occurred. The significant change of the redox properties of cytochrome b upon the removal of PL (Yu et al., 1979b) also indicates the close

relation between cytochrome *b* and PL. Cytochrome c_1 probably is not directly involved in this change since the redox behavior of isolated cytochrome c_1 is not affected by PL (Chiang & King, 1979) and the change in circular dichroism spectra of cytochrome c_1 in the Soret region by PL has not been reported, although an increase in molar ellipticity in the far-UV region has been observed when isolated cytochrome c_1 is incorporated into PL vesicles. No correlation of the circular dichroism spectral changes in the Soret and far-UV regions has been made. The significance of the increase in far-UV ellipticity, therefore, remains to be elucidated.

The treatment of the cytochrome b–c_1 III complex with phospholipase A_2 clearly demonstrated that phospholipids are required in this particular region of the electron-transfer chain. The activity of the phospholipase A_2 treated cytochrome b–c_1 III complex can be fully restored by the addition of asolectin, indicating that the digested products, lysophospholipids, still stabilized the treated protein even though they were no longer able to interact with the protein to form a active enzyme complex. Further study on the conformational change resulting from phospholipase A_2 treatment should yield useful information regarding the protein–PL interaction and is currently in progress.

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