The Role of Phospholipid in Succinate Cytochrome *c* Reductase†

Linda Yu, Chang-an Yu, and Tsoo E. King*

ABSTRACT: A lipid-depleted succinate cytochrome *c* reductase was prepared by fractionation of succinate ctyochrome *c* reductase in the presence of 0.5% cholate and 40% ammonium sulfate. The lipid-depleted preparation possessed the same content of cytochromes *b* and c_1 as the intact reductase, but showed only 40% of the succinate phenazine methosulfate activity and no succinate cytochrome *c* reductase activity. The depleted preparation in solution form was stable only for a few hours in contrast to the intact reductase which retained full activity for at least 1 week. Upon the addition of coenzyme Q (CoQ) and phospholipids derived from the 40% supernatant fraction, the succinate cytochrome *c* activity of the depleted reductase was restored. Complete restoration was also effected by addition of pure phosphatidylethanolamine and cardiolipin in the presence of CoQ. However, neither of the phospholipids alone showed maximal activity. The action of CoQ was visible only when the phospholipids were present. The restored activity was also antimycin A sensitive. Titration of the lipid-depleted reductase with phospholipids gave a sigmoidal curve. The maximum reductase activity was restored to the lipid-depleted preparation after a 90-min incubation with the phospholipids from the 40% supernatant fraction and CoQ at the concentration of 0.28 mg and 10 μg per mg of protein, respectively. Addition of the phospholipids also restored the succinate phenazine methosulfate activity up to the same level as that found in the intact succinate cytochrome *c* reductase. This restoration could be completely effected by cardiolipin alone without CoQ. A very small portion of cytochrome *b* in the lipid-depleted reductase was succinate reducible. Addition of the phospholipids also restored succinate reducibility of cytochrome *b* to almost the same level as that found in the intact succinate cytochrome *c* reductase.

One approach to probe the role of lipids in the mitochondrial respiratory chain involves the removal of lipids from mitochondrial or submitochondrial particles, followed by the reincorporation of lipids to the lipid-depleted preparation to restore its original function. Although this approach has been used extensively in the submitochondrial particles pioneered by Fleischer and coworkers (1962), it has not yet been widely applied in the purified respiratory segments. Fleischer *et al.* (1962) have shown that removal of phospholipids from mitochondrial particles, by aqueous acetone extraction or phospholipase digestion, leads to inactivation of respiration which can be restored by the addition of phospholipids. Lester and Fleischer (1959), Szarkowska (1966), and Ernster *et al.* (1969), among others, have claimed that extraction of ubiquinone from beef heart submitochondrial particles results in an inactivation of NADH and succinate oxidase activities which is reversible by the reincorporation of ubiquinone.

We have succeeded in removing phospholipids from succinate cytochrome *c* reductase by a simple, mild procedure. The lipid-depleted reductase shows no succinate cytochrome *c* activity and impaired succinate phenazine methosulfate action. This paper reports the details of these facets including the potency of purified phospholipids in the restoration of the enzymic activities and the apparent effect of phospholipids on the succinate reducibility of the cytochrome *b* in the system. The latter effect may be related to the multiplicity of *b* cytochromes.

Experimental Procedure

Material. Horse cytochrome *c*, type III, crystalline bovine serum albumin, and CoQ_6[1] were procured from Sigma; sodium cholate, sodium deoxycholate, Triton X-100 and Tween-80 from Mann; chromatographically pure cardiolipin, phosphatidylethanolamine, and phosphatidylcholine from General Biochemical; and Asolectin from Associated Concentrates, Inc., Woodside, Long Island. All other chemicals in the highest available purity were purchased commercially. The water used was doubly distilled in an all-glass apparatus from deionized distilled water. The heart muscle preparation was prepared from bovine heart, according to the adaptation (King, 1961, 1967a) used in this laboratory of the original method of Keilin and Hartree (1940). Succinate cytochrome *c* reductase was prepared according to the procedure described previously (Takemori and King, 1964; King 1967b) except the percentage of ammonium sulfate saturation used in the second step was 55% instead of 51% and in the last step was 50% instead of 48%.

Preparation of the Lipid-Depleted Succinate Cytochrome c Reductase. The lipid-depleted succinate cytochrome *c* reductase was prepared from succinate cytochrome *c* reductase by means of ammonium sulfate fractionation in the presence of 0.5% sodium cholate. Succinate cytochrome *c* reductase was dissolved in 0.1 M phosphate buffer containing 0.5% sodium cholate (pH 7.4) to a protein concentration of 10 mg/ml. Neutralized saturated ammonium sulfate solution was added to 40% saturation. After stirring for 20 min at 4°, the solution was centrifuged at 48,000*g* for 20 min. The supernatant solution, designated as "the 40% supernatant," was saved for the preparation of the phospholipids used in the reincorporation experiments. The precipitate thus obtained was dissolved in 0.1 M phosphate buffer (pH 7.4), containing 0.5% cholate. The ammonium sulfate–cholate fractionation was

† From the Department of Chemistry, State University of New York at Albany, Albany, New York 12222. *Received August 18, 1972.* This work was supported by grants from the National Science Foundation and the U. S. Public Health Service.

[1] Abbreviation used is: CoQ, coenzyme Q or ubiquinone.

TABLE I: Comparison of Chemical Composition and Enzymic Activity of Succinate Cytochrome *c* Reductase and Lipid-Depleted Reductase.

Succinate Cytochrome *c* Reductase[a]	Composition				Activity	
	Cytochromes		Lipid		Succinate Cytochrome *c* Reductase (μmol of *c*/min per mg)	Succinate Dehydrogenase[b] (μmol of Succinate/min per mg)
	b (nmol/mg)	c_1 (nmol/mg)	P-lipid (mg/mg)	Ubiquinone (nmol/mg)		
Intact	2.0	1.2	0.16	1.3	3.1	4.8
Lipid depleted	2.3	1.25	0.045	0.9	0.0	1.9

[a] The data given in the table were average values from five batches of succinate cytochrome *c* reductase preparations. [b] The succinate dehydrogenase activities were expressed as V_{max} obtained at infinite concentration of phenazine methosulfate and 36 mM succinate.

repeated once more as before. The precipitate was finally dissolved in 50 mM phosphate buffer (pH 7.4) to a protein concentration of approximately 8 mg/ml.

Preparation of the Phospholipids from the 40% Supernatant. The 40% supernatant was kept at 4° for at least 1 week before use in order to let the residual protein precipitated down. The precipitate, if any, formed during the storage was removed by filtration and the clear solution was then dialyzed against at least 20 volumes of distilled water for 3 days with three changes of water to remove cholate and ammonium sulfate. The dialyzed solution was lyophilized in a Vis-Tris lyophilizer. Dried material (1g), derived from the 40% supernatant, was extracted twice with 100 ml of pentane (Ernster *et al.*, 1969) and then extracted three times with 200 ml of chloroform–methanol mixture (2:1) at room temperature under nitrogen. The chloroform–methanol extracts were combined and nonlipid contaminants were removed by the Folch method of partition (*cf.* Fleischer and Fleischer, 1967). The extract was then evaporated to dryness *in vacuo*. The residual ubiquinone remaining in the chloroform–methanol extract was removed by partitioning the extract between pentane and 95% methanol (Redfearn, 1967). The methanol layer was dried and reextracted once more with chloroform–methanol (2:1) mixture. The final chloroform–methanol extract was then dried *in vacuo* in a rotatory evaporator. The final preparation is designated as "the phospholipids from the 40% supernatant."

Preparation of Lipid Micelles. All the lipid preparations used in this paper were in the "micelle" form. The lipid micelles were prepared by the ultrasonic irradiation method (Fleischer and Fleischer, 1967) at about 4°. Lipid preparations were suspended in water at a concentration of 5 mg/ml and sonicated, intermittently to avoid overheating, under nitrogen in a MSE Sonifier at an output of 8 A for a total time of 5 min. The sonicated mixture was centrifuged at 80,000*g* for 1 hr. After centrifugation, the clear supernatant solution was stored at 4° under nitrogen until used. The actual lipid content was determined gravimetrically.

Determination of CoQ in Succinate Cytochrome c Reductase. The CoQ_{10} in succinate cytochrome *c* reductase was extracted repeatedly by pentane (Ernster *et al.*, 1969). Its concentration was then estimated spectrophotometrically using a millimolar extinction coefficient of 122.5 for A^{275}_{ox-red} (Redfearn, 1967).

Enzyme Assay. Succinate cytochrome *c* reductase and succinate phenazine methosulfate activities were assayed according to the methods described previously (Takemori and King, 1964; King and Takemori, 1962). All the assays were carried out in a 1-ml reaction mixture at room temperature, approximately 22°.

Methods. Cytochrome c_1 was determined from the difference spectra of the ascorbate reduced sample minus the ferricyanide oxidized, using a millimolar extinction coefficient of 17.5 for $A^{552.5} - A^{540}$ (Yu *et al.*, 1972a). Cytochrome *b* was estimated from difference spectra of the dithionite reduced sample minus the ascorbate reduced, using a millimolar extinction coefficient of 28.5 for $A^{562} - A^{577}$ (Berden and Slater, 1970). The concentration of CoQ_6 was estimated spectrophotometrically using an extinction coefficient of 206 at 275 nm for $A^{1\%}_{ox-red}$ (Crane, 1960). Protein in hemoprotein was assayed by a biuret method in the presence of hydrogen peroxide (Yonetani, 1961) with crystalline bovine serum albumin as a standard.

Results

Chemical Composition and Properties of the Lipid-Depleted Succinate Cytochrome c Reductase. Table I shows the comparison of chemical composition and enzymic activities between the intact and the lipid-depleted succinate cytochrome *c* reductase. As also reported previously (Takemori and King, 1962, 1964; King and Takemori, 1964), all the succinate cytochrome *c* activity of the reductase was found to be antimycin A sensitive. The lipid-depleted preparation showed 40% of the succinate dehydrogenase activity but practically no succinate cytochrome *c* reductase activity. As shown in Table I, about 70% of the phospholipid and 30% ubiquinone of the reductase were removed during the cholate–ammonium sulfate fractionation. Addition of the 40% supernatant back to the lipid-depleted succinate cytochrome *c* reductase resulted in a *complete* restoration of both the succinate cytochrome *c* reductase and succinate dehydrogenase activities. It was clear that the loss of the enzymic activities in the lipid-depleted preparation was simply due to the removal of some essential components from the reductase in the course of the fractionation and such components remained in the supernatant solution. Treating the supernatant solution with 8 M urea or heating up to 80° for 3 min did not affect its ability in the restoration of the enzymic activity. These results gave a clue of the involvement of lipid rather than protein in the reactivation. As expected, addition of the phospholipids prepared

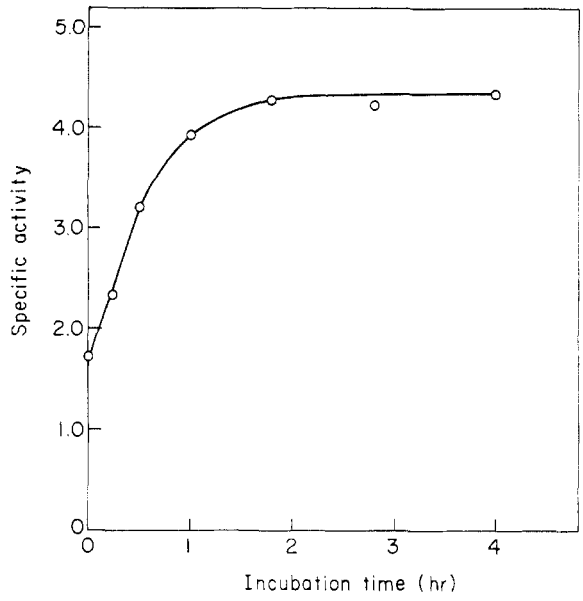

FIGURE 1. Effect of incubation time on the restoration of succinate cytochrome *c* reductase activity; 0.1 ml of the lipid-depleted preparation (7 mg/ml) was mixed with 0.4 ml of phospholipid–CoQ_6 micelle solution containing 0.3 mg of the phospholipids from the 40% supernatant and 31 μg of CoQ_6. The mixture was incubated at 4°. At the indicated times, the aliquots were withdrawn and assayed for the activity. The specific activity was expressed in μmol of cytochrome *c* reduced per min per mg of protein.

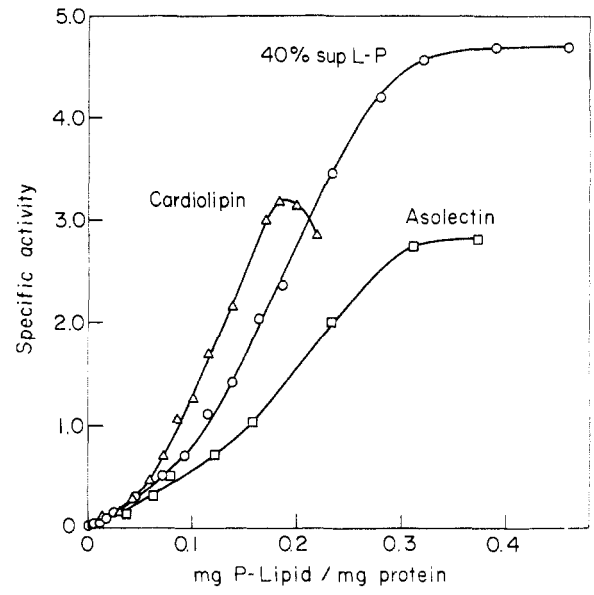

FIGURE 2: Titration of the lipid-depleted succinate cytochrome *c* reductase by phospholipids in the presence of CoQ_6. Aliquots (0.1 ml) of the lipid-depleted preparation (4.3 mg) were mixed with 0.4 ml of the phospholipid–CoQ_6 micelle solution, containing 31 μg of CoQ_6 and the amount of the total phospholipids as shown. The mixtures were incubated at 4° for 2 hr prior to the assay. The specific activity was expressed as μmol of cytochrome *c* reduced per min per mg of protein.

from the 40% supernatant to the lipid-depleted reductase under the following conditions resulted in complete restoration of the enzymic activities.

Restoration of Succinate Cytochrome c Reductase Activity from the Lipid-Depleted Preparation by Reincorporation of the Phospholipids from the 40% Supernatant. A freshly prepared lipid-depleted succinate cytochrome *c* reductase was dissolved in 50 mM phosphate buffer (pH 7.4) to a protein concentration of 8 mg/ml. To a 0.1 ml of the enzyme solution, 0.4 ml of a micelle solution, containing 0.3 mg of phospholipid and 31 μg of CoQ_6, was added. The enzymic activity restored by such treatment was a function of incubation time as shown in Figure 1. The restored activity was directly proportional to the incubation time up to 2 hr, after that the activity became constant. It must be emphasized that the specific activity of succinate cytochrome *c* reductase thus restored was quantitative, *i.e.*, the same as that of the intact reductase. Moreover, as with the intact reductase, the lipid reincorporated reductase was stable; only a slight decrease in activity was observed upon storage at 4° for 24 hr. The lipid reincorporated reductase was also completely sensitive to antimycin A. In order to determine the amount of phospholipid required for the maximal restoration of the activity, titration experiments were conducted. The results are summarized in Figure 2. As can be seen, 0.28 mg of the phospholipids from the 40% supernatant per mg of the lipid-depleted reductase protein was required for complete restoration of the enzymic activity. For the pure cardiolipin and purified Asolectin, 0.18 and 0.28 mg, respectively, per mg of the reductase protein were required for the maximal restoration of activity. It might be significant to note that all the titrations showed a sigmoidal characteristic with a distinct lag, even though the maximal restoration for different lipids tested was quite different. The sigmoidal characteristics of titration curves might be caused by a small amount of the residual cholate remaining in the preparation so that the phospholipids might have first to compete for the cholate "sites." This possibility is not likely, because the titration curves obtained at different concentrations of cholate were practically the same. Increase of the cholate in the system did not increase the lag of the curves or affect their sigmoidal behavior.

Both Asolectin and cardiolipin were only about 60% as effective as the phospholipids from the 40% supernatant. The amount of phospholipid required to restore maximal activity was significantly higher than the lipid content of the intact reductase. This fact would indicate that not all the phospholipids added could be properly utilized as efficiently as the endogenous entity. This explanation seems to be substantiated by the sigmoidal behavior of the titration. The latter may also suggest the complicated action of the phospholipids in the multiple site participation for the possible alignment of specific respiratory protein components for their proper conformations. These conformations might be obligatory for their appropriate functions.

Specificity of Phospholipids on the Restoration of Succinate Cytochrome c Reductase Activity from the Lipid-Depleted Preparation. Various phospholipids were tested for their ability in restoring the succinate cytochrome *c* reductase activity from the lipid-depleted preparation. The results are summarized in Table II. The phospholipids prepared from succinate cytochrome *c* reductase possessed the same effectiveness as that prepared from the 40% supernatant. Thin-layer chromatographic analysis of the phospholipids from the 40% supernatant revealed the presence of cardiolipin, phosphatidylethanolamine, and lecithin as three major components. Similar chromatograms were also obtained for lipid prepared from the reductase. In order to identify which phospholipid was essential for the succinate cytochrome *c* reductase activity, the chromatographically pure phospholipids were tested for their ability in restoring enzymic activity from

TABLE II: Effect of Various Phospholipids on the Succinate Cytochrome *c* Reductase Activity of the Lipid-Depleted Reductase.[a]

Reductase	Addition	Succinate Cytochrome *c* Reductase Act.	
		μmol of *c* min^{-1} mg^{-1}	%[b]
Lipid depleted	1. None	0.004	0
	2. Coenzyme Q_6	0.004	0
	3. Phospholipid from 40% supernatant	1.8	40
	4. Phospholipid from 40% supernatant + CoQ_6	4.5	100
	5. Asolectin	0.67	15
	6. Asolectin + CoQ_6	2.7	60
	7. Cardiolipin	1.3	30
	8. Cardiolipin + CoQ_6	2.9	65
	9. Phosphatidylethanolamine	0.67	15
	10. Phosphatidylethanolamine + CoQ_6	2.0	45
	11. Lecithin	0.04	1
	12. Lecithin + CoQ_6	0.18	4
	13. Cardiolipin + phosphatidylethanolamine + CoQ_6	4.4	98
Intact	14. None	3.1–4.5	
	15. Phospholipid from 40% supernatant + CoQ_6	4.5	100

[a] Lipid-depleted succinate cytochrome *c* reductase was mixed with phospholipid in the presence or absence of CoQ_6. The amount of phospholipids added, based on 1 mg of protein, was 0.4, 0.35, 0.18, 0.43, and 0.72 mg of the phospholipid from the 40% supernatant, Asolectin, cardiolipin, phosphatidylethanolamine, and lecithin, respectively. CoQ_6 was added at the concentration of 50 μg/mg of protein. In the intact reductase 0.1 mg of the phospholipid of 40% supernatant was added per mg of reductase protein. All the phospholipid concentrations used in the experiment were the amount required to obtain the maximal activity. The activity was determined after incubation of the mixture at 4° for 2 hr. [b] Per cent of the maximal activity, or 4.5 μmol of cytochrome *c* reduced per mg per min.

TABLE III: Effect of Detergents on the Restoration of Succinate Cytochrome *c* Reductase Activity from the Lipid-Depleted Preparation.[a]

Reductase	Addition	Act.	
		μmol of *c* min^{-1} mg^{-1}	%
Intact	None	3.1	
	Triton X-100 + CoQ_6	4.2	100
Lipid-depleted	None	0.0	0
	Triton X-100 + CoQ_6	0.6	15
	Tween-80 + CoQ_6	0.1	3
	Sodium deoxycholate + CoQ_6	0.2	4
	Sodium cholate + CoQ_6	0.2	4

[a] The lipid-depleted preparation was mixed with various detergents in the presence of CoQ_6. The final concentration of detergents based on mg of protein was 0.092, 0.1, 0.39, and 1.56 mg of Triton X-100, Tween-80, sodium deoxycholate, and sodium cholate, respectively. The concentration of detergents indicated above was the amount required in achieving the maximal activity for each detergent used, which were obtained from preliminary experiments. The CoQ_6 was added at the concentration of 50 μg/mg of protein. The activity was assayed after 2-hr incubation at 4°.

the lipid-depleted preparation. The effectiveness of the tested phospholipids was found to be in the order of cardiolipin, phosphatidylethanolamine and lecithin. Although the reductase contains these three major phospholipids, maximal restoration could be achieved without the addition of lecithin.

Effect of Detergents on the Restoration of Succinate Cytochrome c Reductase Activity of the Lipid-Depleted Reductase. It has been reported that the addition of certain detergents to lipid-deficient cytochrome oxidase restores the oxidase activity to the same level as that achieved with phospholipids (Morrison *et al.*, 1960). However, as shown in Table III, we found only a partial restoration of reductase activity by the addition of detergents and CoQ_6 to the lipid-depleted reductase. The concentrations given in the table were obtained from titration curves at which the maximal restoring activity was reached. Among the detergents tested, Triton X-100 was most effective. However, the maximal activity restored by Triton X-100 was still only about 20% of that attained with phospholipids. A slight stimulation on the intact succinate cytochrome *c* reductase activity upon addition of Triton X-100 was also observed (*cf.* also Takemori and King, 1964).

The Requirement of CoQ in the Restoration of Succinate Cytochrome c Reductase Activity from the Lipid-Depleted Preparation. As indicated in Table II, only 40% of the reductase activity was restored upon addition of the phospholipids from the 40% supernatant in the absence of CoQ. Addition of CoQ_6 resulted in a full restoration. A titration curve of the lipid-depleted reductase with CoQ_6, in the presence of a sufficient amount of phospholipid, is given in Figure 3. To avoid the uncertainty of residual CoQ in the phospholipid preparation, chromatographically pure cardiolipin and purified Asolectin were also used in the titration experiment. A similar result for CoQ_6 titrations was obtained for the purified phospholipids tested. Ten micrograms of CoQ_6 per milligram of protein was found to be the saturating concentration. As indicated in Figure 3 and Table II, the fact that a substantial amount of activity was restored by phospholipids alone without the addition of CoQ_6 suggests that a significant amount of ubiquinone was still present in the lipid-depleted preparation. Indeed, the direct chemical analysis, as shown in Table I, showed a major portion of the ubiquinone to have remained in the depleted preparation. However, no succinate cytochrome *c* reductase activity was restored upon addition of CoQ *alone*.

Effect of Phospholipid on the Succinate-Reducible Cytochrome b in the Lipid-Depleted Succinate Cytochrome c Reductase. As shown in Table IV, the lipid-depleted preparation pos-

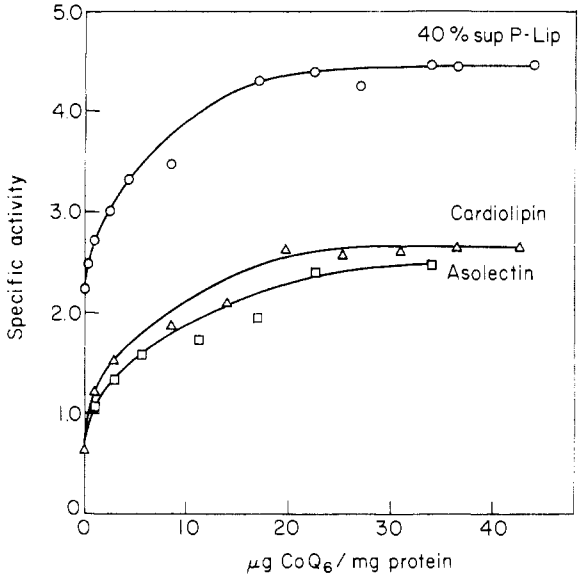

FIGURE 3: Titration of the lipid-depleted succinate cytochrome *c* reductase by CoQ_6 in the presence of phospholipids. Conditions were the same as those described in Figure 2, except the amounts of the phospholipids used were: 0.4, 0.18, and 0.3 mg per mg of protein for the phospholipids from the 40% supernatant, cardiolipin, and Asolectin, respectively. The amounts of CoQ_6 as indicated were mixed with phospholipids before addition to the enzyme preparation.

TABLE IV: Effect of Phospholipids and CoQ on the Succinate Reducibility of Cytochrome *b* of the Lipid-Depleted Succinate Cytochrome *c* Reductase.[a]

Addition	Cytochrome *b*		
	Reduced by Dithionite (μM)	Reduced by Succinate (μM)	Succinate Reducible *b*: Dithionite Reducible *b* (%)
None	13	0.9	7
+Phospholipids	14	6.4	48
+CoQ_6	13	2.1	17
+Phospholipids + CoQ_6	12	5.4	47

[a] The system contained 11.4 mg of the lipid-depleted reductase in 2 ml of 50 mM phosphate buffer (pH 7.4)–0.25 M sucrose. The lipid-depleted reductase was incubated for 2 hr at 4° with, as indicated, 4.6 mg of the phospholipids from the 40% supernatant and 45 μg of CoQ_6.

sesses a very small percentage of the succinate-reducible cytochrome *b*, whereas the dithionite-reducible cytochrome *b* was practically the same as that of the intact reductase, as expected. Addition of phospholipids resulted in an increase of the succinate reducibility of cytochrome *b*. The amount of succinate-reducible cytochrome *b* reached a level slightly lower than that of the intact reductase. CoQ alone also resulted in a small increase of succinate-reducible cytochrome *b* but with no restoration of succinate cytochrome *c* reductase activity (*cf.* Table II). Addition of the phospholipids in the presence of CoQ did not further increase the succinate-reducible cytochrome *b* beyond the level reached with phospholipids alone, although the enzymic activity was enhanced. Furthermore, our unpublished results from the experiments of the titration of the lipid depleted reductase by the phospholipids in the absence of CoQ have shown that the amount of succinate reducible cytochrome *b* is a function of the phospholipid present in the system. The titration curves exhibit a plateau at phospholipid concentration of 0.25 mg/mg of protein. The phospholipid-restored succinate-reducible cytochrome *b* shows the same characteristic as that of the original cytochrome *b* in intact succinate cytochrome *c* reductase. Difference spectra obtained between partially reduced and "completely" reduced forms also show three distinguishable α peaks as described in our previous reports (Yu *et al.*, 1972b). Over the years of experience in the preparation of succinate cytochrome *c* reductase, we have found that the cytochrome *b* in the reductase which is reducible by succinate, varies from 60 to 75% of the dithionite-reducible *b*, but no correlation could be established with the enzymic activity.

From these results may be deduced, among others, two salient points which in turn may explain some rather important observations. The succinate-reducible *b* is type II cytochrome *b* as we reported previously (Yu *et al.*, 1972b) or sometimes known as b_k. In spite of a number of papers on the multiplicity of *b* that have appeared (*cf.* Yu *et al.*, 1972b; Slater, 1972; Wilson and Dutton, 1971; and the references cited), the most basic question cannot be resolved due to the nature of the experiments reported. The question is whether the multiplicity of *b* is due to (i) the existence of different proteins (three different *b* cytochromes), or (ii) to one protein (one cytochrome *b*) in different environments or ligands or, (iii), although less likely, to multiple proteins with only one of them sensitive to environments or ligands. The first possibility may or may not be likely because the succinate-reducible *b* is a function of the phospholipid and CoQ in the system. Looking from another angle of the problem, it is clear that the oxidation–reduction potential is dependent heavily upon the microenvironment of the respiratory proteins. The existence of multiple sites which require phospholipid is probably responsible for the lack of complete parallelism between the phospholipid (or CoQ) restoration of succinate cytochrome *c* reductase activity and of the succinate-reducible *b*. In other words, the enzymic activity (the reduction of cytochrome *c*) is the total manifestation of the functionality of the system, whereas the succinate reducibility of cytochrome *b* is only a part of the total. That is why CoQ alone can increase the latter but not the phospholipids titratable succinate cytochrome *c* reductase activity.

Effect of Removal and Reincorporation of Phospholipids on Succinate Dehydrogenase Activity toward Artificial Electron Acceptors. As indicated in Table I, about 60% of the succinate dehydrogenase activity toward phenazine methosulfate was inactivated after removal of the phospholipids from the succinate cytochrome *c* reductase. The dehydrogenase activity was fully restored upon the reincorporation of the phospholipids from the 40% supernatant or cardiolipin (see Table V). Although the phenazine methosulfate activity varied in different batches of the reductase, the effect of phospholipid removal and reincorporation were consistent. In the study of the succinate dehydrogenase in the lipid-depleted reductase, we also compared the K_m for succinate and the K_m for phenazine methosulfate in the presence and absence of phospholipid. The results are summarized in Table V. It may be seen

TABLE V: Effect of Phospholipids on the Succinate Phenazine Methosulfate Activity of the Lipid-Depleted Succinate Cytochrome *c* Reductase.[a]

Reductase	K_m^{suc} (mM)	K_m^{PMS} (mM)	V_{max} (mM^{-1} min^{-1} mg^{-1})
1. Intact reductase	0.46	0.48	4.6
2. Lipid-depleted reductase	0.20	0.23	1.9
3. (2) + CoQ_6	0.21	0.20	1.7
4. (2) + cardiolipin	0.45	0.48	4.4
5. (2) + phospholipids from 40% supernatant	0.45	0.48	4.5

[a] The reincorporation of cardiolipin, phospholipids from 40% supernatant, and CoQ_6 was performed as described in the legend of Table II. K_m^{PMS} was obtained from experiments with 0.06–0.6 mM of phenazine methosulfate in the presence of 36 mM succinate and K_m^{suc} from 0.06 to 0.20 mM of succinate.

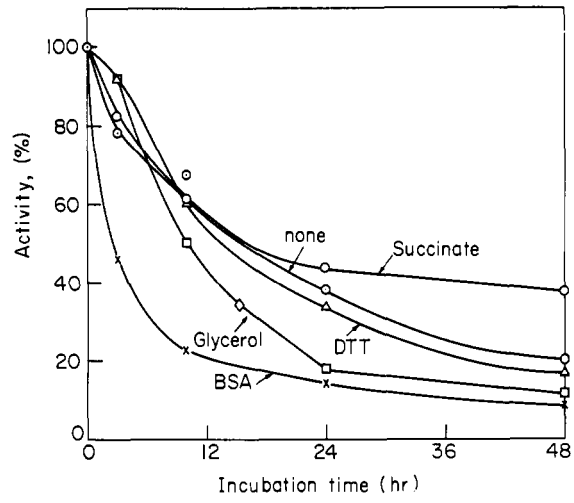

FIGURE 4: Stability of lipid-depleted succinate cytochrome *c* reductase. The lipid-depleted preparations were suspended at protein concentration of 7 mg/ml in 50 mM phosphate buffer (pH 7.4), wherever indicated also containing 20 mM succinate, 10% glycerol, 1% dithiothreitol, or 1% bovine serum albumin. At the indicated time, 0.1-ml aliquots were withdrawn and mixed with 0.4 ml of a phospholipid–CoQ_6 micelle solution, which contained 0.3 mg of the phospholipids from the 40% supernatant and 31 μg of CoQ_6, and incubated at 4° for 2 hr prior to the assay.

that the lipid depleted reductase shows a lower K_m for both succinate and the artificial electron acceptor than the intact reductase does. The phospholipid not only recovered the succinate dehydrogenase activity but also restored the K_m values. Of interest, in contrast to the succinate cytochrome *c* reductase activity, which requires cardiolipin, phosphatidylethanolamine and CoQ for recovery of activity, cardiolipin alone suffices to recapture the artificial activity. Indeed, CoQ is completely ineffective.

Stability of the Lipid-Depleted Succinate Cytochrome c Reductase. The lipid-depleted succinate cytochrome *c* reductase was found to be more stable in its precipitate form than in the solution form with respect to the restoration of its succinate cytochrome *c* activity by phospholipids. When the lipid-depleted reductase was kept in the precipitate form, at 4°, the activity could be preserved overnight, whereas in solution the activity lasted only for a few hours. As indicated in Figure 4, none of the tested compounds, including succinate, dithiothreitol, glycerol, and bovine serum albumin, were effective in preserving the activity of the lipid-depleted preparation. In contrast, the intact reductase was very stable. These facts may suggest that one of the functions of the phospholipids in the reductase is to maintain the respiratory components in a conformation resistent to inactivation. This may also explain why the depleted reductase is more stable in the precipitate (because of the more intimate contact with the remaining lipid) and more unstable in the presence of serum protein (because of the competition between the reductase and the albumin for the lipid). It recalls that lipid-free succinate dehydrogenase in the soluble form is very unstable whereas the dehydrogenase in submitochondrial particles or related particulate forms linked with lipid is stable (*cf.* King, 1966).

Discussion

The results described in this paper demonstrate the requirement of phospholipids in the succinate cytochrome *c* reductase activity. Removal of 70% of the phospholipids completely abolishes the succinate cytochrome *c* reductase activity. Similar phenomena have been observed in the submitochondrial particles by Fleischer and coworkers (1962). However, using cholate–ammonium sulfate precipitation to remove lipid for the study of lipid requirement in a specific electron transfer segment may possess advantages over using the complete system by organic sovent extraction or phospholipase digestion. For example, most of the electron transport components in the respiratory chain are more sensitive to the organic solvents used than to cholate. Usually organic solvents easily denature many enzymes, especially the cytochromes. Although phospholipase digestion does not cause denaturation of protein components as drastically as organic solvents do, the digestion products, especially free fatty acids, are rather detrimental to certain enzymic actions such as the succinate cytochrome *c* reductase activity. Therefore, the results obtained from the phospholipase digestion, even after extensive washing with serum albumin, must be interpreted with caution. Cholate and ammonium sulfate treatment, on the other hand, does not have such harsh effects as the depleted preparation is completely inactive, whereas the activity is fully restored upon the reincorporation of phospholipids.

Divergent views have been expressed as to the role of ubiquinone in the mitochondrial respiratory chain (for example, Hatefi, 1966; Green, 1966; Mitchell, 1966; Kröger and Klingenberg, 1967; Lee *et al.*, 1965; Redfearn, 1966; Storey, 1968). Our present studies on succinate cytochrome *c* reductase have shown that CoQ cannot restore the antimycin A sensitive succinate cytochrome *c* reductase activity from its lipid-depleted preparation without the addition of the phospholipids. Phospholipids alone can restore a part of the activity, but the full restoration is obligated to both CoQ and the phospholipids. If CoQ alone does not restore succinate cytochrome *c* reductase activity because of the insolubility of the CoQ in water, then it would be expected that the addition of CoQ in the presence of detergents such as Triton X-100 would enhance, at least in part, the restoration of activity. But this is not the case.

It has been reported (King, 1963) that the K_m for phenazine methosulfate for soluble succinate dehydrogenase decreased as its reconstitution activity decreased. Rossi *et al.* (1970) have found that the removal of ubiquinone from submitochondrial particles leads to a decrease in activity of succinate dehydrogenase and a decrease in the K_m for succinate as well as for phenazine methosulfate. Cerletti *et al.* (1967) have shown that preincubation with phospholipids stimulates the activity of isolated succinate dehydrogenase, suggesting that the lipid environment is an important factor in regulating the catalytic properties of the enzyme. They (Cerletti *et al.*, 1969) have substantiated their conclusion by the results obtained with phospholipase digestion of intact mitochondria. Similar results have been found in the succinate dehydrogenase activity of succinate cytochrome *c* reductase in the present study. Lipid produced a significant effect on the kinetic behavior of the dehydrogenase activity in the lipid-depleted succinate cytochrome *c* reductase.

References

Berden, J. A., and Slater, E. C. (1970), *Biochim. Biophys. Acta 216*, 237.

Cerletti, P., Caiafa, P., Giordano, M. G., and Giovenco, M. A. (1969), *Biochim. Biophys. Acta 191*, 502.

Cerletti, P., Giovenco, M. A., Giordano, M. G., Giovenco, S., and Strom, R. (1967), *Biochim. Biophys. Acta 146*, 380.

Crane, F. L. (1960), *in* Quinones in Electron Transport, Walstenholme, G. E. W., Ed., CIBA Foundation Symposium, Boston, Mass., Little, Brown and Co., p 36.

Ernster, L., Lee, I. Y., Norling, B., and Persson, B. (1969), *Eur. J. Biochem. 9*, 299.

Fleischer, S., Brierley, G., Klouwen, H., and Slautterback, D. B. (1962), *J. Biol. Chem. 237*, 3264.

Fleischer, S., and Fleischer, B. (1967), *Methods Enzymol. 10*, 423.

Green, D. E. (1966), *Comp. Biochem. 14*, 309.

Hatefi, Y. (1966), *Comp. Biochem. 14*, 199.

Keilin, D., and Hartree, E. F. (1940), *Proc. Roy. Soc., Ser. B 129*, 277.

King, T. E. (1961), *J. Biol. Chem. 236*, 2342.

King, T. E. (1963), *J. Biol. Chem. 238*, 4037.

King, T. E. (1966), *Advan. Enzymol. 28*, 155.

King, T. E. (1967a) *Methods Enzymol. 10*, 202.

King, T. E. (1967b), *Methods Enzymol. 10*, 216.

King, T. E., and Takemori, S. (1964), *J. Biol. Chem. 239*, 3559.

Kröger, A., and Klingenberg, M. (1967), *Curr. Top. Bioenerg. 2*, 176.

Lee, C. P., Estabrook, R. W., and Chance, B. (1965), *Biochim. Biophys. Acta 99*, 32.

Lester, R. L., and Fleischer, S. (1959), *Arch. Biochem. Biophys. 80*, 470.

Mitchell, P. (1966), Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Bodmin, Cornwall, Glynn Research Ltd.

Morrison, M., Bright, J., and Rouser, G. (1960), *Arch. Biochem. Biophys. 114*, 50.

Redfearn, E. R. (1966), *Vitamins Hormones 24*, 465.

Redfearn, E. R. (1967), *Methods Enzymol. 10*, 381.

Rossi, E., Norling, B., Persson, B., and Ernster, L. (1970), *Eur. J. Biochem. 16*, 508.

Slater, E. C. (1972), *Proc. Mech. Bioenerg.* (in press).

Storey, B. T. (1968), *Arch. Biochem. Biophys. 126*, 585.

Szarkowska, L. (1966), *Arch. Biochem. Biophys. 113*, 519.

Takemori, S., and King, T. E. (1962), *Biochim. Biophys. Acta 64*, 194.

Takemori, S., and King, T. E. (1964), *J. Biol. Chem. 239*, 3546.

Wilson, D. F., and Dutton, P. L. (1971), *in* Electron and Coupled Energy Transfer in Biological Systems, Vol. 1, Part A, King, T. E., and Klingenberg, M., Ed., New York, N. Y., Marcel Dekker, p 221.

Yonetani, T. (1961), *J. Biol. Chem. 236*, 1680.

Yu, C. A., Yu, L., and King, T. E. (1972a), *J. Biol. Chem. 247*, 1012.

Yu, C. A., Yu, L., and King, T. E. (1972b), *Biochim. Biophys. Acta 267*, 300.