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Kinetic Studies of the Lipid Requirement of Mitochondrial Cytochrome *c* Oxidase*

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Abstract

The lipid requirement of cytochrome *c* oxidase was reinvestigated using both acetone and phospholipase A to deplete mitochondria of lipid. Removal of lipid resulted in a decrease in both the apparent K_m for cytochrome *c* and apparent V_{max} when compared to control mitochondria. Addition of phospholipid to the assay mixture reactivated the enzyme. For both treatments the K_m returned to the control value. With phospholipase A treated mitochondria the V_{max} increased to near the control value, while acetone extracted mitochondria could be restored to a V_{max} of 1/2 that of the control. Detergent does not substitute for phospholipid and inhibits the reactivation with phospholipid.

The removal of lipid from mitochondria with aqueous acetone[1] has been used to demonstrate a phospholipid requirement for electron transport.[2,3] It could be shown that phospholipid is required for each segment of the electron transfer chain between succinate and oxygen, including cytochrome *c* oxidase.[4] Subsequent reports have confirmed this requirement for cytochrome *c* oxidase activity in purified preparations.[5,6] Treatment of mitochondria with phospholipase A is a milder procedure for removal of phospholipid,[7] and has allowed the demonstration of a lipid requirement in DPNH oxidase as well as confirm the requirement in succinate oxidase.[7–9]

Morrison *et al.*[10] reported a preparation of cytochrome *c* oxidase which contained little lipid, yet retained significant amounts of activity. The cholate used for purification and remaining with the enzyme was apparently sufficient to maintain cytochrome *c* oxidase activity in their preparation. How can these results be reconciled with the lipid requirement of cytochrome *c* oxidase in the mitochondria? In order to clarify this question and further elucidate the role of lipid in the cytochrome *c* oxidase reaction we have reinvestigated the lipid requirement of cytochrome *c* oxidase in mitochondria using the phospholipase A method[7] as well as solvent extraction to remove phospholipid. We have confirmed the requirement for lipid in the cytochrome *c* oxidase reaction in mitochondria; detergent does not substitute for phospholipid in reactivation of the enzyme. The lack of specificity in the isolated cytochrome *c* oxidase would appear to reflect an altered behavior.

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Experimental Procedure

Phospholipase A treatment of mitochondria to remove phospholipid was carried out as described by Fleischer and Fleischer.[7] Beef heart mitochondria (1·25 ml, 25 mg/ml) were mixed sequentially with 1·0 ml of 0·05 M glycyl glycine, pH 7·4, and 0·025 ml of 0·4 M $CaCl_2$. Each sample was preincubated for 2 minutes at 37° prior to the addition of 1·0 ml of 1% phospholipase A in 0·05 M glycyl glycine, pH 7·4. The reaction was terminated after 30 minutes by dilution with 22 ml of wash solution (1% BSA; 0·05 M glycyl glycine, pH 7·4; 0·25 M sucrose and 0·001 M EDTA). The mitochondria were collected by centrifugation at 30,000 rpm for 15 minutes in a Spinco model L using a #50 Titanium rotor. The pellet was washed four times with 25 ml of wash solution and once with 25 ml of 0·25 M sucrose. The final wash was centrifuged at 40,000 rpm for 10 minutes and the pellet was resuspended in 5 ml of 0·25 M sucrose. As a control, beef heart mitochondria were carried through the same procedure but 1 ml of 1% BSA was substituted for the phospholipase A. All samples were stored frozen until use. Using the above procedure, phospholipase A treatment was calibrated by varying the amount of phospholipase A and the time of reaction to give minimal residual activity and a maximal reactivation with phospholipid.

Lipid was also removed by extracting beef heart mitochondria with acetone [10% water, 90% acetone (v/v)].[7] Mitochondrial phospholipid (MPL) was isolated as previously described.[7] Reactivation of cytochrome *c* oxidase was performed using aqueous microdispersions of MPL prepared by the method of Fleischer and Klouwen.[2]

Cytochrome *c* Oxidase Assay: A mixture containing 0·1 ml of 0·62 M phosphate, pH 7·0, and 1 mM EDTA; 5·9 μg of mitochondrial protein and the desired amount of MPL was incubated for 10 minutes at 32°. After incubation, the solution was diluted and reduced cytochrome *c* was added to start the reaction. For standard assay conditions the final concentration of reduced cytochrome *c* was 25·8 μM. A final volume of 1·0 ml was used in all assays. The reaction was recorded on a Gilford recording spectrophotometer by following the decrease in OD at 550 mμ for about 2 minutes. Initial velocity was expressed as μmoles of cytochrome *c* oxidized per minute per mg protein using a millimolar extinction coefficient of 18·5 at 550 mμ for reduced to oxidized cytochrome *c*. Reduced cytochrome *c* was prepared as described by Wharton and Tzagaloff.[11]

Results

Removal of lipid from mitochondria results in a loss of cytochrome *c* oxidase activity as shown in Table I. Acetone removes most of the lipid as judged by the phosphorus to protein ratio and reduces the rate of cytochrome oxidase to about 10% of the control (approximately 1·4 μg P/mg protein is not extractable with lipid solvents).[3] The phospholipase A procedure[7] removes less lipid than acetone extraction, and results in a smaller decrease in enzymatic activity.

Reactivation of cytochrome *c* oxidase in lipid-depleted mitochondria as a function of increasing amounts of added mitochondrial phospholipid (MPL) is presented in Fig. 1. In the case of phospholipase A-treated mitochondria, the activity returned to nearly 100% of the control when about 1·4 μg of lipid phosphorus were added to the assay mixture containing 5·9 μg mitochondrial protein. The addition of larger amounts of

TABLE I. Removal of Lipid From Mitochondria

Treatment	Bound Phosphorus Total P	Lipid P* (μg P/mg protein)	% P remaining	Cytochrome *c* Oxidase μMoles/min/mg	% Activity
Control†	15·8	13	100	2·29	100
Phospholipase A	5·9	4·5	35	0·61	27
Acetone	3·6	2·2	17	0·27	12

* Each value in this column is corrected for the 1·4 μg P per mg protein which is not extractable with organic solvents (3). The value for heart mitochondria is taken from reference 3.

† The control was treated the same as phospholipase A treated mitochondria except that the phospholipase A was omitted.

lipid did not produce further increase in the velocity. The same amount of liqid also reactivated cytochrome *c* oxidase in acetone-treated mitochondria, however, maximum activation was only 40% of the control. The addition of MPL to untreated mitochondria did not affect the rate of cytochrome *c* oxidase.

To further elucidate the role of lipid in cytochrome *c* oxidase, initial velocity studies were carried out on lipid-depleted mitochondria in the presence and absence of added phospholipid. The apparent K_m for cytochrome *c*, the apparent maximum velocity (V_{max}) and their respective standard deviations were determined by a computer fit of the kinetic data to a rectangular hyperbola as described by Cleland.[12] The kinetic constants for lipid-depleted and control mitochondria are summarized in Table II. The corresponding double reciprocal plots of the data are shown in Fig. 2. Treatment with

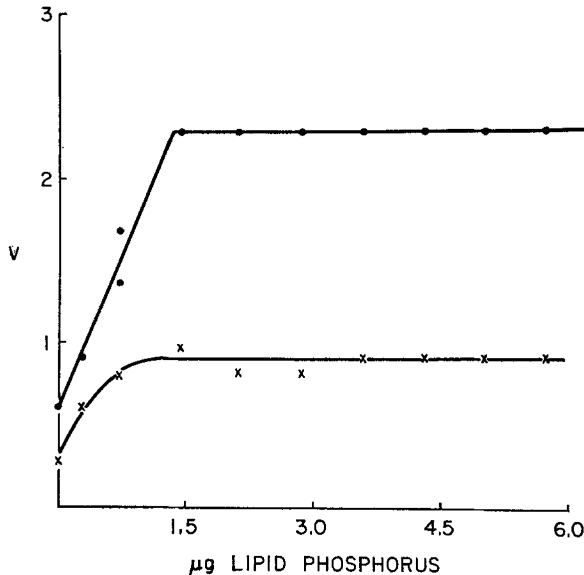

Figure 1. Reactivation of cytochrome *c* oxidase after treatment with acetone and phospholipase A. 5·9 μg of mitochondrial protein, and the indicated amount of MPL were preincubated for 10 min. Initial velocity (v) is expressed as μmoles of cytochrome *c* oxidized per minute per mg protein. Micrograms phospholipid can be obtained by multiplying μg P by 25. •——• Phospholipase A treated mitochondria. x——x Acetone extracted mitochondria.

TABLE II. Kinetic Constants for Cytochrome *c* Oxidase

Sample	μg P/mg*	Apparent K_m (μM)	Apparent V_{max} (μmoles/min/mg)
Control	15·8	73 ± 22†	6·5 ± 1·1
	16·8	114 ± 35‡	11·1 ± 2·1
Phospholipase A treated	5·9	35 ± 9†	1·3 ± 0·1
	4·0	41 ± 10‡	1·0 ± 0·1
Acetone treated	3·6	30 ± 3	0·41 ± 0·02
Phospholipase A treated + MPL§	—	79 ± 15†	5·6 ± 0·6
		162 ± 47‡	11·8 ± 2·3
Acetone treated + MPL§	—	104 ± 24	3·2 ± 0·4

* μg phosphorus per mg protein.
† determined with the same mitochondrial preparation.
‡ determined with the same mitochondrial preparation.
§ MPL (3 μg lipid phosphorus per assay) were preincubated with the lipid-depleted mitochondria.

phospholipase A or acetone results in a decrease in both the apparent V_{max} and the apparent K_m for cytochrome *c*. Acetone extraction results in a greater decrease in V_{max} than phospholipase A treatment. When MPL is added to the assay medium, the K_m returns to the control value and the V_{max} is increased. Determinations carried out with different preparations of mitochondria show some variation in the kinetic constants, particularly the apparent V_{max}. The apparent K_m and V_{max} of cytochrome *c* oxidase in control mitochondria were not appreciably changed by the addition of MPL to the assay mixture.

The rebinding of phospholipid microdispersions to lipid-deficient mitochondria was also carried out.[7] The mitochondria were reisolated by centrifugation and then assayed

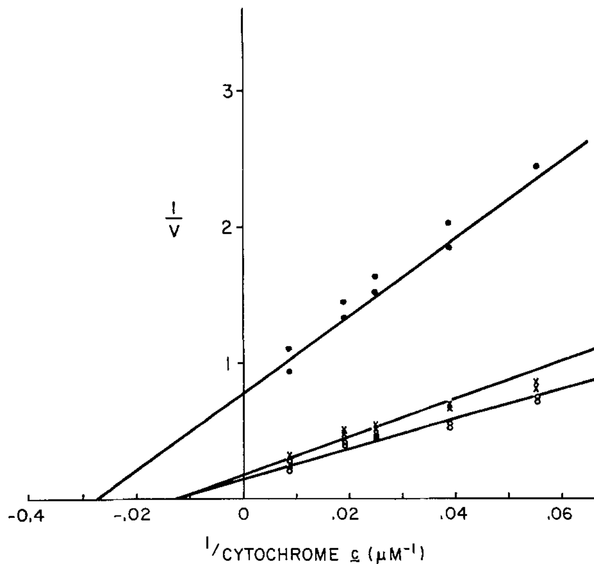

Figure 2A.

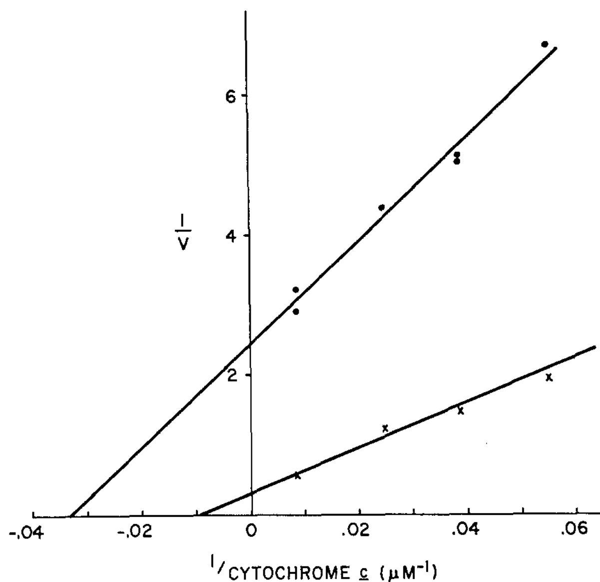

Figure 2B.

Figure 2. Double reciprocal plots of the velocity of cytochrome *c* oxidase activity as a function of reduced cytochrome *c*. 5.9 μg of mitochondrial protein was used. The points are initial velocity data and the lines are from the computer fit of the data. 1/v = min-mg/μmole cytochrome *c* oxidized. A. Effect of Phospholipase A: •——• Phospholipase A treated mitochondria; x——x Phospholipase A treated mitochondria assayed in the presence of MPL (3 μg lipid P per assay); o——o Control mitochondria. B. Effect of Acetone: •——• Acetone-extracted mitochondria; x——x Acetone-extracted mitochondria assayed in the presence of MPL (3 μg lipid P per assay).

for cytochrome *c* oxidase activity. The V_{max} for phospholipase A and acetone treated preparations could be restored to 5·6 and 2·3 (μmoles/min/mg) respectively. The K_m's of these preparations increased as well, although the results were somewhat variable.

Since it has been suggested that detergents can substitute for phospholipid in cytochrome *c* oxidase, several detergents were added to the standard assay mixture instead of phospholipid and the resulting initial velocity measured. As seen in Table III, Triton X-100, Tween-80 and oleic acid caused only a small increase in activity at low concen-

TABLE III. Effect of Detergents on Lipid-Depleted Cytochrome *c* Oxidase

Amount Added (μg)* →	0	5	10	20	50
Detergent		Initial Rates (μmoles/min/mg)			
Triton X-100	0·61	0·69	0·79	0·37	0·40
Tween-80	0·61	0·73	1·02	0·79	0·79
Oleic Acid	0·61	0·77	0·73	0·80	0·46
Sodium Dodecyl Sulfate	0·61	0·61	0·61	0·45	0·55
Deoxycholate	0·61	0·61	0·55	0·69	0·61
Cholate	0·61	0·46	0·61	0·61	0·68

* Addition to standard assay mixture.

The phospholipase A depleted preparation shown in Table I was used. For comparison, the reactivation of this preparation with phospholipid microdispersions is shown in Fig. 1. The initial rate in the presence of lipid is 2·3 μmoles/min/mg.

trations and Triton X-100 and oleic acid inhibited at high concentrations. Sodium lauryl sulfate, deoxycholate and cholate gave no activation or inhibited the reaction. Varying amounts of deoxycholate were also added to an assay containing 3 μg phospholipid phosphorus and phospholipase A treated mitochondria, (Table IV). In all cases, the deoxycholate inhibited reactivation as compared to a control without deoxycholate.

TABLE IV. Effect of Deoxycholate on Lipid Reactivation of Cytochrome *c* Oxidase Activity

Addition*	Amount Added (μg)	Initial Velocity (μmoles/min/mg)
None	—	2·29
Deoxycholate	10	1·59
Deoxycholate	50	1·08
Deoxycholate	200	1·23

* Standard assay condition, with the addition of MPL (3 μg lipid phosphorus per assay). The phospholipase A depleted preparation shown in Table I was used.

Discussion

The results presented here confirm the requirement of lipid for the cytochrome *c* oxidase activity in mitochondria.[2,3] Removal of phospholipid by either phospholipase A or acetone results in loss of activity. In both cases the enzyme can be reactivated by the addition of phospholipid to the assay medium. Acetone extraction is found to cause the greatest reduction in activity, however, cytochrome *c* oxidase so treated cannot be fully reactivated by the addition of lipid. It appears that acetone causes some irreversible alteration of the enzyme, as well as the removal of phospholipid. This is supported by the changes in the kinetic parameters since the apparent K_m returns to the control value whereas the apparent V_{max}, which is a function of enzyme concentration, does not. The milder conditions of phospholipase A treatment allows nearly 100% recovery of activity when phospholipid is added to the assay. The apparent K_m for cytochrome *c* reported here for beef heart is similar to that reported by Smith and Camerino.[13]

Morrison *et al.*[10] have questioned the requirement for phospholipid in the cytochrome *c* oxidase activity. These authors purify cytochrome *c* oxidase complex by solubilization with detergents and obtain a near lipid-free preparation which retains activity. Our studies, described here confirm the lipid requirement for mitochondrial cytochrome *c* oxidase. In the membrane-bound enzyme, detergent does not substitute for phospholipid but instead inhibits reactivation by lipid. The most likely explanation of this apparent discrepancy is that solubilization and isolation of cytochrome *c* oxidase results in alteration of the enzyme. Kirkpatrick and Jacobs[14] report that solubilization of cytochrome *c* oxidase in the reduced states causes conformational changes in the enzymes as compared to the membrane bound form. Since Morrison and coworkers used reducing conditions throughout their preparation,[15] the conformational changes described by Kirkpatrick and Jacobs[14] resulting from solubilization, may be related to the loss of specificity for phospholipids.

Smith and Camerino[13] and Mackler and Green[16] have reported that treatment of mitochondria with deoxycholate causes a large increase in cytochrome *c* oxidase activity. This has been interpreted as an "opening phenomenon", whereby the cytochrome *c* is more accessible to the enzyme. The assay conditions used in this study are hypotonic and adjusted to give maximally "opened rates".

The kinetic data for cytochrome *c* oxidase allow further insight into the role of lipid in this reaction. The results show that the apparent K_m, as well as the apparent V_{max}, decreases when lipid is removed and returns to the control value when lipid is added to the assay mixture. It is unlikely that these changes in the kinetic constants are due to their dependence on oxygen since high concentrations of oxygen were used (saturating at 32°). The catalytic activity of the enzyme is given by the ratio V_{max}/K_m which is equal to the apparent first order rate constant at low substrate concentrations. The apparent V_{max} decreases more than the apparent K_m, thus, catalysis rather than substrate binding is most affected by the removal of lipid. These results indicate that lipid is directly involved in the catalytic function of the enzyme.

Acknowledgments

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