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CYTOCHROME *C* OXIDASE: THE EFFECTS OF LIPIDES AND SURFACE ACTIVE AGENTS ON ENZYMIC ACTIVITY

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SUMMARY

Cytochrome *c* oxidase prepared by fractionation of DPNH oxidase loses up to 90% of its activity when suspended in 5% sucrose. This loss of activity can be restored by surface active agents, deoxycholate and unsaturated lysolecithin. When frozen cytochrome *c* oxidase is thawed it separates into three layers which have different original activities, but which can be reactivated to the same level by these compounds. Similarly particles can be separated by centrifugation which have different initial activities, but which can be stimulated by lysolecithin to the same final activity.

Extraction of cytochrome *c* oxidase with acetone or mixtures of acetone and ethanol results in removal of some phosphorus and marked diminution of activity. This inactivation can be reversed to some degree by a number of phospholipides in the presence of deoxycholate. Much better reactivation is obtained with yeast lysolecithin. In no case is the degree of reactivation greater than the percentage of original phospholipide remaining in the preparations of enzyme.

INTRODUCTION

In recent studies HATEFI[1] has shown that cytochrome *c* oxidase, prepared by treating mitochondria with *tert.*-amyl alcohol, is stimulated by a lipoprotein isolated from mitochondria. Small increases in activity were observed when the system was supplemented with crude preparations of individual phospholipides. GREENLEES AND WAINIO[2], using cytochrome oxidase isolated from a KEILIN AND HARTREE preparation, showed that a number of crude phospholipides were capable of activating the enzyme. Earlier WITTER[3] had demonstrated that treatment of mitochondria with lysolecithin increased the cytochrome *c* oxidase activity and at the same time "uncoupled" oxidative phosphorylation.

The present report describes the effects of a variety of lipides and surface active agents on the enzymic activity of cytochrome *c* oxidase. The effects of these agents on acetone extracted cytochrome *c* oxidase is also presented.

EXPERIMENTAL

Cytochrome *c* oxidase was prepared as described previously[4] from reduced diphosphopyridine nucleotide oxidase (DPNH oxidase). In 30 % sucrose the most active preparations catalyze the oxidation of approx. 15 to 20 μmoles of reduced cytochrome *c*/mg protein/min. Phosphorus determinations were performed by the method of FISKE AND SUBBAROW[5]. Protein was determined by the biuret technique as described previously[6]. Ferrocytochrome *c* was prepared from Sigma Chemical Company cytochrome *c* (Type III) by reduction with sodium dithionite. Excess dithionite was removed by aeration. Ferrocytochrome *c* oxidase activities were determined spectrophotometrically as described previously[7]. Yeast lecithin, yeast lysolecithin, and derivatives of yeast lecithin were prepared by HANAHAN AND JAYKO[8]. The phospholipides from DPNH oxidase were prepared by HANAHAN using an unpublished chromatographic technique. Tweens No. 20, 40, 60, and 81 were obtained from the Atlas Powder Company and tris(hydroxymethyl)aminoethane from Sigma Chemical Company.

Cytochrome *c* oxidase was lyophilized and then extracted with acetone as follows: 100 mg of the powdered enzyme were placed in a 50-ml centrifuge tube and 40 ml of cold acetone was added. The mixture was shaken repeatedly for 1 to 4 h and then centrifuged. This was repeated 4 to 6 times so that extraction time varied from 4 to 24 h. The entire procedure was performed at 4°. After the last extraction the suspension was filtered quickly in a Buchner funnel and the residue was taken up immediately in 0.02 *M* Tris buffer pH 7.5. In order to remove additional phospholipide, concentrations of 5, 10, 20, and 40 % ethanol were added to the acetone in other experiments.

In the reactivation studies the compound to be tested (dissolved in chloroform) was placed in a tapered centrifuge tube. The chloroform was evaporated and the residue suspended in 0.1 ml of 0.02 *M* phosphate buffer pH 7.5. To this mixture was added 0.1 ml of enzyme (5 mg protein/ml). Necessary dilutions were made with 0.02 *M* phosphate buffer pH 7.5 and reaction rates were carried out.

RESULTS

Table I shows the maximal stimulating effects of yeast lecithin, deoxycholate, yeast lecithin plus deoxycholate, and yeast lysolecithin on preparations of cytochrome

TABLE I

THE EFFECTS OF VARIOUS AGENTS ON THE ACTIVITY OF PREPARATIONS OF CYTOCHROME *c* OXIDASE

All activities expressed as μmole cytochrome *c* oxidized/mg protein/min.

Preparation	*Initial activity*	*Substance used in stimulating activity*			
		Yeast lecithin	*Deoxycholate*	*Yeast lecithin plus deoxycholate*	*Yeast lysolecithin*
1	1.2	2.3	7.0	7.0	
2	1.0	2.1	6.3	10.8	
3	0.6	1.0	3.9	3.6	
4	1.2		3.8		10.8
5	1.0		1.3		11.2
6	1.0		4.3		10.7

c oxidase. The enzyme preparations were stored in 5% sucrose and had low initial activities. Yeast lecithin increased the activity to nearly twice the initial level. Deoxycholate and deoxycholate plus lecithin stimulated the enzymic activity 5 to 10 times. Combinations of yeast lecithin and deoxycholate were not consistently superior to deoxycholate alone. The amounts of deoxycholate and/or yeast lecithin required for maximal stimulation varied from preparation to preparation. The most remarkable and consistent stimulation of the enzyme was obtained by adding yeast lysolecithin. Again the amount necessary varied with the preparation. When the turbid enzyme suspension was added to the lysolecithin or deoxycholate the suspension clarified. Maximum stimulation of enzymic activity was present when the suspension cleared. Because deoxycholate and lysolecithin had similar effects on solubility of suspensions of cytochrome *c* oxidase and on the catalytic activity of the enzyme, the influence of lysolecithin on DPNH oxidase was tested. Lysolecithin "opened" DPNH oxidase in

TABLE II

ACTIVITIES OF LAYERS OF CYTOCHROME *c* OXIDASE RESULTING FROM FREEZING AND THAWING OR CENTRIFUGING

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Layer	*Initial activity*	*Activity after addition of yeast lysolecithin*
	Freezing and thawing	
Top		
Hazy olive-green	4.0	14.2
Middle		
Hazy green-brown	2.6	14.5
Bottom		
Clear green-brown	6.7	14.0
	Centrifugation	
1. Supernatant	6.0	12.4
Residue	1.5	11.0
2. Supernatant	4.0	10.0
Residue	1.0	11.4

the presence of histidine buffer in the same manner as has previously been reported for deoxycholate[7].

When frozen preparations of cytochrome oxidase were thawed, 3 distinct layers were observed. Table II shows the activities of these layers before and after stimulation with lysolecithin. The enzyme preparations used in the study were stored frozen in 0.02 *M* Tris buffer and did not show the marked diminution of activity that occurs with preparations stored in 5 % sucrose. The top layer was olive green in color and cloudy in appearance; the middle layer a cloudy greenish brown and the bottom layer greenish brown and clear. Although the various layers had different initial activities, the final stimulated activities were the same. Also shown in Table II, when cytochrome *c* oxidase suspended in Tris buffer is centrifuged at 124,000 × *g* for 10 min, particles are separated which have a lower specific activity than the supernatant enzyme. On stimulation with lysolecithin both fractions have the same catalytic activity.

TABLE III

THE EFFECTS OF A VARIETY OF COMPOUNDS ON THE ACTIVITY OF ACETONE-TREATED CYTOCHROME *c* OXIDASE

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

	Acetone-treated
Activity prior to acetone treatment	12.0
Activating compound	
None	0.2
Yeast lysolecithin	4.0
Yeast lecithin	0.4
Deoxycholate	0.4
Deoxycholate plus yeast lecithin	0.7
Ubiquinone	0.2
α-tocopherol	0.3
Digitonin	0.2
Saponin	0.2
Phospholipid from DPNH oxidase	0.4

Table III shows the effects of a number of substances on the reactivation of acetone treated cytochrome *c* oxidase. Acetone extraction reduced the activity of cytochrome oxidase to approximately 2 % of the original value. Subsequent treatment with deoxycholate, yeast lecithin, a variety of lipides from DPNH oxidase and combinations of lecithin and deoxycholate produced a 2- to 3-fold increase in the activity of the acetone-treated preparations. Upon exposure of the cytochrome *c* oxidase to yeast lysolecithin the activity increased to one-third of the original value. Ubiquinone, α-tocopherol, digitonin, and saponin had no appreciable effect. A saturated lysolecithin prepared from egg lecithin and lysolecithinic acid were approximately one-half as effective as the unsaturated yeast lysolecithin. Choline and glycerylphosphorylcholine were ineffective.

Table IV shows the concentrations of total phosphorus in four acetone extracted preparations of cytochrome oxidase and the maximally stimulated activities of the preparations. Neither the duration of extraction nor the number of changes of acetone were directly related to the amount of phosphorus remaining in the preparations.

TABLE IV

THE ACTIVITY OF ACETONE-TREATED CYTOCHROME *c* OXIDASE AND TOTAL ENZYME PHOSPHORUS

All activities expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Preparation	*Stimulated* activity before extraction*	*Total P before extraction (mg/100 mg protein)*	*Activity after extraction*	*Total P after extraction (mg/100 mg protein)*	*Stimulated* activity after extraction*	*% of original P remaining after extraction*	*% of original stimulated activity remaining*
1	12.5	0.67	0.31	0.40	4.3	60	34
2	12.5	0.67	0.22	0.24	3.1	36	25
3	14.1	0.65	3.0	0.54	5.0	83	36
4	11.5	0.75	0.53	0.21	1.6	28	14

* Maximum activity obtained with lysolecithin added to enzyme.

TABLE V

A COMPARISON OF THE ACTIVITIES AND LIPIDE PHOSPHORUS OF TWO PREPARATIONS OF CYTOCHROME *c* OXIDASE EXTRACTED WITH ACETONE AND MIXTURES OF ACETONE AND ETHANOL

All activities represent the maximum activity obtained with lysolecithin stimulation and are expressed as μmoles cytochrome *c* oxidized/mg protein/min.

Enzyme	*Maximum stimulated activity*	*Lipide-extractable P (mg/100 mg protein)*	*% P remaining*	*% activity remaining*
Before extraction	7.3	0.28	100	100
Extracted with:				
acetone	5.1	0.20	71	69
acetone + 5% ethanol	1.2	0.04	14	16
acetone + 10% ethanol	0.7	0.02	7	10
acetone + 20% ethanol	0.05	0.012	3.6	0.7
acetone + 40% ethanol	0.03	0.004	1.4	0.4
Before extraction	11.2	0.28	100	100
Extracted with:				
acetone	4.0	0.16	56	37
acetone + 5% ethanol	1.2	0.12	42	11
acetone + 10% ethanol	0.4	0.01	5	3.7
acetone + 20% ethanol	0.04	0.006	2	0.4
acetone + 40% ethanol	0.005	0.001	0.3	0.05

The percentage decrease in activity after acetone extraction was greater in all instances than the percentage of total phosphorus removed. In no case was the percentage of original activity restored equal to the percentage of original total phosphorus remaining.

Table V shows a comparison of the lipide phosphorus to maximal recovered activity. Here the percentages of lipide phosphorus and percentages of enzymic activity remaining after extraction are parallel and suggest the possibility of a relationship between enzymic activity and the presence of phospholipides in the enzyme preparations.

DISCUSSION

It has been shown that two surface active agents, deoxycholate and unsaturated (yeast) lysolecithin, will stimulate cytochrome *c* oxidase. Associated with the stimulation is a physical change, namely, solubilization, marked by clearing of the enzyme solution. This would suggest that possibly the stimulation is related to the size or configuration of the enzyme particles. Support for such an explanation is given by the results of experiments which show first, that different layers of frozen and thawed enzyme have different original activities but can be stimulated to the same level by lysolecithin, and second, that enzyme fractions which differ in centrifugal properties and activities are reactivated to the same level.

Cytochrome *c* oxidase treated with acetone and alcohol loses most of its activity and some of its phospholipide. Reactivation with the most effective agent, yeast lysolecithin, does not bring the activity of the enzyme up to the level of the phospholipide remaining in the preparations. It would appear that such stimulation does not reactivate the enzyme fraction from which phospholipide has been removed but makes the remaining intact enzyme more available for catalysis.

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