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Reconstitution of Cytochrome Oxidase Vesicles and Conferral of Sensitivity to Energy Transfer Inhibitors

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Summary. 1. Phospholipids and cytochrome oxidase solubilized with cholate were reconstituted either by dialysis or by dilution of the detergent. The reconstituted cytochrome oxidase vesicles oxidized ascorbate-cytochrome *c* at a rate which was low, insensitive to energy transfer inhibitors and markedly stimulated by uncouplers of oxidative phosphorylation. The rate of reconstitution was dependent on pH, on the concentration of cholate and on the presence of high concentrations of monovalent ions or low concentrations of divalent ions. The integrity of the cytochrome oxidase vesicles was retained after freeze-drying, provided sucrose was present during the process. 2. Reconstitution with pure phospholipids revealed that cardiolipin was required for the marked stimulation of respiration by uncouplers. 3. Cytochrome oxidase vesicles were reconstituted in the presence of hydrophobic mitochondrial proteins which contained oligomycin-sensitive ATPase. The resulting vesicles oxidized ascorbate-cytochrome *c* at a rapid rate which was not enhanced by uncouplers. Addition of an energy transfer inhibitor such as rutamycin resulted in a partial inhibition of respiration which was released by uncouplers. 4. Cytochrome oxidase vesicles reconstituted in the presence of phenol red were rather impermeable to protons and became very permeable on addition of uncouplers. When the reconstitution was performed in the presence of the hydrophobic proteins from mitochondria, proton translocation became partially sensitive to rutamycin. 5.These observations are consistent with some of the formulations of the chemiosmotic hypothesis.

We have shown recently [2] that vesicles reconstituted with cytochrome oxidase and phospholipids oxidized ascorbate in the presence of cytochrome *c* at a rate which was stimulated three- to fivefold by uncouplers of oxidative phosphorylation or by ionophorous agents. During respiration in the presence of valinomycin, the medium became acidic and K^+ ions were translocated into the vesicles. These ion movements, which were abolished by uncouplers, resembled those observed in intact mitochondria.

According to Mitchell [9] protons are translocated across the membrane via the oxidation chain in one direction and via $CF_0 - F_1$ [4], the oligomycin-

sensitive ATPase, in the opposite direction. The latter process is coupled to the formation of ATP and is inhibited by energy transfer inhibitors [10].

It is the purpose of this paper to describe experiments on the reconstitution of cytochrome oxidase vesicles in the absence or presence of hydrophobic proteins from mitochondria which contain the oligomycin-sensitive ATPase. It will be shown that the presence of this fraction results in a loss of respiratory control and renders proton translocation sensitive to energy transfer inhibitors.

Experimental Procedure

Materials and Methods

The materials and the procedure for the reconstitution of cytochrome oxidase vesicles were essentially as described previously [2] but with some variations in the buffers, pH and in the phospholipids as described in the legends to tables and figures. The preparation of the hydrophobic proteins was carried out as described previously [5]. Following exactly the same procedure, a similar preparation of hydrophobic proteins was obtained from spinach chloroplasts by Dr. C. Carmeli *(unpublished experiments)*. Cardiolipin was purchased from General Biochemicals, Inc. The preparations of purified phospholipids and method of sonication in the presence of cholate were as described elsewhere [3].

Assay of Cytochrome Oxidase, "Respiratory Control" and Sensitivity to Rutamycin

Oxygen uptake was measured with a Clark oxygen electrode at room temperature. In a final volume of 1 ml, 50 μmoles of KP_i buffer (pH 7.5), 600 μg of cytochrome *c* (Sigma type VI) and 50 μmoles of Tris ascorbate (pH 6.8) were mixed by a magnetic mixer and readings of oxygen consumption were taken for 2 min prior to the addition of the sample. Cytochrome oxidase vesicles were added next and readings were taken. For measurements of "respiratory control", 2 μliters of a 0.02 M solution of 1799 [1] in ethanol were added. When hydrophobic proteins were present during reconstitution, 2 μliters of a 0.2% ethanolic solution of rutamycin were added prior to the uncoupler. Corrections were made for the rate of autoxidation in the absence of cytochrome oxidase vesicles which amounted to about 20 nmoles of oxygen per min.

Results and Discussion

Time Course of Reconstitution of Cytochrome Oxidase Vesicles with Respiratory Control

In the preliminary communication [2], we have described a procedure for the preparation of cytochrome oxidase vesicles which was based on the method of reconstituting $^{32}P_i$-ATP exchange vesicles [5] and oxidative phosphorylation at the third site [12]. We have reported that optimal rates

1 A 2:1 adduct of hexafluoroacetone and acetone.

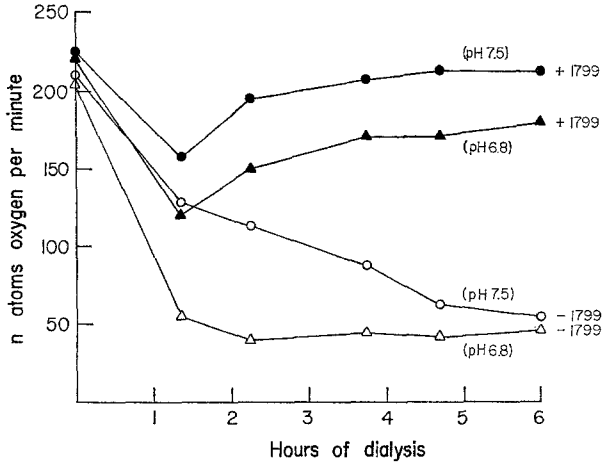

Fig. 1. Time course of reconstitution of cytochrome oxidase vesicles at pH 7.5 and 6.8. In a final volume of 0.5 ml, 0.3 ml of a sonicated suspension of soybean phospholipids (38 μmoles per ml) in 2% cholate-50 mM KP_i buffer at the indicated pH were mixed with 600 μg of cytochrome oxidase and dialyzed at 4 °C in an open ($\frac{1}{4}$ inch diameter) cellulose bag against 1 liter of 50 mM KP_i buffer at the indicated pH. Samples (10 μliters) were taken out at intervals and assayed for cytochrome oxidase as described under Experimental Procedure

of phosphorylation were obtained after removal of cholate by dialysis for at least 16 to 18 hr at pH 8.0. An analysis of the effect of pH and time of dialysis during reconstitution of cytochrome oxidase vesicles revealed an entirely different pattern. As shown in Fig. 1, the development of respiratory control was favored at more acid pH. At pH 6.8, a marked decrease of ascorbate oxidation with external cytochrome *c* was observed in the first sample taken after 80 min of dialysis. Addition of uncoupler stimulated the rate of oxidation. At pH 7.5, the development of respiratory control was considerably slower. Lowering the pH below 6.5 prevented the appearance of respiratory control. Thus, a fairly sharp pH optimum can be observed for reconstitution. As shown in Fig. 2, at pH 7.1 pronounced respiratory control was already observed after 30 min of dialysis. There are two features in these experiments which require discussion. One is the apparent loss of cytochrome oxidase activity even in the presence of uncoupler after about 1 hr of dialysis (Fig. 1). Such loss of activity was actually expected if the reconstitution of cytochrome oxidase is transmembranous with some cytochrome *a* orientated toward the inside and some toward the outside. Cytochrome oxidase with cytochrome *a* on the inside of the vesicles should not be accessible to reduced cytochrome *c* added externally. That this indeed has happened was indicated by a further stimulation of oxygen uptake over that in the presence of uncoupler on addition of cholate. A quantitative

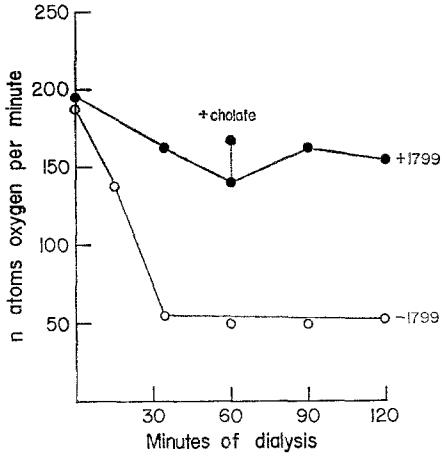

Fig. 2. Time course of reconstitution of cytochrome oxidase vesicles at pH 7.1. Experimental conditions were as described in the legend of Fig. 1

evaluation of these somewhat variable observations was difficult because strong detergents such as cholate inhibit cytochrome oxidase activity and milder detergents such as Tween-80 were not very effective. With time, the respiration of the reconstituted cytochrome oxidase vesicles in the presence of uncoupler approached the initial values, indicating that the orientation of cytochrome oxidase was mainly unidirectional, with cytochrome *a* on the outside. It may be no accident that this actually is the natural orientation of cytochrome oxidase in mitochondria. That the major orientation of cytochrome oxidase is mainly unidirectional is also in line with earlier observations [12] that the rate of oxidation with internal cytochrome *c* is quite low compared with the rates reported in this paper.

Reconstitution of Cytochrome Oxidase Vesicles without Dialysis

The rapid rate of oxidation with external cytochrome *c* is at least partly responsible for the fact that respiratory control can be observed after a few hours whereas $^{32}P_i$-ATP exchange requires at least 12 to 16 hr of dialysis [5]. Analysis with [^{14}COOH]-labeled cholate [3] revealed that under our experimental conditions only about one-half of the cholate was removed by 2 hr of dialysis. Since the starting concentration of cholate in the reconstitution of cytochrome oxidase vesicles was 1.2% and since the samples were diluted 100-fold for the assay of cytochrome oxidase activity, the final concentration of cholate was well below that required to eliminate respiratory control [11]. These considerations suggested that dialysis might not be required if the

appropriate concentrations of cholate were selected for reconstitution. It can be seen from Fig. 3 that this is indeed the case. After 1 hr, pronounced stimulation of cytochrome oxidase by uncoupler was seen after incubation with 1% cholate and after 2 hr with 0.75% cholate. Although the final control ratios in these experiments were not as high as those obtained after prolonged dialysis and removal of cholate, they were reproducible and suitable for short term experiments.

Effect of Salt and Cholate Concentration on Reconstitution

Reconstitution at low salt concentrations yielded cytochrome oxidase vesicles with low respiratory control. As shown in Table 1, Experiment 1,

Table 1. Effect of monovalent and divalent cations on reconstitution of cytochrome oxidase vesicles

Additions during reconstitution	Oxygen uptake				
	After 2 min		After 3 hr		
	−1799 (natoms/min)	+1799 (natoms/min)	−1799 (natoms/min)	+1799 (natoms/min)	RC[d]
Exp. 1[a]					
None	270	276	278	290	1.16
100 mM KP_i (pH 7.5)	225	280	108	284	2.63
6 mM $MgSO_4$	213	264	105	240	2.29
2 mM $MgSO_4$	278	292	90	264	2.93
			After 2 hr		
			−1799 (natoms/min)	+1799 (natoms/min)	RC
Exp. 2[b]					
None[c]			135	176	1.30
0.8 mM $MgSO_4$			112	171	1.52
1.6 mM $MgSO_4$			81	167	2.06
3.2 mM $MgSO_4$			36	123	3.40

[a] Experiment 1: In a final volume of 0.5 ml, 0.2 ml of a sonicated suspension of phospholipid (38 μmoles per ml) in 2% sodium cholate-5 mM Tricine (pH 7.5) were mixed with 600 μg of cytochrome oxidase and the indicated amounts of KP_i buffer or $MgSO_4$.

[b] Experiment 2: In a final volume of 0.25 ml, 0.1 ml of a sonicated suspension of phospholipids (38 μmoles per ml) in 2% cholate-50 mM KP_i (pH 7.5) were mixed with 150 μg of cytochrome oxidase and the indicated amounts of $MgSO_4$.

Assays of cytochrome oxidase activity with ascorbate-cytochrome *c* were performed as described under Experimental Procedure with 10-μliter samples after the indicated period of incubation at 4 °C.

[c] 20 mM KP_i was introduced with the phospholipid suspension.

[d] RC = respiratory control.

Table 2. Effect of cholate concentration on reconstitution of cytochrome oxidase vesicles

Cholate concentration	Oxygen uptake		
	−1799 (natoms/min)	+1799	RC
0.2%	118	135	1.14
0.4%	105	147	1.39
0.8%	63	147	2.34
1.2%	78	156	2.0

In a final volume of 0.5 ml, 0.2 ml of a sonicated suspension of phospholipids (38 μmoles per ml) in 50 mM KP_i (pH 7.5) were incubated with 300 μg of cytochrome oxidase in the presence of 2 mM $MgSO_4$ and the indicated amounts of sodium cholate for 2 hr at 4 °C and then tested as described under Experimental Procedure.

only reconstitution in the presence of high concentrations of monovalent cations or of low concentrations of $MgSO_4$ yielded vesicles which responded well to uncouplers. In the presence of 20 mM KP_i (pH 7.5), which by itself gave rise to a low "respiratory control" ratio, increasing the Mg^+ concentration yielded higher control ratios (Table 1, Experiment 2). At Mg^+ concentrations above 3 mM, very high ratios were obtained but the final cytochrome oxidase activity was consistently lower than at low Mg^+ concentrations. Attempts to regain the missing activity by addition of detergents usually resulted in either no or only slight stimulation of the oxidation rate.

The effect of cholate concentration during reconstitution is shown in Table 2. This experiment was carried out in the presence of 2 mM $MgSO_4$ and readings were taken after 2 hr of incubation at 4 °C. It can be seen that under these experimental conditions an optimal respiratory control ratio was obtained in the presence of 0.8% cholate and little control at 0.2% cholate. Interestingly enough, without any cholate the control ratio was consistently somewhat higher than with low cholate, possibly due to some lysolecithin present in the crude phospholipid preparation.

Reconstitution of Cytochrome Oxidase Vesicles with Pure Phospholipids

Substituting crude soybean phospholipids with single purified phospholipids during reconstitution yielded cytochrome oxidase vesicles with respiratory control values below 2 (Table 3). However, cardiolipin with either phosphatidyl choline (PC) or a mixture of phosphatidyl choline and phosphatidyl ethanolamine (PE) gave respiratory control ratios of 3.8. The effect of cardiolipin on respiratory control was the first clue that acidic

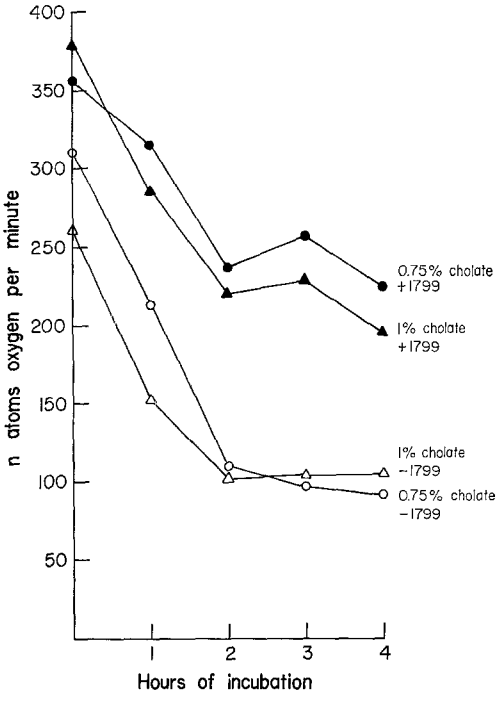

Fig. 3

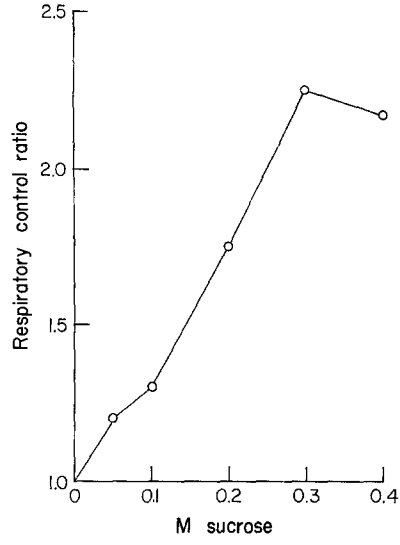

Fig. 4

Fig. 3. Reconstitution of cytochrome oxidase vesicles without dialysis. Experimental conditions were as described in the legend of Fig. 1 except that 0.2 ml of sonicated phospholipids (38 μmoles per ml) in KP_i (pH 7.1) were used and cholate was added prior to reconstitution at the indicated concentration. The mixtures were incubated at 4 °C and samples (10 μliters) assayed at intervals

Fig. 4. Effect of freeze-drying with and without sucrose on cytochrome oxidase vesicles. Cytochrome oxidase vesicles were prepared as described under Experimental Procedure, suspended in the indicated concentrations of sucrose and freeze-dried. Water was added to the dried samples to bring them to the original volume and 10-μliter aliquots were assayed for cytochrome oxidase activity and respiratory control

phospholipid may fulfill a specific function. Marked stimulation of $^{32}P_i$-ATP exchange [3] and of the P:O ratio[2] by cardiolipin have also been observed under appropriate conditions of reconstitution of phosphorylating vesicles.

Effect of Freeze-Drying on Respiratory Control of Cytochrome Oxidase Vesicles

For the purpose of analysis by various physical methods (e.g., X-ray diffraction and freeze etching) it was desirable to explore the effect of freeze-

2 E. Racker and A. Kandrach, *unpublished observations.*

Table 3. Reconstitution of cytochrome oxidase vesicles with purified phospholipids

Phospholipid in reconstitution	Oxygen uptake		Respiratory control
	−1799 (natoms/min)	+1799 (natoms/min)	
Phosphatidyl choline (10 μmoles)	102	135	1.32
Phosphatidyl ethanolamine (10 μmoles)	117	147	1.25
PC (5 μmoles) + PE (5 μmoles)	132	162	1.23
Cardiolipin (1.5 μmoles)	90	150	1.66
PE (5 μmoles) + cardiolipin (1.5 μmoles)	78	162	2.08
PC (5 μmoles) + cardiolipin (1.5 μmoles)	54	207	3.8
PC (5 μmoles) + PE (5 μmoles) + cardiolipin (1.5 μmoles)	69	261	3.8

Cytochrome oxidase vesicles were reconstituted with purified phospholipids (with amounts indicated in the table) as follows: The phospholipids were sonicated [3] in the presence of 2% cholate in 50 mM KP_i buffer (pH 7.5) until they were clarified. In a final volume of 0.3 ml at final concentrations of 1% cholate and 50 mM KP_i (pH 7.5), 600 μg of cytochrome oxidase were dialyzed against 1,000 volumes of 50 mM KP_i buffer (pH 7.5) for 4 hr and again for 16 hr. Assay of respiration was performed with 10-μliter samples as described under Experimental Procedure. The uncoupler (2 μliters of 10^{-2} M 1799) was added after 2 min of linear respiration.

drying on cytochrome oxidase vesicles. Lyophilization of cytochrome oxidase vesicles in 50 mM KP_i buffer completely destroyed respiratory control. As can be seen from Fig. 4, inclusion of sucrose during lyophilization protected against the loss of respiratory control. Moreover, the dried preparation retained respiratory control for weeks even at room temperature and could be conveniently shipped to other laboratories. The loss of respiratory control by freeze-drying is of interest as an example for the well-known freezing damage to membranous structures. It is apparent from the experiments on the reconstitution of cytochrome oxidase vesicles that it could be the phospholipid and not the protein which is damaged by freezing since cytochrome oxidase activity was preserved even in the absence of sucrose. That the damage to the phospholipids was not irreversible (e.g., caused by lipoxidation) was shown by the following experiment. When phospholipids were lyophilized in the absence of sucrose, but in the presence of salt, they yielded milky suspensions even when suspended in 2% cholate. However, they could be clarified by sonic oscillation and then used for reconstitution of cytochrome oxidase vesicles giving rise to respiratory control identical to that obtained with phospholipids lyophilized in the presence of sucrose. Thus, it is clear that sucrose is only required during the process of freeze-drying (or freezing) and that the alteration of structure is reversed by sonic

oscillation. These observations are in line with previous findings [7] which suggested that freezing alters the phospholipids. Thus, freezing damage to biological membranes may at least partly be caused by alteration of the structural organization of phospholipids.

Effect of CF_0-F_1 on Respiratory Control of Cytochrome Oxidase Vesicles

It was proposed by Mitchell [9] that the process of proton translocation during respiration and the resulting formation of a membrane potential is responsible for the phenomenon of respiratory control. He also proposed that CF_0-F_1, the oligomycin-sensitive ATPase, catalyzes the translocation of protons in the direction opposite to that of the respiratory chain driving the formation of ATP. In the absence of a functional ATP-generating machinery, this process should result in a loss of respiratory control. Indeed, submitochondrial particles do not have respiratory control unless either coupling factors or an energy transfer inhibitor such as rutamycin is added [1]. When reconstitution of cytochrome oxidase vesicles was carried out in the presence of increasing amounts of a hydrophobic protein fraction which contained CF_0-F_1, respiration was increased proportionately and became correspondingly less stimulated by uncoupler. As shown in Table 4, at concentrations of 500 μg of the fraction per mg of phospholipid, the rate of ascorbate oxidation was about the same in the absence and presence of 1799. The specificity of this stimulation was examined by reconstituting cytochrome oxidase vesicles in the presence of a crude soluble extract obtained by exposing mitochondria to sonic oscillation. Such a crude mixture of mitochondrial proteins had little or no effect on respiratory control of cytochrome oxidase vesicles. However, when these proteins were heat-denatured and thus rendered hydrophobic prior to reconstitution, respiratory control was abolished. Thus, any hydrophobic proteins may interfere with the proper alignment of the phospholipids in the vesicles. It became, therefore, essential to develop an assay specific for the mitochondrial hydrophobic protein responsible for proton translocation. Energy transfer inhibitors are known to confer respiratory control to submitochondrial particles [6] and Mitchell suggested [10] that they act by inhibiting proton translocation via the oligomycin-sensitive ATPase. It can be seen from Table 4 that rutamycin inhibited partly the increased respiration of cytochrome oxidase vesicles reconstituted in the presence of the hydrophobic protein fraction. No effect of rutamycin was observed when heat-denatured proteins were used in reconstitution (Table 4). In view of the fact that the hydro-

Table 4. Effect of hydrophobic protein fraction from mitochondria on respiration of reconstituted cytochrome oxidase vesicles [a]

Additions	Oxygen uptake					
	0	60	100	200	300	500 µg HP [b]
	(natoms/min)					
Exp. 1						
None	32	42	54	83	100	116
1799	123	115	120	123	127	123
	0		500 µg HP		500 µg heated HP	
	(natoms oxygen/min)					
Exp. 2						
None	40		182		93	
Rutamycin	38		126		93	
1799	182		176		142	

[a] An aqueous suspension of 4% soybean phospholipid was sonicated as described [2] in the presence of 2% cholate in the presence of 5 mM Tricine (pH 8.0) and 150 mM KCl. To 0.3 ml of the clarified preparation, 600 µg of cytochrome oxidase and the indicated amounts of hydrophobic proteins [5] expressed in µg per mg phospholipids were added. The final volume was adjusted to 0.5 ml with distilled water and the mixture dialyzed as described [2] except that in this experiment dialysis was performed against two changes of 500 volumes of 5 mM Tricine (pH 8.0) containing 50 mM NaCl. Measurements were performed as described under Experimental Procedure.

[b] µg hydrophobic protein from mitochondria [5] added per mg of phospholipid.

Table 5. Effect of spinach hydrophobic protein on respiration of cytochrome oxidase vesicles [a]

Additions	Oxygen uptake				
	0	0	125 µg	250 µg	250 µg CHP [b]
	(natoms/min)				
None	37	35	60	75	79
DCCD	52	–	52	55	–
Rutamycin	–	33	–	–	79
1799	109	115	94	97	119

[a] Experimental conditions were as described in the legend of Table 4 except that 100 mM KP_i buffer (pH 7.1) was used instead of Tricine-KCl. One µliter of a 0.1 M solution of DCCD in ethanol was added where indicated.

[b] CHP = hydrophobic proteins from spinach chloroplasts prepared by the procedure described for the mitochondrial hydrophobic proteins [5].

phobic protein fraction was still rather crude and perhaps partly denatured, it is not surprising that the inhibition by rutamycin was usually not greater than 30 to 40%.

As shown in Table 5, a preparation of hydrophobic proteins prepared from spinach chloroplasts by the procedure used for isolation of this fraction from mitochondria [5] also stimulated the rate of ferrocytochrome *c* oxidation. Since it was shown previously [8] that DCCD [3] but not rutamycin is an energy transfer inhibitor of chloroplasts, it was not surprising to find that rutamycin had no effect on the cytochrome vesicles reconstituted with the chloroplast proteins. However, DCCD inhibited respiration partially and the inhibition was released by uncoupler.

Effect of Mitochondrial Hydrophobic Protein on Proton Translocation in Reconstituted Cytochrome Oxidase Vesicles

When cytochrome oxidase vesicles were reconstituted in the presence of phenol red, respiratory control was not affected. Addition of HCl did not alter the appearance of these red vesicles, which turned instantaneously yellow on addition of an uncoupler. While this experiment serves admirably in a classroom demonstration, a more quantitative approach was taken with the aid of a dual wavelength Aminco spectrophotometer. On addition of acid, an initial drop in absorbance at 560 to 590 nm was detected, caused by the small amount of residual phenol red on the outside of the vesicles. On addition of valinomycin, there was a slow decrease in absorbance, which was not altered on addition of rutamycin, indicative of the low permeability of these vesicles to protons (Table 6). On addition of an uncoupler, the red

Table 6. Proton translocation of cytochrome oxidase vesicles reconstituted in the presence of mitochondrial hydrophobic proteins

Additions	Reconstituted vesicles	
	−Hydrophobic protein	+Hydrophobic protein
HCl	<0.1	0.2
Valinomycin	0.8	2.6
Rutamycin	0.8	2.0
1799	>30	>30

Cytochrome oxidase vesicles were reconstituted with or without 100 µg hydrophobic proteins per mg of phospholipid as described in the legend of Table 4. Dialysis against several changes of the Tricine-KCl buffer was continued until insignificant amounts of phenol red appeared in the outside fluid. A sample corresponding to 120 µg of cytochrome oxidase was placed in a dual wavelength spectrophotometer and recorded at 560 − 590 nm in a final volume of 3 ml containing 5 mM Tricine (pH 8.0) − 50 mM KCl. After 6 µliters of 0.5 N HCl, 2 µliters of a 0.01% solution of valinomycin in ethanol, 2 µliters of a 0.4% solution of rutamycin in ethanol and 1 µliter of a 0.01 M solution of 1799 in ethanol were sequentially added to the reaction mixture. The changes in absorbance are expressed in arbitrary units as cm per min.

3 DCCD = N,N′-dicyclohexylcarbodiimide.

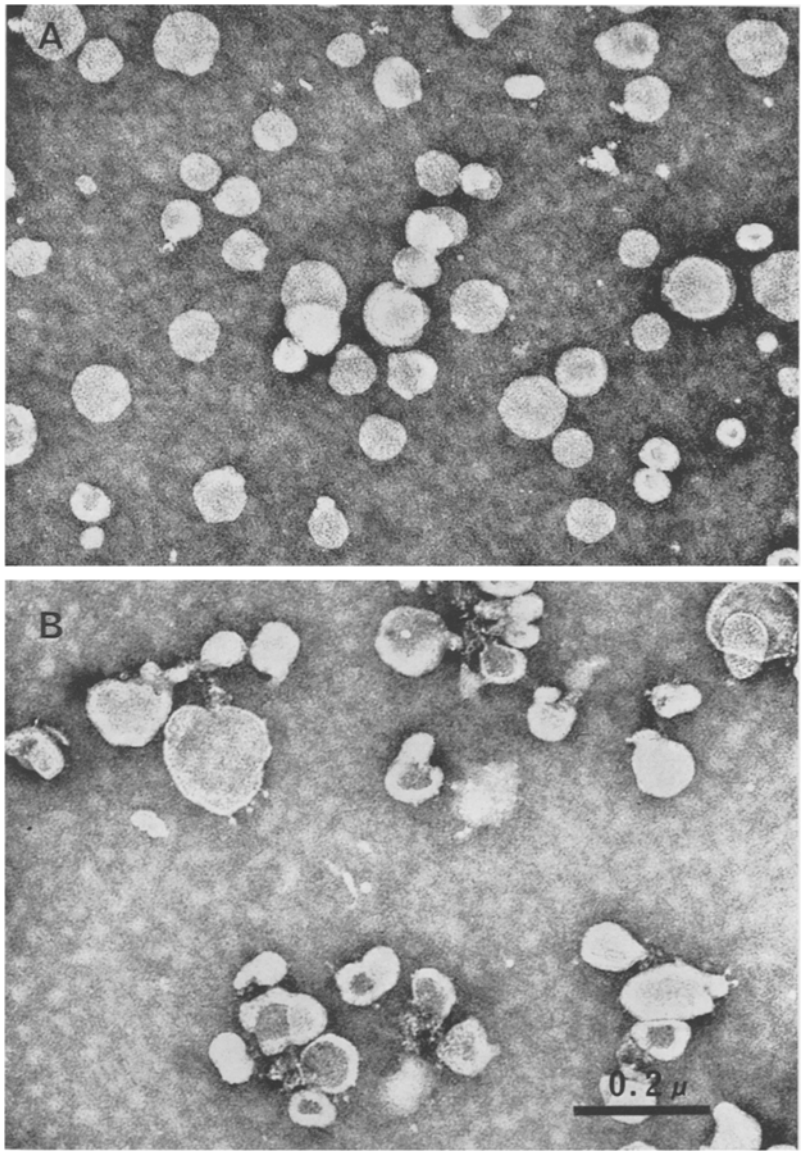

Fig. 5. Electron-micrographs of reconstituted cytochrome oxidase vesicles. Negative staining with phosphotungstate. Final magnification 120,000 × . (*A*) Cytochrome oxidase vesicles reconstituted with crude soybean phospholipids. (*B*) Same as (*A*) but reconstituted in the presence of hydrophobic proteins

color disappeared almost instantaneously. If reconstitution of the cytochrome oxidase vesicles was performed in the presence of the hydrophobic proteins, the rate of absorbance change was distinctly inhibited on addition

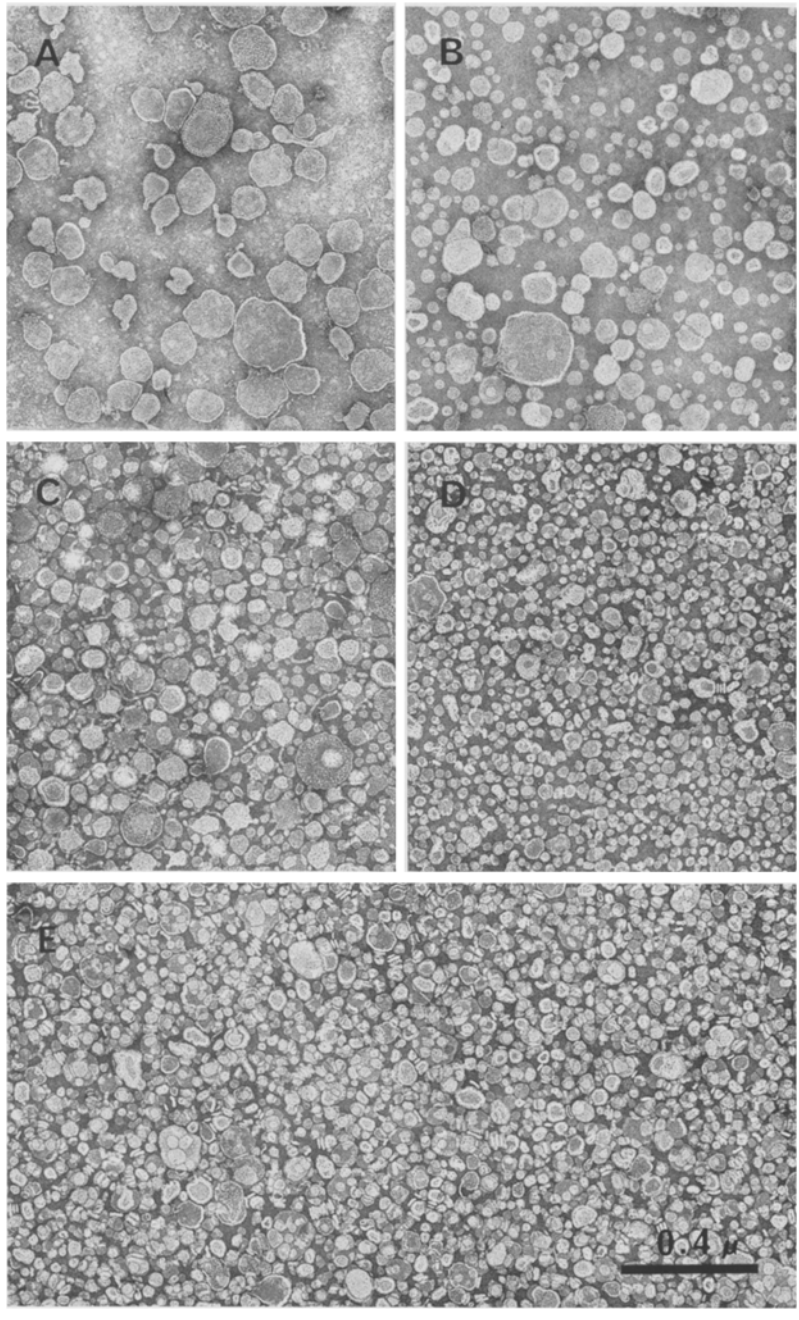

Fig. 6. Electron-micrographs of reconstituted cytochrome oxidase vesicles. Negative staining with phosphotungstate. Final magnification 60,000 ×. Reconstitution with: (*A*) phosphatidyl choline; (*B*) phosphatidyl ethanolamine; (*C*) phosphatidyl choline + phosphatidyl ethanolamine; (*D*) phosphatidyl choline + cardiolipin; (*E*) phosphatidyl choline + phosphatidyl ethanolamine + cardiolipin

of rutamycin. As in the measurements of oxygen uptake the inhibition of rutamycin was only partial.

Electron-Microscopy

Electron-micrographs of cytochrome oxidase vesicles reconstituted with crude soybean phospholipids are shown in Fig. 5*A*. The vesicles are variable in size (500 to 1,500 Å) compared with the somewhat smaller phospholipid vesicles (400 to 800 Å) reconstituted under the same conditions without any protein. The vesicles reconstituted in the presence of hydrophobic proteins were more heterogenous in appearance. Occasionally, inner membrane spheres (F_1) were seen on the periphery of vesicles which resembled submitochondrial particles (Fig. 5*B*).

The experiments on the reconstitution of cytochrome oxidase vesicles with purified phospholipids yielded marked differences depending on the properties of the phospholipids. As can be seen from Fig. 6, also, the appearance of these vesicles varied markedly with the different phospholipids used for reconstitution. The most striking differences were seen with cardiolipin which in combination with phosphatidyl choline yielded small and more homogenous vesicles. These were the vesicles which were most responsive to stimulation of respiration by uncouplers.

Conclusion

Cytochrome oxidase vesicles reconstituted from phospholipids and cytochrome oxidase appear to be valuable tools for the study of respiratory control. We are using at present the loss of respiratory control as an assay during the isolation of a proton transporter from the fraction of mitochondrial hydrophobic proteins. We are also using these vesicles for the study of pumps which catalyze the translocation of ions other than protons.

The experiments on respiratory control and proton translocation described in this paper were designed on the basis of Mitchell's chemiosmotic hypothesis. The fact that the data came out as predicted does not constitute proof, but once more establishes its value as a working hypothesis.

Generous gifts of 1799 by Dr. P. Heytler and of rutamycin by Dr. R. J. Horley are gratefully acknowledged. I wish to thank Dr. Y. Kagawa for preparations of the hydrophobic protein fraction from mitochondria, Dr. C. Carmeli for a preparation of the hydrophobic protein fraction from chloroplasts and Dr. J. Telford for the electron micrographs.

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