INCORPORATION OF BIOLOGICALLY ACTIVE PROTEINS INTO LIPOSOMES

G. EYTAN, M. J. MATHESON and E. RACKER
Section of Biochemistry, Molecular and Cell Biology, Wing Hall, Cornell University, Ithaca, N.Y., U.S.A.

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1. Introduction

Three procedures for reconstitution of biologically active phospholipid vesicles described previously [1–3] involve major disorganization of the liposomes. In the procedure described in the present paper, membrane proteins are allowed to penetrate into preformed liposomes which contain lysolecithin. Lysolecithin, which was used in this work to facilitate the incorporation of various mitochondrial proteins into liposomes, is present in mitochondria [4]. Phospholipase A_2 which catalyzes the conversion of phosphatidyl choline into lysolecithin, is present in mitochondrial membranes [5,6]. Thus the reconstitution system described here may serve as a model for the incorporation of proteins into preexisting membranes.

2. Materials and methods

Bovine heart phosphatidylcholine and phosphatidylethanolamine [7], cytochrome oxidase [8,9], complex III [10], hydrophobic protein [1] and F_1 [11] were prepared as described in the references. Lysolecithin and cardiolipin were purchased from General Biochemical Inc., Chagrin Falls, Ohio. Cytochrome oxidase [12], coenzyme Q cytochrome *c* reductase [13] and $^{32}P_i$-ATP exchange [1] were measured as described. Respiratory control ratio was defined as the ratio of the rate measured in the presence of valinomycin (0.5 μg/ml) and 1799 (20 μM) to that measured in the absence of uncouplers.

2.1. *Incorporation of proteins into phospholipid vesicles*

A solution of Phospholipids (including lysolecithin, where indicated) in chloroform–methanol (2 : 1) was dried down in a test tube under a stream of nitrogen, dissolved in a small volume of ether and dried again. Potassium Pi buffer (50 mM pH 7.0) was added to a final lipid concentration of 2% and the suspension was sonicated to clarity. The resulting liposomes were incubated at 0°C with the protein (0.05 mg/ml) and samples of 1 and 25 μl were assayed for complex III and cytochrome oxidase activities respectively.

3. Results and discussion

3.1. *Incorporation of cytochrome oxidase into liposomes*

Cytochrome oxidase was incubated with liposomes containing lysolecithin (10% of total phospholipids). A decrease in the rate of oxygen consumption with ascorbate–cytochrome *c* was observed which was reversed by uncouplers as shown in fig.1. Addition of 3% Tween 80 which disrupts liposomes without inhibiting cytochrome oxidase (R. Carroll and E. Racker, unpubl. obs.) did not further stimulate the respiration rate. Since neither cytochrome *c* nor ascorbate penetrate the vesicles, this observation implies that most of the cytochrome oxidase is oriented unidirectionally with its *a* side exposed to the external medium. This unidirectional orientation of cytochrome oxidase can be explained by assuming that the a_3 side of the enzyme is more hydrophobic and thus is the first to be incorporated. As the enzyme is added to the outside of the preformed liposomes this would result in all the enzyme molecules having their *a* side exposed to the medium. Indications that the a_3 side is indeed more hydrophobic were obtained using the surface probe diazobenzene sulfonate [9]. It was shown that

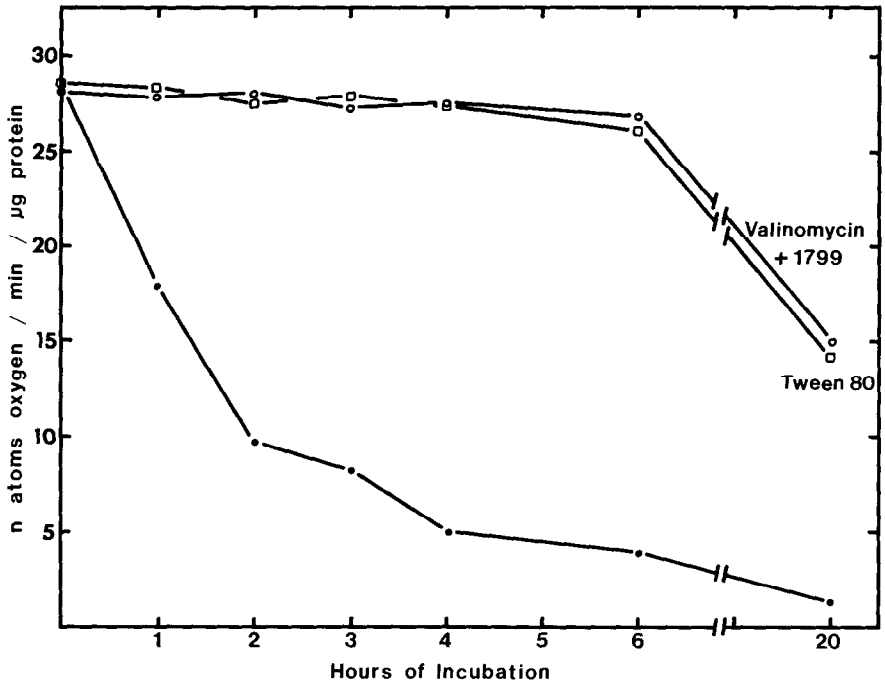

Fig. 1. Incorporation of cytochrome oxidase into liposomes. Cytochrome oxidase was incorporated into phospholipid vesicles containing phosphatidylethanolamine : phosphatidylcholine : cardiolipin : lysolecithin (1 : 1 : 0.15 : 0.25). Samples were withdrawn at the times indicated and assayed either in absence or presence of Tween 80 (3%) or valinomycin (0.5 µg/ml) plus 1799 (20 µM).

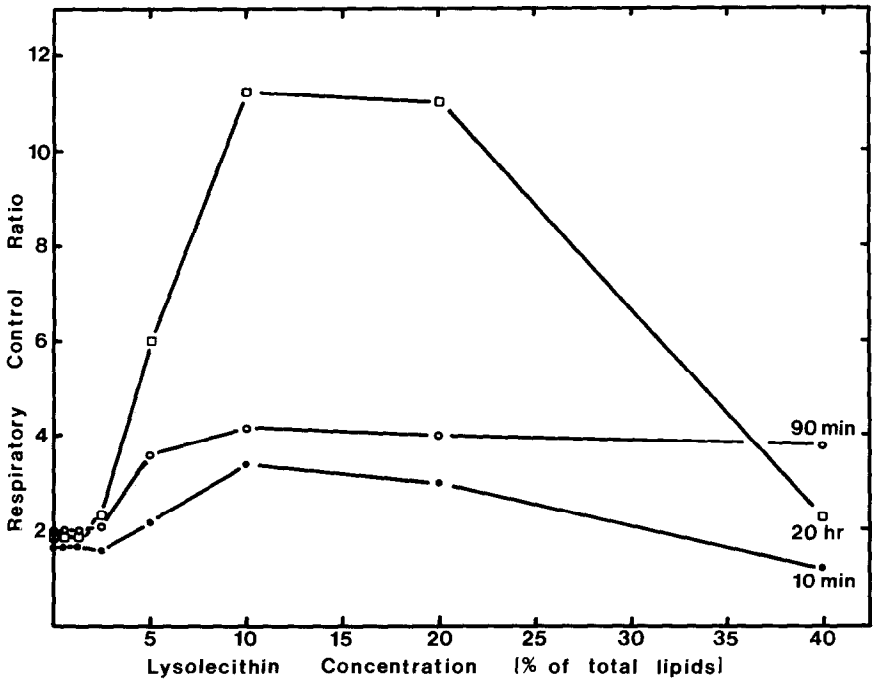

Fig. 2.

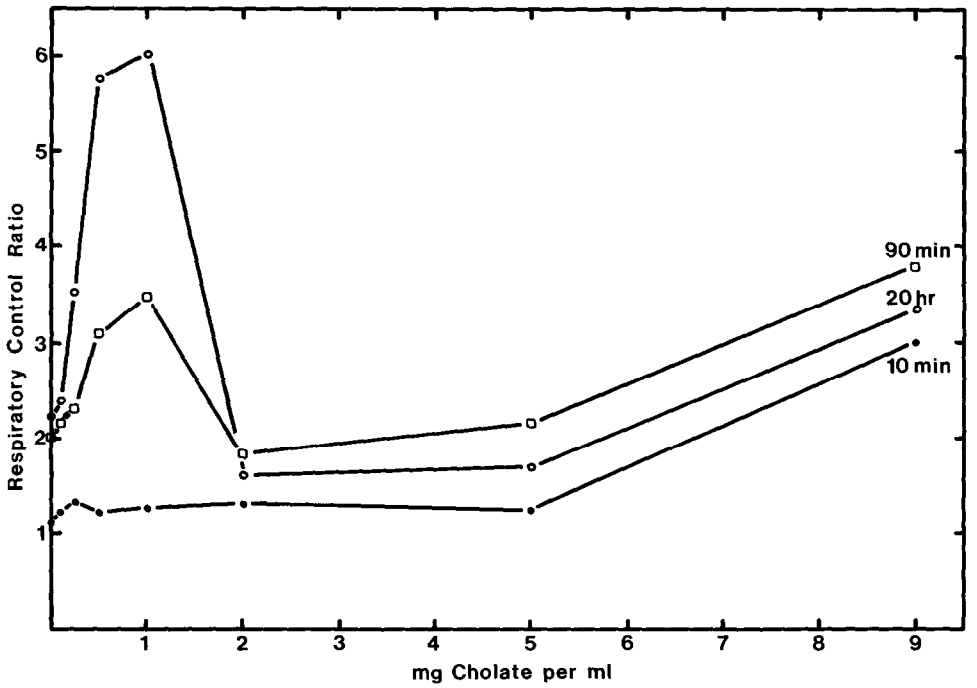

Fig.3. Effect of cholate on the oncorporation of cytochrome oxidase. Phosphatidylethanolamine : phosphatidylcholine : cardiolipin (1 : 1 : 0.15) suspension was sonicated to clarity in KP_i buffer 50 mM (pH 7.0). Samples containing 2% phospholipids were incubated at 0°C with cytochrome oxidase and varying amounts of potassium cholate.

while three of the six subunits of the enzyme are exposed to the aqueous phase at the *a* side, only one is exposed at the a_3 side. This explanation for the insertion of the protein into the liposomes can be applied also to vesicles formed by the cholate procedure, which have the same unidirectional orientation (Carrol R. and Racker, E., unpubl. obs.), however, it is then necessary to assume that during the dialysis the liposomes are formed prior to the incorporation of proteins.

Fig.2 shows that the optimal amount of lysolecithin required for incorporation of cytochrome oxidase is between 10 and 20% of the total lipids. The concentration of lysolecithin (10%), which we routinely use for the incorporation procedure did not dissolve or disrupt the liposomes as shown by experiments with radioactive rubidium. Liposomes containing lysolecithin were prepared in 50 mM KP_i. ^{86}Rb was added and cytochrome oxidase was incorporated according to the usual procedure. The vesicles were separated from excess ^{86}Rb on Sephadex G-50 column. No ^{86}Rb was found in the vesicles fraction. In another experiment the vesicles were first loaded with ^{86}Rb, separated on a column, and then incubated with or without cytochrome oxidase. There was only very little leakage of Rb^+ during cytochrome oxidase incorporation and no more than in the control.

Crude preparation of either soybean or mitochondrial phospholipids which contained substantial amounts of lysolecithin could be used for the incorporation of cytochrome oxidase. The respiratory control ratio of these vesicles was above 3. Further addition of

Fig.2. Effect of lysolecithin on the incorporation of cytochrome oxidase. Phospholipid suspensions containing various amounts of lysolecithin and phosphatidylethanolamine : phosphatidylcholine : cardiolipin (1 : 1 : 0.15) were sonicated to clarity and then incubated with cytochrome oxidase at 0°C.

lysolecithin to these lipids did not improve this ratio.

Cholate can be substituted for lysolecithin in the incorporation of cytochrome oxidase. As shown in fig.3 there are two different mechanisms of cytochrome oxidase vesicles formation in the presence of cholate. One mechanism takes place at a high (1%) cholate concentration [3] and probably involves opening of the vesicles. At a low concentration (0.1%) of cholate the mechanism is probably similar to that with lysolecithin, referred to as incorporation, because no evidence for opening of the vesicles as indicated by massive ion leakage could be detected. In contrast to the cholate-dilution procedure which is virtually completed within 10 min, the incorporation at 0°C of cytochrome oxidase is a slow process requiring hours for completion. An interesting point is the narrow range of cholate concentrations (about 0.1%) which is optimal for the incorporation of the enzyme. The inhibition of the incorporation by higher cholate concentrations (0.2–0.5%) is difficult to explain in view of the fact that at even higher concentrations (1%) reconstitution can also take place.

3.2. *Incorporation of complex III into liposomes*

Complex III was reconstituted into liposomes by a procedure similar to that used for the incorporation of cytochrome oxidase. The optimal lysolecithin concentration was again 10% of total phospholipid (fig.4). However, in this case cholate substituted for lysolecithin only at high cholate concentrations.

3.3. *Reconstitution of the $^{32}P_i$-ATP exchange and the Ca^{++}-pump by the incorporation procedure*

Hydrophobic protein fraction [1] was incorporated into liposomes containing lysolecithin. In the presence of F_1 these liposomes catalyzed a rapid $^{32}P_i$-ATP exchange which was inhibited by rutamycin and uncouplers (table 1). The optimal concentration of hydrophobic protein was about 0.2 mg/ml. Similar to complex III, the P_i-ATP exchange was not reconstituted at low cholate concentrations. At high concentrations reconstitution occured by the cholate-dilution procedure [3].

Attempts to incorporate the Ca^{++} ATPase from sarcoplasmic reticulum into liposomes and measure

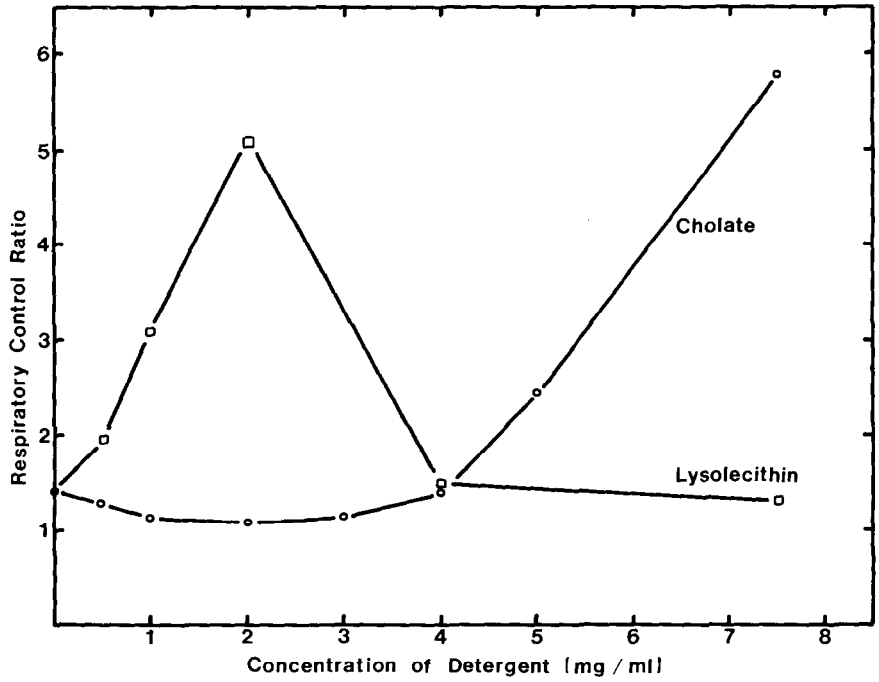

Fig.4. Effect of cholate and lysolecithin on the incorporation of complex III into liposomes. Phosphatidylethanolamine : phosphatidylcholine : cardiolipin (1 : 1 : 0.075) suspensions containing varying amounts of either cholate or lysolecithin were sonicated to clarity and incubated with complex III at 0°C for different time periods.

Table 1
Reconstitution of P_i-ATP exchange vesicles by the incorporation procedure

Concentration of hydrophobic protein (mg/ml)	Detergent used in incorporation	Inhibitor added to assay mixture	$^{32}P_i$-ATP exchange (nmoles/min/mg protein)
0.05	Lysolecithin		40
0.1	Lysolecithin		50
0.2	Lysolecithin		70
0.3	Lysolecithin		30
0.2	Lysolecithin	Rutamycin (4 μg/ml)	8
0.2	Lysolecithin	1799 (20 μM)	4
0.2	Cholate (0.05%)		0
0.2	Cholate (0.1%)		0
0.2	Cholate (0.2%)		0
0.2	Cholate (0.6%)		140
1.0	Cholate (0.6%)		150

A mixture of phosphatidylethanolamine : phosphatidylcholine : cardiolipin (1 : 1 : 0.03) and lysolecithin where indicated were washed with ether, dried and sonicated to clarity in tricine KOH, 10 mM pH 8.0. The phospholipids were incubated for 2 hr at 0°C with the amounts of hydrophobic protein and cholate indicated in the table. Samples (0.1 ml) were withdrawn and incubated for 20 min at room temperature with 40 μg F_1 and then assayed for $^{32}P_i$-ATP exchange as described [1].

the Ca^{++}-pump met with partial success. The best rates obtained using lysolecithin liposomes were about 25% of those obtained by the cholate-dialysis or dilution procedures.

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