Incorporation of Mitochondrial Membrane Proteins into Liposomes Containing Acidic Phospholipids*

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Studies of lipid-protein interactions in artificial systems were hampered for many years by the lack of biological assays for the function and orientation of the proteins in the membrane. In the last few years procedures have been developed for reconstitution of biologically active vesicles either with detergents (1-13) or by sonication (14-16) of the membrane proteins with phospholipids. A Ca2+ pump was reconstituted with the ATPase from sarcoplasmic reticulum (2), a Na+ pump with the (Na+ -K+)-ATPase isolated from the electric eel (15) or from other sources (3, 4), and a proton pump with bacteriorhodopsin (5). The reversible proton translocating ATPases from the inner mitochondrial membrane (1, 6) and from thermophilic bacteria (17) were reconstituted. The three sites of oxidative phosphorylation (7-9), vesicles with respiratory control (10-12), and the adenine nucleotide transport (13) were reconstituted with purified proteins from the inner mitochondrial membrane.

These reconstitution procedures provided membrane biochemists with tools to attack problems such as (a) the isolation of reconstitutively active membrane proteins, (b) the nature and function of reconstitutively active proteins, (c) the specificity and role of phospholipids in reconstituted systems (18, 19), e.g. the influence of composition of fatty acids and head groups, and (d) elucidation of reaction mechanisms, e.g. of oxidative phosphorylation by the reconstitution of a proton pump from Halobacter halobium (10) together with the oligomycin-sensitive ATPase from the inner mitochondrial membrane (5).

These experiments served as a model for the insertion of proteins in vivo and demonstrated the feasibility of the chemiosmotic hypothesis (20) for oxidative phosphorylation.

However, these reconstitution procedures involved either dissolving the liposomes with detergents or disrupting them by sonication. Recently (21), we have described briefly a procedure for incorporation of proteins into liposomes in which 10% of the total phospholipids was lysolecithin. This incorporation took place with no disruption of the liposomes but still had the disadvantage of including unnaturally high concentrations of lysolecithin which might affect the properties of the lipid bilayer. In the present work we describe the incorporation of mitochondrial proteins into liposomes containing acidic phospholipids. The incorporation occurred without added detergent or sonication. This incorporation procedure may serve as a model for the insertion of proteins in vivo.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome oxidase was prepared according to Yonetani (22) as modified by Eytan et al. (23). In some experiments the presence of Emasol was avoided by preparing the cytochrome oxidase with cholate according to Yu et al. (24). Delipidated cytochrome oxidase was prepared by suspending the enzyme (10 mg/ml) in 2% cholate, 50 mM KPi (pH 7.0) and precipitating it three times at 35% saturation of ammonium sulfate. The pellet was collected by centrifugation at 10,000 x g for 10 min. QH2-cytochrome c reductase (25), oligomycin-sensitive ATPase (18), bacteriorhodopsin (26), phosphatidylethanolamine, and phosphatidylcholine (18) were prepared as described. Phosphatidylserine was donated by Dr. C. Miller, Cornell University, who prepared it from ox brain according to Papahadjopoulos and Miller (27). Cardiolipin and phosphatidylserinolstol were purchased from General Biochemical Inc., Chagrin Falls, Ohio. All lipids used in this work exhibited single spots upon analysis by thin layer chromatography; their concentration are expressed as micromoles of

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**RESULTS**

**Incorporation of Cytochrome Oxidase into Liposomes containing Acidic Lipids**—Isolated cytochrome oxidase was incorporated into liposomes containing 30 mol% of phosphatidylserine. Upon mixing the enzyme with the liposomes, the cytochrome oxidase activity decreased within minutes and could be recovered by addition of uncouplers or detergents (Fig. 1). We expressed the degree of incorporation in terms of the respiratory control ratio as defined above. For example, respiratory control ratio of 5 implies that at least 80% of the cytochrome oxidase molecules were incorporated into the vesicles. Since the uncoupled rate assayed in the presence of valinomycin and 1799, or of Tween 80, was comparable to that of the isolated enzyme assayed in the presence of soybean phospholipids, the incorporated enzyme was oriented with its cytochrome c binding site exposed to the medium. This unidirectional orientation corresponds to that observed in mitochondria (28).

Fig. 1. Time course of cytochrome oxidase incorporation. Phospholipid vesicles were formed with phosphatidylethanolamine (13.2 μmoles), phosphatidylcholine (14.4 μmoles), and phosphatidylserine (7.4 μmoles) by sonication to clarity in 1 ml of 50 mM KP, buffer (pH 7.0) containing 10 mM MgCl₂. Purified cytochrome oxidase (1 mg) was added and the mixture was incubated at room temperature. Samples were withdrawn and assayed in absence or presence of either Tween 80 (3%) or valinomycin (0.5 μg/ml) plus 1799 (20 μM).

and in cytochrome oxidase vesicles reconstituted by the cholate dialysis procedure (10).

The incorporation of cytochrome oxidase can actually be observed under the conditions of assay. In that case low amounts of cytochrome oxidase (10 μg) were added to the reaction mixture containing, in addition to cytochrome c and ascorbate, 1.2 mM phospholipid vesicles consisting of phosphatidylserine:phosphatidylethanolamine:phosphatidylycholine at the ratios of 1:1:1. As shown in Fig. 2, oxygen uptake decreased progressively as the enzyme was incorporated. Full activity was recovered by addition of uncoupling agents. The respiratory control ratio attained in such experiments was somewhat lower (3 to 4) than that obtained by the procedure described in the legend of Fig. 1.

**Phospholipid Requirements for Incorporation of Cytochrome Oxidase**—About 30 mole % of phosphatidylserine was optimal for cytochrome oxidase incorporation (Fig. 3a). The inhibition observed at higher concentrations of phosphatidylserine is probably caused by a greater proton permeability of the vesicles (29). Two other acidic phospholipids, phosphatidylinositol and cardiolipin, also promoted incorporation. Cardiolipin was the most effective, allowing incorporation at 10 mole % (Fig. 3b). Phosphatidylinositol, like phosphatidylserine, was optimal at 20 to 30 mole %. As will be shown later, Mg³⁺ accelerated the rate of incorporation. It should be noted, however (Fig. 3, a and b), that incorporation took place in the presence of EDTA.

In liposomes containing 30% phosphatidylserine, the optimal phospholipid ratio for incorporation was phosphatidylethanolamine:phosphatidylycholine (4:1) in the absence of divalent cations (Fig. 4a). In the presence of Mg³⁺ the optimal ratio of phosphatidylethanolamine to phosphatidylycholine was lower (3:2). It can be seen from Fig. 4b that in liposomes containing cardiolipin instead of phosphatidylserine the optimal ratio of phosphatidylethanolamine to phosphatidylycholine was reversed (1:3), but even in the absence of phosphatidylethanolamine high respiratory control ratios were observed.

![Fig. 1. Time course of cytochrome oxidase incorporation.](image1)

![Fig. 2. Incorporation of cytochrome oxidase during assay conditions.](image2)

The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
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**Fig. 3.** Incorporation of cytochrome oxidase into liposomes with varying concentrations of phosphatidylserine or cardiolipin. Liposomes were prepared from phosphatidylethanolamine and phosphatidylcholine (at the ratio of 3:1) and various amounts of phosphatidylserine (a) or phosphatidyl inositol (c) or from phosphatidylethanolamine and phosphatidylcholine (at the ratio of 1:3) and various amounts of cardiolipin (b). The liposomes were incubated at a concentration of 25 mM with cytochrome oxidase (1 mg/ml) in Hepes (10 mM, pH 7.0), KCl (40 mM), and either MgCl₂ (10 mM) or EDTA (10 mM). The respiratory control ratio was assayed after 30 min of incubation at room temperature.

**Fig. 4.** Optimal phosphatidylethanolamine (PE) to phosphatidylcholine (PC) ratios for incorporation of cytochrome oxidase. Liposomes were prepared by drying down the amounts of phosphatidylethanolamine and phosphatidylcholine indicated below the figures, together with either phosphatidylserine (a, 7.6 μmoles, 30% of total phospholipids), cardiolipin (6, 5 μmoles, 20% of total), or phosphatidylinositol (c, 7.6 μmoles). The lipids were sonicated to clarity in 1 ml of Hepes (10 mM, pH 7.0), KCl (40 mM), and either MgCl₂ (10 mM) or EDTA (10 mM). Cytochrome oxidase (1 mg) was added and the respiratory control assayed after 30 min at room temperature.
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With phosphatidylinositol-containing liposomes reconstitution was not influenced by Mg\(^{2+}\) (Fig. 4c) and in this case phosphatidylcholine was not required. Cytochrome oxidase was also incorporated into liposomes prepared from a crude mixture of phospholipids from bovine heart mitochondria, yielding a respiratory control ratio of 3:4. This finding is in line with the known phospholipid composition of inner mitochondrial membranes, phosphatidylethanolamine:phosphatidylcholine:cardiolipin ratio of 2:2:1 (30).

The degree of incorporation was dependent on the ratio of phospholipid to protein. Below a ratio of 20:1 (w:w) the respiratory control ratio obtained by incorporation dropped rapidly. This is similar to observations with other procedures of reconstitution with cholate (10) or by sonication (14). Over a wide range absolute concentrations of the protein and lipids did not affect the final respiratory control ratio obtained by incorporation, but the rate of incorporation was faster at higher phospholipid and protein concentrations.

Purified cytochrome oxidase contains 20% (w/w) phospholipids (24). The role of the phospholipids in incorporation was studied by substitution with pure phospholipids. The lipids were removed by repeated ammonium sulfate precipitations of the enzyme from a 2% cholate solution. After three cycles the enzyme contained less than 1% phospholipids (w/w) and its oxidase activity was enhanced 30- to 40-fold by added phospholipids. This delipidated enzyme was incorporated into liposomes containing acidic phospholipids as shown in Fig. 5. When the delipidated enzyme was incubated first with low amounts of either phosphatidylcholine or phosphatidylethanolamine, incorporation was strongly inhibited, whereas phosphatidylserine did not inhibit (data not shown). The control enzyme was not inhibited by the neutral phospholipids.

Thus, the composition of the phospholipids associated with the enzyme plays a major role in its incorporation into liposomes.

**Effect of Divalent Cations on Incorporation of Cytochrome Oxidase** As shown in Fig. 6, Mg\(^{2+}\) accelerated the rate of incorporation of cytochrome oxidase at room temperature without affecting the final level attained. The effect of Mg\(^{2+}\) was similar with liposomes containing phosphatidylserine (Fig. 6), cardiolipin or phosphatidylinositol.

Mn\(^{2+}\) or Ca\(^{2+}\) substituted for Mg\(^{2+}\) in accelerating the rate of incorporation. At the conditions tested (20-min incubation at 10\(^{\circ}\)) the optimal concentrations of Mg\(^{2+}\) and Mn\(^{2+}\) were about 10 mM and of Ca\(^{2+}\) about 2 mM. Higher concentrations of the cations inhibited the incorporation, probably by causing aggregation of the liposomes.

**Are Detergents Required for Incorporation?**—Incorporation of cytochrome oxidase into liposomes containing acidic phospholipids did not require the addition of detergents. In fact, addition of either cholate or lysolecithin inhibited incorporation at very low concentrations (Fig. 7). The detergents did not interfere with the assay since they had no effect on respiratory control when added at these concentrations to reconstituted vesicles. Next, the possibility was considered that the small amount of detergent which is present in the purified enzyme plays a role in the incorporation procedure. Yu et al. (24) described recently a procedure for the purification of cytochrome oxidase with cholate as the only detergent. We followed this procedure using radioactive cholate (see Fig. 8). The isolated 1% solution of the enzyme contained 1 mg of cholate/mg of enzyme. After two precipitations with ammonium sulfate the cholate concentration fell to 100 &mg of protein, but the resuspended enzyme was no longer soluble. Similar results were obtained when the enzyme was washed with either toluene or hexane containing 1% soybean phospholipids. As shown in Fig. 8 the removal of cholate had only a small effect on the incorporation. After the second precipitation, there was some reduction in respiratory control which was probably caused by lack of incorporation due to the aggregation of the enzyme.
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**FIG. 7.** Inhibition of the incorporation by lysolecithin and cholate. Liposomes (25 mM) prepared from phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol at the ratios of 3:1:2 were incubated at room temperature for 30 min with cytochrome oxidase (1 mg/ml) in the presence of various concentrations of either cholate or lysolecithin. The cholate was added directly to the incubation medium, the lysolecithin was added to the phospholipids before sonication.

**FIG. 8.** Removal of cholate from cytochrome oxidase does not impair its subsequent incorporation into liposomes. Cytochrome oxidase (10 mg) was prepared according to Yu et al. (24) except that [14C]cholate (40 μCi/m mole) was used. The purified enzyme was resuspended in 1 ml of 50 mM KP (pH 7.0), 0.4 M KCl and then precipitated twice at 35% ammonium sulfate saturation by centrifugation at 100,000 × g for 20 min. After these washes the resuspended enzyme (10 mg/ml) was insoluble and further washes were carried out by pelleting the enzyme without ammonium sulfate. After every wash the pellet was resuspended in 1 ml of the above buffer and samples were withdrawn for radioactive determinations and incorporation into liposomes as described in the legend to Fig. 1.

**Incorporation of Oligomycin-sensitive ATPase and QH$_2$-Cytochrome c Reductase—**The oligomycin-sensitive ATPase from bovine heart mitochondria was incorporated into liposomes containing acidic phospholipids. The degree of incorporation was monitored by assaying the $^{32}$P$_7$-ATP exchange. The lipids and cation requirements were similar to those of cytochrome oxidase (Fig 9). The oligomycin-sensitive ATPase could be incorporated at higher protein to lipid ratio (up to 1:5) than cytochrome oxidase.

**TABLE I**

<table>
<thead>
<tr>
<th>Mode of reconstitution</th>
<th>ATPase activity</th>
<th>$^{32}$P$_7$-ATP exchange activity</th>
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<tbody>
<tr>
<td></td>
<td>Emasol</td>
<td>± Emasol</td>
</tr>
<tr>
<td>Incorporation</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Cholate dilution</td>
<td>2.6</td>
<td>4.4</td>
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The orientation of the oligomycin-sensitive ATPase in the liposomes was determined by assaying their ATPase activity in the presence or absence of Emasol 1130. Since liposomes are impermeable to ATP, any ATPase oriented with its active site inside the liposomes would not contribute to ATP hydrolysis in the assay. We have found that 3% Emasol 1130 rendered the liposomes permeable to nucleotides without substantially inhibiting the enzyme. As shown in Table I, liposomes that were reconstituted by the cholate dilution procedure were oriented bidirectionally, since a marked stimulation of ATPase activity was observed on addition of Emasol 1130. On the other hand, the enzyme in vesicles prepared by incorporation was unidirectionally oriented, as indicated by the lack of stimulation of ATPase by Emasol.

The incorporation of oligomycin-sensitive ATPase can be
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utilized to reconstitute bacteriorhodopsin-catalyzed photophosphorylation. Bacteriorhodopsin was incorporated by the sonication procedure into liposomes consisting of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinerine in equal amounts. Subsequently, the ATPase was incorporated into these vesicles. The presence of light-driven phosphorylation (Fig. 10) by this procedure demonstrates that some of the ATPase was incorporated into the vesicles containing bacteriorhodopsin. QH₂-cytochrome c reductase was incorporated into liposomes with a phospholipid composition similar to that used for incorporation of cytochrome oxidase. High respiratory control ratios could be obtained at phospholipid:protein ratios considerably lower than those required for cytochrome oxidase incorporation.

**DISCUSSION**

Incorporation of cytochrome oxidase, QH₂-cytochrome c reductase, and oligomycin-sensitive ATPase into acidic phospholipid containing liposomes is different from previously described procedures of reconstitution (1–16). It does not involve perturbation of the liposomes and the proteins by either addition of detergents or by sonication. It is rapid and mild, taking only a few minutes of incubation at room temperature. The incorporation of cytochrome oxidase and of QH₂-cytochrome c reductase is very efficient, most of the protein added being unidirectionally incorporated into the liposomes. We used the respiratory control ratios as indices of the extent of incorporation, a respiratory control ratio of 5, for example, indicating 80%. This is a minimal estimate assuming complete proton impermeability of the liposomes, which obviously is not the case. We do not have a comparable quantitative index for the incorporation of the oligomycin-sensitive ATPase, but a relative evaluation can be made from the ratio of ³²P-ATP exchange/ATPase activity. It would thus appear from the data shown in Table I that the cholate dilution procedure is somewhat more effective but less specific in the reconstitution of the ATPase complex into the liposomes.

The fact that incorporation does not involve the disruption of liposome structure (21) makes it useful in the elucidation of certain aspects of reconstitution such as the effects of size, composition, and asymmetry of the liposomes. We have also used the incorporation procedure to study the effect of the presence of one protein in a liposome on the incorporation of additional proteins. The oligomycin-sensitive ATPase exhibits a much higher affinity to liposomes containing cytochrome oxidase than to liposomes with no protein, whereas cytochrome oxidase is more readily incorporated into protein-free liposomes than into ATPase-containing liposomes.

The requirement for acidic phospholipids raises the question of the relationship between incorporation and fusion. Fusion between liposomes dependent on Ca²⁺ and phosphatidylinerine (or other acidic phospholipids) was recently demonstrated (31, 32). An analysis (29) of the role of the phospholipid composition of liposomes in the fusion process revealed that phosphatidylethanolamine accelerate fusion and that about 30% phosphatidylinerine was required. Cardiolipin but not phosphatidylinositol substituted for phosphatidylinerine in the presence of phosphatidylethanolamine. There are important differences between incorporation and fusion. Phosphatidylinositol did not facilitate fusion but did facilitate incorporation. The fusion of liposomes was completely dependent on divalent cations, whereas incorporation was not.

We suggest that the incorporation procedure is an adequate model for study of problems involving the incorporation in vivo of proteins into biological membranes. The unidirectional orientation of the proteins, the high specificity for phospholipid composition, and its resemblance to that of natural membranes support this conclusion. Moreover, the system lends itself to the study of sequential incorporation of membrane proteins.

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