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Morphology of Proteoliposomes Containing Fluorescein-Phosphatidylethanolamine Reconstituted with Native and Subunit III-Depleted Cytochrome *c* Oxidase[1]

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Abstract

Beef heart cytochrome *c* oxidase was reconstituted in asolectin liposomes containing the pH indicator fluorescein-phosphatidylethanolamine (FPE) by the cholate-dialysis procedure. The influence of FPE on the asolectin liposome size and of the removal of subunit III from the complex on its incorporation into liposomes was analyzed by freeze-fracture electron microscopy. Samples were frozen without the addition of cryoprotectants. The vesicle size distribution of native enzyme reconstituted into asolectin liposomes was homogeneous, 84% of the population having a diameter of 14–37 ± 7.5 mm. The preparation containing FPE had a similar vesicle size distribution, but with bigger diameter range (20–50 nm). In all three different types of proteoliposome preparations the majority of particles containing vesicles was found to have 1 particle (42–81%). The absence of subunit III did not influence the incorporation of the enzyme into the liposomes and was as good as the preparation with native enzyme (> 99%). Therefore we conclude that the suppression of the proton pump activity was due to the intrinsic properties of subunit III and not to defective incorporation into artificial membrane systems.

Key Words: Cytochrome *c* oxidase; fluorescein-phosphatidylethanolamine; proteoliposomes; rapid freezing; freeze-fracture size analysis.

Introduction

Mammalian cytochrome *c* oxidase is a complex membrane protein which catalyzes electron transfer from cytochrome *c* to oxygen and proton translocation from the matrix to the cytoplasmic side of mitochondria (for a review see Azzi, 1980, Wikström *et al.*, 1981, Capaldi *et al.*, 1983, and

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Azzi *et al.*, 1985). Its complex function is currently under investigation in natural membranes as well using the isolated and purified enzyme reconstituted in phospholipid vesicles (Wikström, 1981; Casey *et al.*, 1984; Thelen *et al.*, 1985a). To measure internal pH changes in this system, a new experimental approach was recently developed (Thelen *et al.*, 1984) consisting in the incorporation of the pH indicator fluorescein-phosphatidylethanolamine, FPE,[3] into phospholipid vesicles during reconstitution. The absorbance changes of the indicator was shown to indicate, under appropriate conditions, internal pH changes. This experimental setup was used to make a quantitative analysis of the net internal alkalinization upon addition of ferrocytochrome *c* to native and subunit III-depleted cytochrome oxidase reconstituted into proteoliposomes (Thelen *et al.*, 1985b). The differences in the ability of the native and subunit III-depleted enzyme to pump H^+ could be explained in two ways. Subunit III is required for the function of the proton pump or it has a structural function, for instance being necessary for the insertion of the enzyme into liposomes. To elucidate this point a structural study was carried out to compare, by using a freeze-fracture technique, the degree of insertion of proteins into the lipid membrane. Another aspect that had to be clarified was the structural perturbation of FPE on the formation and size of lipid vesicles. We present here a freeze-fracture analysis of proteoliposomes with native and subunit III-depleted cytochrome oxidase reconstituted with and without FPE. The vesicle preparations we analyzed were the same preparation used by Thelen *et al.* (1985b) for the H^+/e^- stoichiometry measurements.

Experimental

Enzyme and Vesicle Preparations

Bovine heart cytochrome oxidase was prepared according to Yu *et al.* (1975), and stored frozen at $-70°C$ as a stock solution (300 μM). The concentration of cytochrome aa_3 was calculated from spectra (dithionite-reduced minus air-oxidized) taken with an Aminco DW-2a spectrophotometer using $\Delta\varepsilon_{605-630\,nm} = 27\,mM^{-1} \cdot cm^{-1}$. Subunit III was removed from the native enzyme essentially as described by Bill and Azzi (1982) and as modified by Thelen *et al.* (1985b).

Cytochrome oxidase was reconstituted into liposomes using the cholate dialysis procedure as described by Thelen *et al.* (1985b). The asolectin/FPE

[3] Abbreviations: FPE, fluorescein-phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

weight ratio was 40 and the final lipid/protein weight ratio was approximately 30 using 40 mg lipid/ml. Potentiometric measurements, orientation of the enzyme, and measurements of the respiratory control and of the molecular activity are reported in detail elsewhere (Thelen *et al.*, 1985b).

Electron Microscopy

Samples (approximately 0.1 μl) on a gold grid were frozen between two thin copper holders with liquid propane cooled by liquid nitrogen using a Balzer's Cryo-jet prototype (Müller *et al.*, 1980). The very high cooling rate (approximately 10^4 °C/s) avoids the use of cryoprotectants. Freeze-fracturing was carried out in a Balzer's BAF 300 freeze-etching apparatus at 123°K and a pressure of 10^{-5} Pa. Specimens were replicated with Pt/C and were examined in a Philiphs EM 301 electron microscope at 100 kV. Micrographs were taken on Agfa Scientia 23 D56 film.

Results and Discussion

Freeze-Fracture of Proteoliposomes (Native Cytochrome Oxidase) without FPE

Fracture patterns of proteoliposome suspensions after cholate dialysis showed distinct, spherical vesicles randomly distributed over the fracture surface (Fig. 1A). Practically all vesicles (> 99%) were unilamellar. In samples from which not all cholate had been removed by dialysis, aggregated vesicles were observed (not shown). At higher magnification (Fig. 1B), intramembrane particles were clearly observed on the fracture faces of the proteoliposomes, without any difference of distribution between concave and convex surfaces. In vesicles with two or more particles, these were randomly distributed, thus not forming pairs or higher aggregates. The almost complete absence of particles in the background (even if it would be difficult to identify them as a protein–lipid detergent complex in solution) indicated that practically all cytochromes oxidase molecules (> 99%) were incorporated into liposomes during reconstitution. The smooth background ice between liposomes indicated also that the high cooling rate during freezing stopped the water-crystal growth to dimensions (approximately 1–2 nm) which do not induce aggregation or damage of vesicles.

Size analysis showed that approximately 84% of the vesicles (n = 305) had a diameter of 14–37 $\pm$ 7.5 nm (Fig. 2). Table I shows that 8.1% of the total vesicles (n = 4841) contained 1–4 particles. This percentage corresponds to the vesicles effectively seen in the micrographs, without correction for the invisible fracture faces. More than half (58%) of the

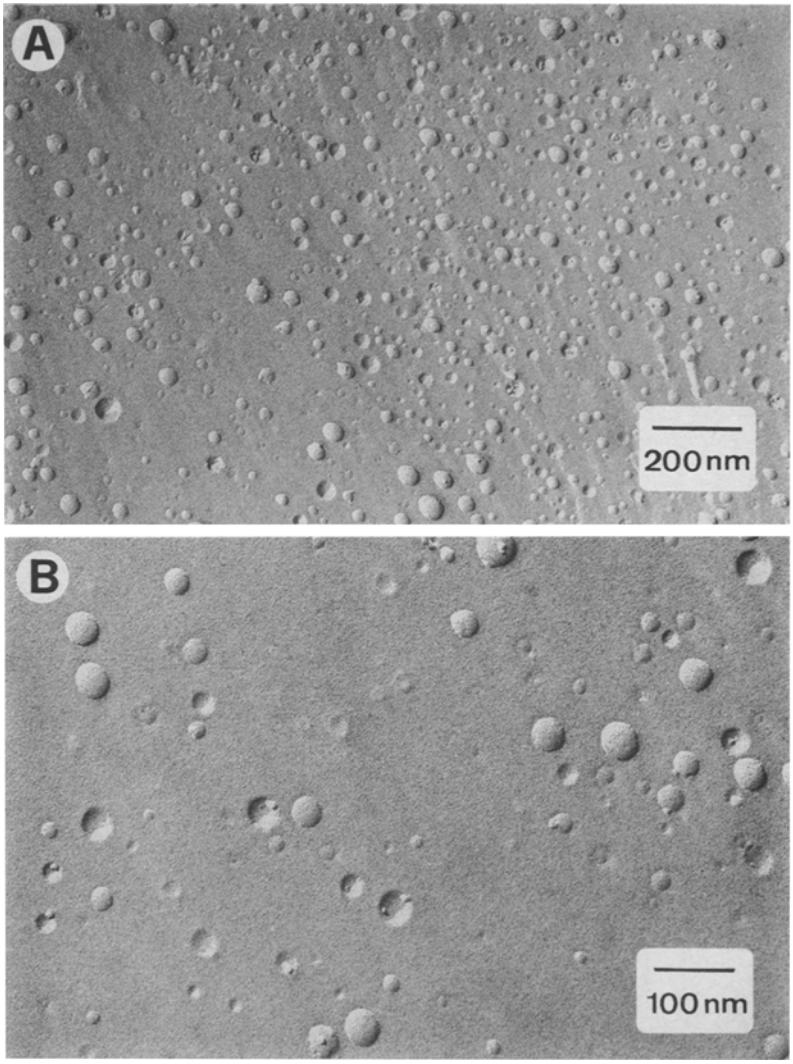

Fig. 1. Freeze-fracture electron micrographs of proteoliposomes reconstituted with cytochrome *c* oxidase by the cholate dialysis procedure. (A) Proteoliposomes (at low magnification) containing cytochrome oxidase at a lipid-to-protein weight ratio of approximately 27 (40 mg of lipid/ml) in 200 mM sucrose, 50 mM KCl, 0.25 mM Hepes, pH 7.3. Practically all proteoliposomes (> 99%) are spherical, unilamellar vesicles. (B) Higher magnification of the same specimen, in a region adjacent to that shown in (A). The intramembrane particles were equally distributed in convex and concave surfaces.

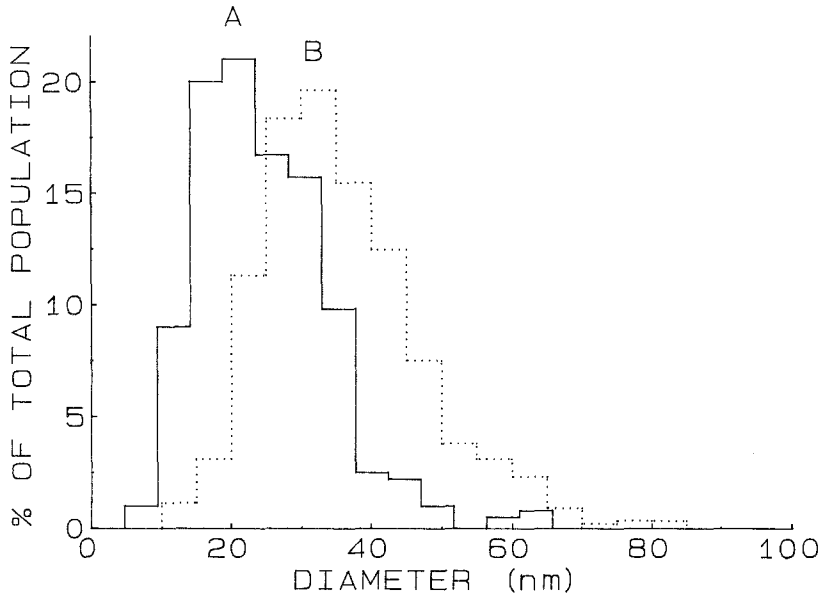

Fig. 2. Vesicle size analysis of reconstituted proteoliposomes. (A) Size distribution of proteoliposomes (n = 305) reconstituted with native enzyme and without FPE; (B) size distribution of proteoliposomes (n = 867) reconstituted with native enzyme in the presence of FPE (1 mg/40 mg asolectin).

Table I. Particle Distribution in the Protein-Containing Vesicles

	Total vesicles	Total vesicles with protein	Number of particles per vesicle[b]				Total particles
			1	2	3	4	
Asolectin liposomes with native enzyme	4841	392(8.1)[a]	228(58)	104(26.5)	43(11)	17(4.5)	633
FPE–asolectin liposomes with native enzyme	867	108(12.5)[a]	46(42.6)	38(35.2)	14(13)	10(9.2)	204
FPE–asolectin liposomes with subunit III-depleted enzyme	600	86(14.3)[a]	70(81.4)	12(14)	4(4.6)	–	105

[a]Percent of total vesicle population.
[b]Percent of vesicles having particles.

vesicles containing particles showed 1 particle, and only 5% of them showed 4 particles or more.

Comparison of Proteoliposomes with and without FPE (Native Cytochrome Oxidase)

Comparison of Fig. 1B with Fig. 3A revealed no substantial differences between the two vesicle preparations. A more accurate analysis of the vesicle size and particle distribution showed some differences. Although the homogeneity of the two vesicle preparations was almost identical (approximately 84% of the vesicles were in a diameter range of approximately 30 nm), the largest number of vesicles containing FPE had a diameter of 30–35 nm, approximately 10 nm bigger than the conventional preparation (17–22 nm; Fig. 2).

The FPE-proteoliposomes showed also a higher percentage of vesicles having 2, 3, 4, and more particles (+15%) relative to the conventional preparation, which contained more vesicles having a single particle (+15%; Table I).

Comparison of Fluorescent Proteoliposomes with Native and Subunit III-Depleted Cytochrome Oxidase

The removal of subunit III from the whole complex (13 subunits) did not modify the size distribution of the total vesicle population (not shown) nor that of the vesicles having intramembrane particles (Fig. 4) relative to the proteoliposomes containing native enzyme. Interestingly, with the subunit III-depleted preparation, vesicles containing 1 intramembrane particle were more abundant (81%; Table I) and vesicles with 4 or more particles were absent. Removal of subunit III, which is accompanied by loss of several other small subunits, results in a considerable change in the total mass of the enzyme and, in particular, of that in contact with the bilayer. Such a change, however, could not be appreciated in the freeze-fracture images due to the resolution limits of the system.

Conclusions

Indirect evidence has indicated that reconstitution by the dialysis technique, under strictly controlled conditions of lipid and protein concentration, dialysis time and changes, etc., yielded a population of vesicles homogeneous and containing one enzyme molecule per liposome. In the present study more direct evidence indicates that largely the population of cytochrome oxidase liposomes consists of empty vesicles. Only a small part of the population

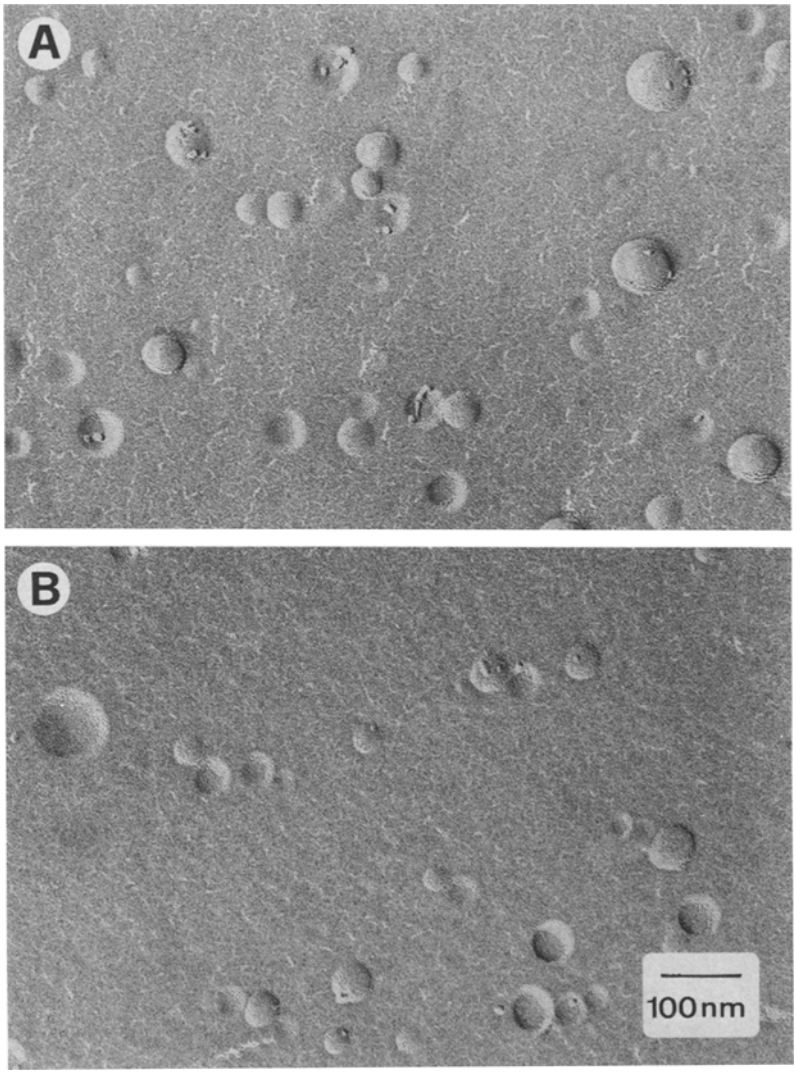

Fig. 3. Freeze-fracture pictures of fluorescent proteoliposomes. The vesicles were reconstituted by the same procedure as in Fig. 1, but the final medium was 125 mM KCl, 25 mM sucrose, 1.8 mM $CaCl_2$, 100 μM Hepes, pH 7.3. These vesicles were used for internal pH measurements (Thelen *et al.*, 1985a, b). (A) Native enzyme; (B) subunit III-depleted enzyme.

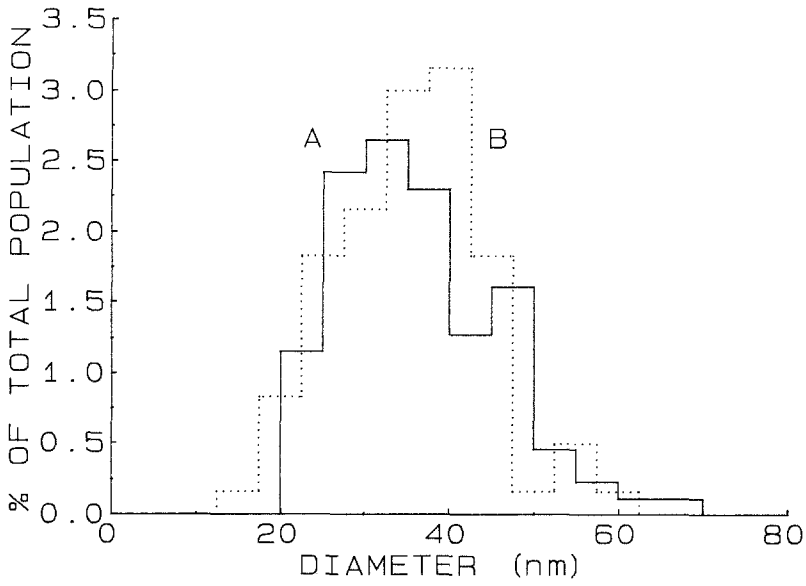

Fig. 4. Vesicle size analysis of reconstituted fluorescent proteoliposomes containing intramembrane particles. (A) Size distribution of proteoliposomes (n = 108) reconstituted with native enzyme; (B) size distribution of proteoliposomes (n = 86) reconstituted with subunit III-depleted enzyme.

contained protein particles. It is remarkable that a majority of them contained also one single protein particle.

It is clear therefore that, although the expectations of a pure homogeneous population of proteoliposomes were not verified, a fractionation of the vesicles in two parts, those containing proteins and the empty ones, would be of great advantage. The second conclusion which may be drawn from the above study is that even small perturbations of the experimental conditions necessary to obtain the liposomes result in a significant change in the type and shape of liposomes.

As shown in Fig. 2 the incorporation of FPE into the reconstituted proteoliposomes and the dialysis carried out in the presence of 1.8–1.9 mM $CaCl_2$ lead to the formation of proteoliposomes of approximately 10 nm larger in diameter than that reconstituted without FPE. The presence of $CaCl_2$ was shown to be necessary to decrease the surface potential of the membrane and consequently to avoid the possibility of FPE responding to membrane potential changes instead of to pH changes (Thelen *et al.*, 1984). The presence of Ca^{2+} may have been also responsible for the increase in vesicle number containing 2 or more particles. The third conclusion which

may be drawn from this study is that the incorporation of a subunit III-depleted enzyme into liposomes occurs very similarly to the native enzyme. In both cases, the absence of nonincorporated enzyme is clear. Thus, the observation that cytochrome oxidase depleted of subunit III has a proton pump which is diminished or absent cannot be attributed to a defective insertion of the enzyme into the membrane. Rather it should be attributed to the lack of a component responsible for the H^+ ejection of the enzyme.

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