EFFECT OF LYSOLECITHIN TREATMENT ON THE STRUCTURE AND FUNCTIONS OF THE MITOCHONDRIAL INNER MEMBRANE

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SUMMARY: Lysolecithin treatment of electron transport particles (ETP) generated membrane fragments capable of catalyzing ATP-$^{32}P_i$ exchange, which was resistant to the uncoupling action of Valinomycin plus Nigericin or Valinomycin plus Monensin A in the presence of K+. Electron micrographs of ultrathin, positively stained sections of lysolecithin treated ETP were virtually devoid of circular patterns characteristic of closed vesicles. The results suggest that the closed vesicular structure of the mitochondrial inner membrane demanded by the chemiosmotic hypothesis of energy transduction (1) may not be essential for the ATP-$^{32}P_i$ exchange reaction.

The evidence that intrinsic proteins of the mitochondrial inner membrane are associated in functional domains has been recently reviewed by Capaldi and Green (2), and membrane fragments of sizes intermediate between those of electron transport particles and those of individual complexes have been obtained by sonication of inner membrane preparations (3,4). As sonication often results in release of coupling factors (5-7), we looked for a milder method for fragmentation of the mitochondrial inner membrane to study the structural requirements for energy transduction.

In the present communication, we wish to report the results of experiments on the effect of lysolecithin treatment on the structure and functions of the mitochondrial inner membrane. Lysolecithin as a disruptive agent is known to inhibit respiration and to uncouple phosphorylation (8,9), but we found that lysolecithin treatment of electron transport particles (ETP) resulted in membrane fragments capable of catalyzing ATP-$^{32}P_i$ exchange.

MATERIALS AND METHODS

ETP were prepared according to the method of Linnane and Ziegler (10)

for the preparation of ETP_H in a medium 0.25M in sucrose and 10mM in Tris-HCl, pH 7.8 without, however, addition of either ATP or Mg^{++}. For electron microscopic studies, negatively stained specimens were prepared by placing a drop of 2% ammonium molybdate (pH 7.4) on a film of the sample suspension previously dried on a carbon coated grid. The positively stained, ultrathin section were prepared by the method of Green *et al.* (11) with the following modifications: acrolein was omitted from the fixative, and glutaraldehyde fixed material was centrifuged at 105,000 x g for 30 minutes. For post-fixation, 1% OsO_4 was used. A Hitachi HU-11E electron microscope was used. The reaction mixture for the assay of the ATP-$^{32}P_i$ exchange reaction contained, in a volume of 1 ml, sucrose, 0.25M; Tris-HCl, pH 7.4, 10mM; $MgCl_2$, 10mM; ATP, 10mM; and $^{32}P_i$ containing potassium phosphate, pH 7.4, 10mM (about 2 x 10^5 cpm/μmole); particles (about 1 mg protein) were added to start the reaction. The exchange reaction was carried out at 30°, and after 5 minutes incubation, inorganic phosphate was separated (12) and ^{32}P in organic phosphate was determined. Radioactivity of ^{32}P was measured in Aquasol (New England Nuclear). A Packard TRI-CARB liquid scintillation spectrometer was used. NADH oxidase activity was measured spectrophotometrically as described by Mackler (13) except the assay was carried out at 25° instead of 38°. Protein was determined by the method of Lowry *et al.* (14), and the cytochrome contents were estimated according to Williams (15). A Cary 14 recording spectrophotometer was used for spectra measurements, and a Beckman DU spectrophotometer with or without Gilford attachment was also used for absorbance measurements at fixed wave lengths. Lysolecithin prepared from egg lecithin (a product of Sigma) was used. Nigericin and Monensin A were a generous gift from Dr. George A. Blondin and Dr. James E. Southard. All the other chemicals used were obtained from commercial sources.

RESULTS AND DISCUSSION

Addition of lysolecithin to a suspension of ETP resulted in rapid decrease in turbidity measured as absorbance at 520mμ as reported by Honjo and Ozawa (9). Turbidimetric titration of ETP with lysolecithin (Fig. 1) indicated a stoichio-

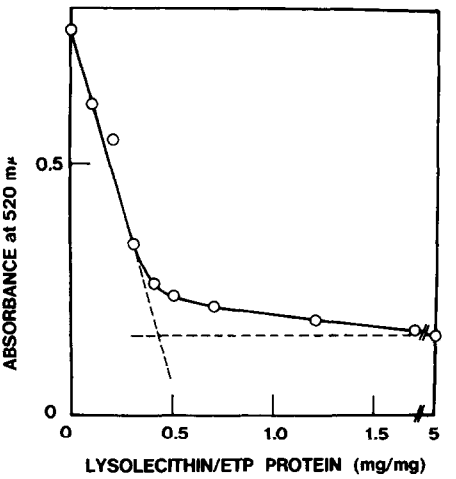

Figure 1. Turbidimetric titration of ETP_H with lysolecithin.

One milliliter of ETP_H (2 mg/ml) in a medium 0.25 in sucrose and 10 mM in Tris-HCl, pH 7.8 was titrated with lysolecithin dissolved in the sucrose-Tris medium. Absorbance at 520 mμ was recorded 5-10 minutes after each addition of lysolecithin.

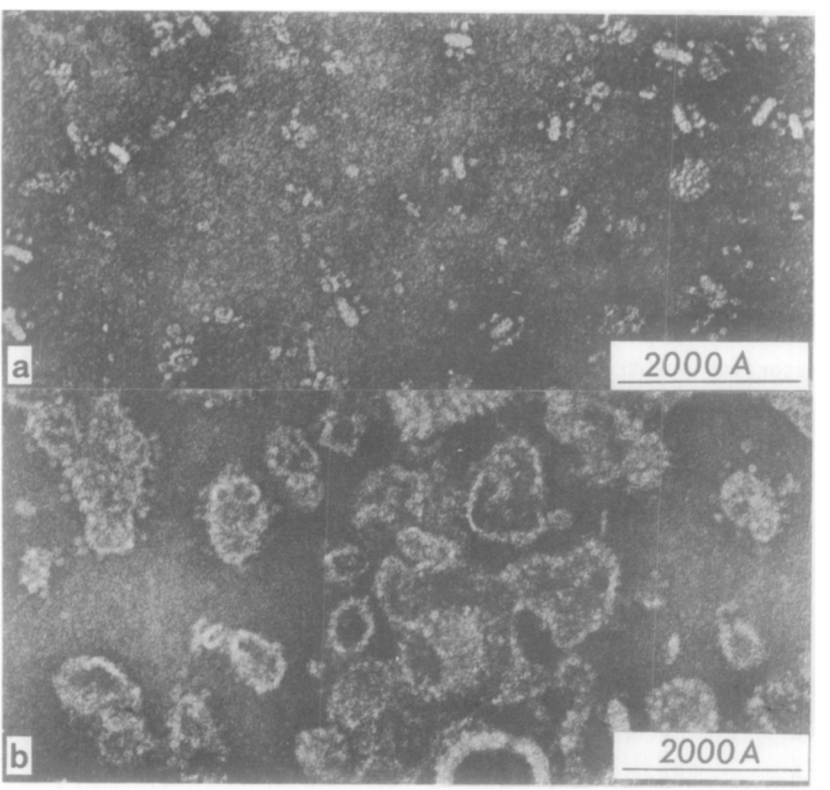

Figure 2. Electron micrographs of negatively stained specimens of (2a) lysolecithin treated ETP_H and (2b) ETP_H. Magnification: x 150,000.

metry of 0.43 mg lysolecithin/mg ETP protein. Since the shape of the titration curve indicated appreciable dissociation, we routinely used an excess amount of lysolecithin. Thus, ETP (10 mg/ml) suspended in a medium 0.25M in sucrose and 10mM in Tris-HCl, pH 7.8, were treated with lysolecithin (1.2 mg/mg protein) at 0° for 30 minutes, and centrifuged at 105,000 x g for 30 minutes to remove large fragments (approximately 25% of the protein was pelleted).

As can be seen in Fig. 2a, electron micrographs of negatively stained specimens of the resulting supernatant (S_1) showed a profusion of small membrane fragments with 4-6 headpieces (F_1). For comparison, an electron micrograph of a negatively stained specimen of untreated ETP is also shown (Fig. 2b). The dimensions of the small membrane fragments produced by the action of lysolecithin on ETP were 130 x 300-400Å (excluding F_1), suggesting that they may be collapsed annuli. To avoid possible rearrangement in the membrane structure that may accompany the negative staining procedure, S_1 was fixed with glutaraldehyde and ultrathin sections were preared from the fixed material. Electron micrographs of an ultrathin section of S_1 and untreated ETP, respectively, indicated the absence of the circular pattern characteristic of closed vesicular structure in the former and the abundance of such patterns in the latter (Fig. 3a, 3b). The result indicated that lysolecithin treatment of ETP generated non-vesicular membrane fragments in line with the known properties of lysolecithin. Thus, the wedge-shaped structure of the lysolecithin molecule favors globular micelles as compared to bilayer structure when lysolecithin is mixed with water (16-18), and incorporation of lysolecithin into lipid bilayers results in beading of lamellae or formation of discs (19). Partial destruction of vesicular structure of mitochondrial inner membrane by phospholipase A treatment (*in situ* formation of lysophosphatides) has been reported by Berezney *et al.* (20).

For the study of the composition of the membrane fragments, the S_1 fraction was centrifuged for an additional 3 hours at 105,000 x g and the pellet (R_2) was resuspended in the sucrose-Tris medium (30 to 50% of the original ETP protein was in this fraction). Analysis of the cytochrome composition indi-

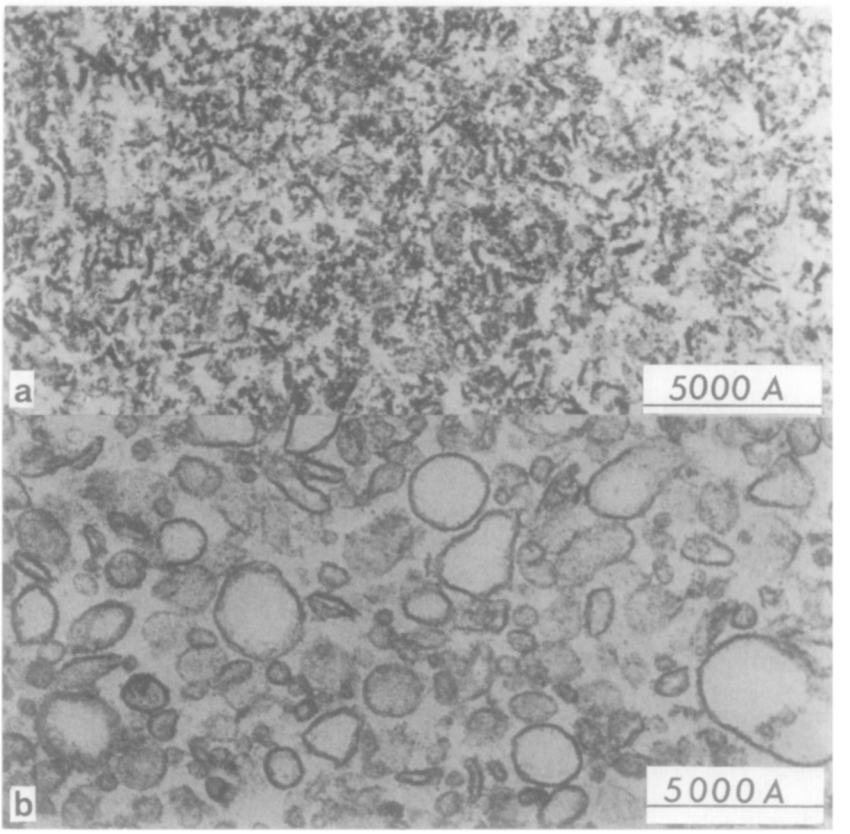

Figure 3. Electron micrographs of positively stained, ultrathin sections of (3a) lysolecithin treated ETP_H and (3b) ETP_H. Magnification: x 60,000.

cated that all the cytochromes were present in the R_2 fraction, although the contents of both cytochrome *a*, a_3 and *b* were somewhat lower than the corresponding value for ETP, and the value for cytochrome c_1 was considerably lower (Table I). Electron microscopic examination of negatively stained specimens indicated that the membrane fragments with headpieces observed in S_1 were found in R_2 and not in the supernatant (S_2) after removal of R_2.

It has been known that lysolecithin inhibits electron transport between NADH dehydrogenase and Co-enzyme Q without inhibiting NADH dehydrogenase and cytochrome oxidase (9). Under the experimental condition used in this study, the lysolecithin treated ETP (S_1) had negligible NADH oxidase activity. Because of the strong inhibition of electron transport by lysolecithin, we

TABLE I. Effect of lysolecithin treatment on cytochrome composition of ETP_H.

	Cytochrome Contents (mμmoles/mg)			
	$a + a_3$	c	c_1	b
ETP_H	2.03	0.88	0.40	1.51
Lysolecithin treated ETP_H (R_2)	1.50	0.83	0.17	1.14

TABLE II. Effect of ionophores on ATP-$^{32}P_i$ exchange reaction.

Additions	Relative activity (%)	
	ETP	Lysolecithin treated ETP (S_1)
Experiment A		
None	(100)	(100)
Val (2 μg)	77	86
Nig (2 μg)	75	72
Mon (2 μg)	73	69
Val (2 μg) + Nig (2 μg)	2	74
Val (2 μg) + Mon (2 μg)	7	78
NH_4Cl (3 μmoles)	88	106
Val (2 μg) + NH_4Cl (3 μmoles)	29	92
Experiment B		
None	(100)	(100)
Val (5 μg)	86	100
NH_4Cl (6 μmoles)	84	90
Val (5 μg) + NH_4Cl (6 μmoles)	2	50

Abbreviations: Val = Valinomycin, Nig = Nigericin, Mon = Monensin A.

measured ATP-$^{32}P_i$ exchange activity as an indicator of the energy transducing capability of lysolecithin treated ETP. The lysolecithin treated ETP (S_1) had about 30 mμmoles/min·mg of ATP-$^{32}P_i$ exchange activity (approximately 30% of the activity of untreated ETP)[1]. This activity was sensitive to the uncouplers, carbonyl cyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol, and to Oligomycin, although the sensitivity was some 3 to 5-fold lower than that found with untreated ETP. Interestingly, with lysolecithin treated ETP (S_1) there was a complete absence of synergistic inhibition of the exchange reaction by either Valinomycin plus Nigericin or Valinomycin plus Monensin A in the presence of K^+. The exchange reaction catalyzed by S_1 was susceptible to uncoupling by Valinomycin plus NH_4Cl. The effect of ionophores on ATP-$^{32}P_i$ exchange reaction is summarized in Table II.

Since the synergistic action of the ionophores in the presence of K^+ in uncoupling energy transduction by ETP is probably due to the destruction of the proton and electrochemical gradient across the closed vesicular structure by means of K^+ ion cycling (21), the observed lack of synergistic inhibition on the exchange reaction catalyzed by the lysolecithin treated ETP suggests that such gradients may not be essential for the exchange reaction. The synergistic uncoupling by Valinomycin plus NH_4Cl does not necessarily constitute evidence for the presence of closed vesicular structure, as NH_3 (a lipid soluble weak base) in the presence of Valinomycin which renders the protonated form, NH_4^+, lipid soluble, may act as a classical uncoupler like octylamine (22). The absence of synergistic uncoupling by the ionophores in the presence of K^+ together with the results of electron microscopic studies of the ultrathin sections of the lysolecithin treated ETP suggests that the closed vesicular structure of mitochondrial inner membrane may not be essential for ATP-$^{32}P_i$ exchange reaction. Finally, the recovery in the R_2 fraction of membrane frag-

[1]The exchange activity was measured with S_1, since some aggregation of the membrane fragments were noted upon centrifugation of S_1 at 105,000 x g for 3 hours. The exchange activity in S_1 was recovered in R_2 and not in S_2.

ments with headpieces, ATP-$^{32}P_i$ exchange activity and cytochromes, is indicative of the existence of functional domains of intrinsic proteins in the intact mitochondrial inner membrane (2).

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