ENERGY COUPLING IN LYSOLECITHIN-TREATED SUBMITOCHONDRIAL PARTICLES

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SUMMARY Lysolecithin, at a level of 1.2 mg per mg protein, completely suppressed active transport of K^+ and energized H^+ uptake in ETP_H, as well as eliminating vesicular profiles as determined by electron microscopic examination of positively stained ultrathin sections. Nonetheless, lysolecithin-treated ETP_H showed essentially complete retention of two coupled functions, namely, uncoupler-stimulated ATPase activity and uncoupler-stimulated NADH oxidase activity and partial retention of ATP-P_i exchange activity and phosphorylation coupled to $NADH_2$ oxidation. These results indicate that the capacity to generate a H^+ gradient or a K^+ gradient is not a prerequisite for energy coupling and that the basic postulate of the chemiosmotic hypothesis (1) is therefore invalid.

INTRODUCTION

In previous communications from this laboratory, we have reported that lysolecithin treatment of ETP[1] generated non-vesicular fragments of the inner membrane that retained coupling capability (2,3). The major limitation in our earlier studies was the partial character of the retention of coupling. If the capacity to generate an ion gradient is not essential for energy coupling, it should be possible to find conditions in which retention of coupling is essentially complete in

ABBREVIATIONS

m-ClCCP = carbonylcyanide *m*-chlorophenylhydrazone

FCCP = carbonylcyanide *p*-trifluoromethoxyphenylhydrazone

TTFB = 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

FOOTNOTE 1 Submitochondrial particles prepared by sonication of heavy beef heart mitochondria in a medium 0.25 M in sucrose and 10 mM in Tris-HCl, pH 7.8.

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such treated particles. The present communication is a report of our studies with lysolecithin-treated ETP_H (rather than ETP) in which coupling was examined under conditions in which the capacity for generating a H^+ and a K^+ gradient is completely eliminated.

MATERIALS AND METHODS

Heavy beef heart mitochondria were prepared according to the method of Hatefi and Lester (4), and electron transport particles (ETP_H) were prepared essentially according to the method of Hansen and Smith (5). ETP_H (protein concentration 20-40 mg/ml) in a medium 0.25 M in sucrose, 10 mM in Tris-HCl, pH 7.8, 1 mM in ATP, 1 mM in potassium succinate, and 1 mM in $MgCl_2$, was stored frozen at -20°. Lysolecithin treatment was carried out at 0° for 30 minutes at a protein concentration of 10-15 mg/ml. Lysolecithin dissolved in a medium 0.25 M in sucrose and 10 mM in Tris-HCl, pH 7.8 was used in most experiments. For energized H^+ uptake measurement, lysolecithin was dissolved in a medium 0.25 M in sucrose and 1 mM in Tris-HCl (pH 7.8) and the pH was adjusted to 7.1 with KOH to minimize the amount of buffer introduced with the sample into the reaction mixture.

ATPase activity was measured essentially according to the method described by Tzagoloff *et al.* (6) except that the reaction mixture was 0.25 M in sucrose, 20 mM in Tris-HCl, pH 8.5, 10 mM in ATP, and 1 mM in $MgCl_2$ and contained 0.2 mg/ml of particles. NADH oxidase activity was determined at 30° using a Beckman Oxygen Analyzer in a reaction mixture (4 ml) which was 0.25 M in sucrose, 10 mM in Tris-HCl, pH 7.4, 10 mM in KCl, 1 mM in NADH and contained 0.5 mg particles/ml. ATP-P_i exchange activity was measured as described earlier (2). Oxidative phosphorylation was measured as described previously (3), except that the NADH concentration was 1 mM. Cytochrome oxidase activity was measured polarographically at 30° in a medium 0.25 M in sucrose, 10 mM in Tris-HCl, pH 7.4, 10 mM in KCl, 20 mM in potassium ascorbate, and 10 μM in cytochrome *c*. NADH dehydrogenase activity was measured essentially according to the method of King and Howard (7) with potassium ferricyanide as the electron acceptor. Energized H^+ uptake was measured essentially according to the method described by Southard *et al.* (8). Electron micrographs of ultrathin sections were obtained according to the method described previously (2). Protein concentration was determined by the Biuret method (9), using bovine serum albumin as the standard.

Lysolecithin from egg lecithin (Sigma, Grade I) was routinely used. FCCP was obtained from Pierce, and durohydroquinone from the K & K Laboratories. Nigericin and TTFB were gifts of Dr. Henry A. Lardy and Dr. Brian Beechey respectively.

RESULTS

Figure 1 shows a representative electron micrograph of an ultrathin section of ETP_H and of $lysoETP_H$ respectively. $LysoETP_H$ was generated by exposing ETP_H to 1.2 mg of lysolecithin per mg protein for 30 minutes at 0°. The loss of vesicular profiles in the electron micrograph of $lysoETP_H$ is complete, indicating that $lysoETP_H$

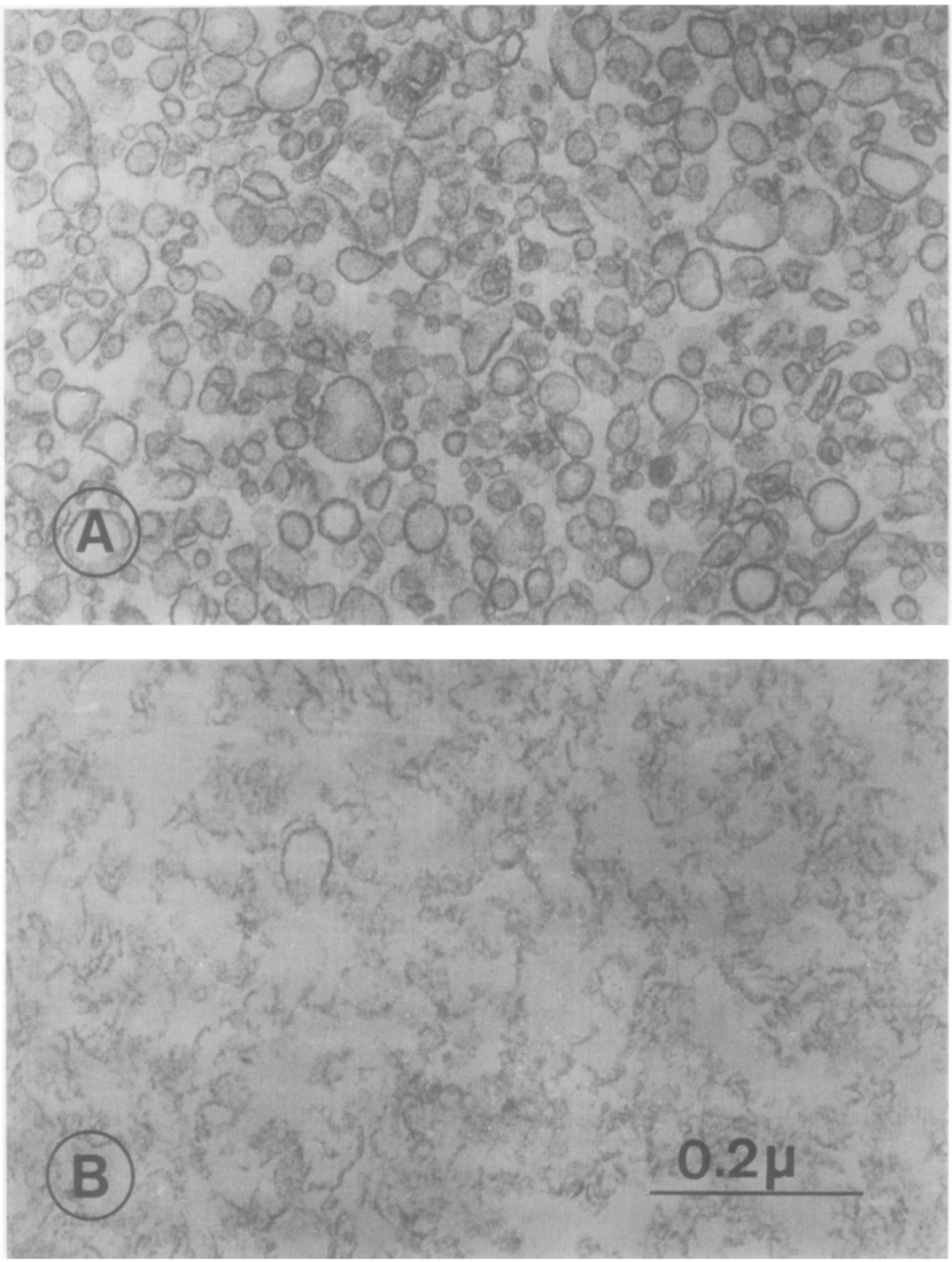

FIGURE 1 Electron micrographs of ultrathin section of: (A) ETP_H and (B) $lysoETP_H$. Magnification: x 51,000.

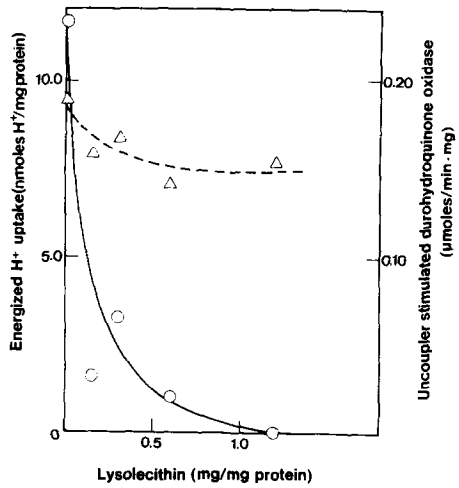

FIGURE 2 Effect of lysolecithin treatment on energized H^+ uptake of ETP_H. ETP_H was incubated with the levels of lysolecithin as indicated for 30 minutes at 0° prior to assay. The reaction mixture for energized H^+ uptake measurement (10 ml) was 0.25 M in sucrose; 1 mM in Tris-HCl, pH 7.1, 10 mM in KNO_3, 1 mM in durohydroquinone, and contained 12.5 μg catalase/ml; and 1.8 mg particles/ml. Energization of particles was effected by generation of O_2 in the anaerobic reaction mixture by the addition of 2.6 μmoles of H_2O_2. Energized H^+ uptake and the corresponding rate of durohydroquinone oxidation (in the absence of FCCP) were recorded simultaneously, and the rate of durohydroquinone oxidation in the presence of FCCP (6 μM) was determined separately. Uncoupler-stimulated durohydroquinone oxidase activity was obtained by subtracting the rate in the absence of FCCP from the rate in the presence of FCCP. Durohydroquinone oxidase activity (in the absence of FCCP) of ETP_H treated with 1.2 mg of lysolecithin per mg protein was 52 nmoles per min per mg (24% of the activity of untreated ETP_H).
O ——— O - Energized H^+ uptake
Δ ——— Δ - Uncoupler-stimulated durohydroquinone oxidase activity

is indistinguishable from lysoETP (3) in respect to the complete absence of electron microscopically detectable closed vesicular structure.

Energized H^+ uptake was inhibited by lysolecithin treatment of ETP_H, and the H^+ uptake was completely suppressed by lysolecithin at a level of 1.2 mg per mg protein (Figure 2). Figure 2 also shows that the loss of energized H^+ uptake was not due to uncoupling by lysolecithin, as FCCP still stimulated durohydroquinone oxidase

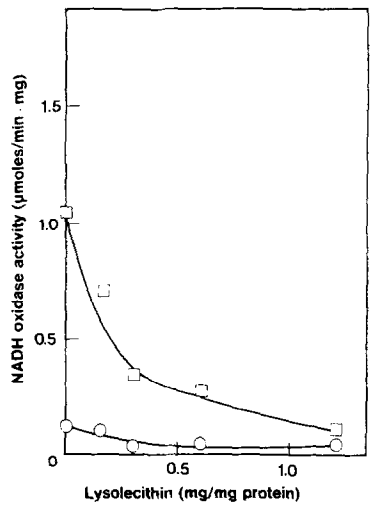

FIGURE 3 Effect of lysolecithin treatment on the stimulation of NADH oxidase activity of ETP_H by ionophores.
○ ——— ○ - no addition
□ ——— □ - valinomycin (1 μg/ml) + nigericin (1 μg/ml)

activity of ETP_H treated with 1.2 mg of lysolecithin per mg protein. A similar result was obtained when energized H^+ uptake measurement was carried out in a valinomycin-containing medium with KCl (50 mM) in place of KNO_3.

The combination of valinomycin and nigericin in the presence of K^+ abolishes both H^+ and K^+ gradient formation (10); the release of respiratory control by the synergistic action of the ionophores constitutes evidence for the presence of a H^+ and K^+ gradient. Figure 3 shows that lysolecithin treatment eliminates the stimulation of NADH oxidase activity of ETP_H by the ionophores, indicating that active transport of K^+ was abolished by lysolecithin and that neither a H^+ nor a K^+ gradient was generated during energization of $lysoETP_H$. The alternative possibility, namely, that H^+ and K^+ gradients are generated in $lysoETP_H$, but nigericin fails to abolish the gradients, seems unlikely in view of the results of energized H^+ uptake measurements described above.

TABLE I. Effect of ionophores on ATP-P_i exchange and oxidative phosphorylation

Particles	Addition	ATP-P_i exchange (nmoles/min.mg)	Oxidative Phosphorylation: Rate of P_i esterification (nmoles/min.mg)	Oxidative Phosphorylation: P/O
ETP_H	None	216	540	1.45
	Val	190	325	1.42
	Nig	169	547	1.43
	Val + Nig	0	109	0.18
$lysoETP_H$	None	50	48	1.09
	Val	48	42	0.88
	Nig	22	50	1.08
	Val + Nig	32	40	0.85

$LysoETP_H$ = ETP_H treated with 1.2 mg of lysolecithin per mg protein for 30 minutes at 0°.

Val = Valinomycin (2.5 μg/mg protein); Nig = Nigericin (0.5 μg/mg protein).

Oxidative phosphorylation was measured with NADH (1 mM) as the substrate.

There is yet another way of demonstrating the generation of a H^+ and K^+ gradient in ETP_H. The combination of valinomycin and nigericin can suppress ATP-P_i exchange or oxidative phosphorylation in ETP_H by inducing cyclic transport of K^+. Table I shows that ETP_H, but not $lysoETP_H$, shows the synergistic inhibition of ATP-P_i exchange and oxidative phosphorylation by the combination of the ionophores.

As can be seen in Figure 4, uncoupler-stimulated ATPase activity was essentially completely retained in $lysoETP_H$ with reference to the same activity in ETP_H. The uncoupler used was TTFB, but similar results were obtained with FCCP. Since the concentration of uncoupler required for maximal stimulation of ATPase activity was not the same for $lysoETP_H$ and for ETP_H respectively, the comparison should be

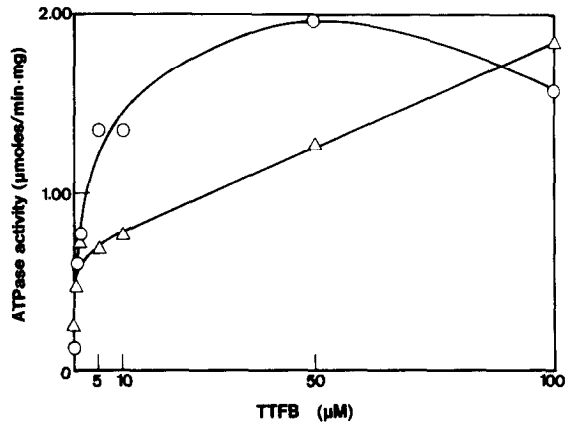

FIGURE 4 TTFB stimulation of ATPase activity of ETP_H and $lysoETP_H$.
O ——— O - ETP_H
Δ ——— Δ - $lysoETP_H$

made of the maximal uncoupler response with either particle, regardless of the exact uncoupler concentration.

Figure 5 shows the effect of varying concentrations of three different uncouplers on the NADH oxidase activity of ETP_H and $lysoETP_H$ respectively. The uncoupler-stimulated NADH oxidase activity (the difference between the activity in the presence and in the absence of an uncoupler) of $lysoETP_H$ was about 50% of that found with ETP_H regardless of the uncoupler used. However, the loss of coupling in $lysoETP_H$ was not due to any factor other than the inactivation of NADH oxidase activity of ETP_H by lysolecithin treatment, since the maximum activity of $lysoETP_H$ measured in the presence of an uncoupler was also about 50% of the corresponding activity of ETP_H. The active NADH oxidase of $lysoETP_H$ is as fully coupled as that of untreated ETP_H. The dissociation of complexes accounts, at least in part, for the inactivation of NADH oxidase in $lysoETP_H$, inasmuch as 20% of NADH dehydrogenase and 25% of cytochrome oxidase activities were found in the supernatant that had no NADH oxidase activity when $lysoETP_H$ was centrifuged at 105,000 x g for 30 minutes.

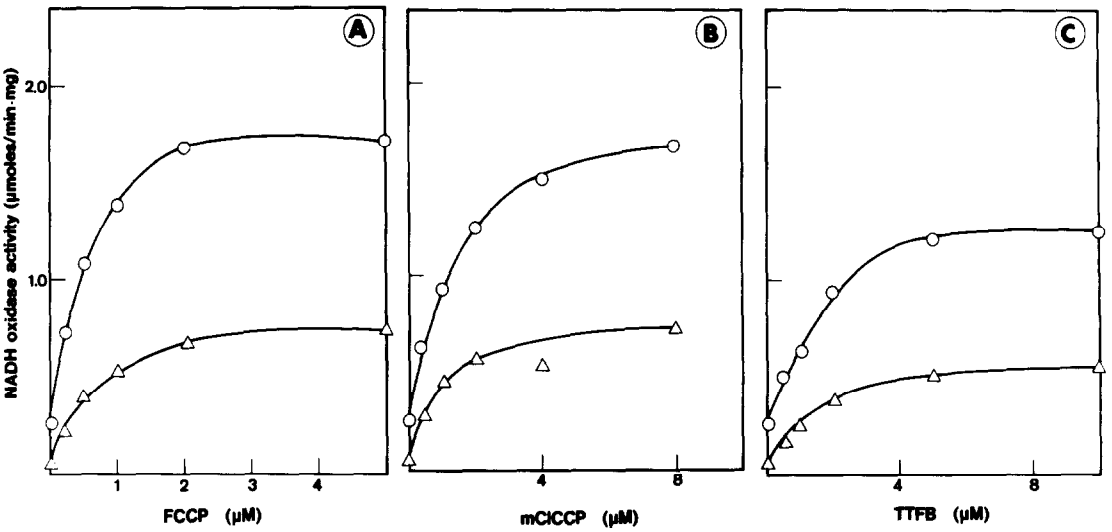

FIGURE 5 Uncoupler stimulation of NADH oxidase activity of ETP_H and $lysoETP_H$
O ——— O - ETP_H
Δ ——— Δ - $lysoETP_H$

DISCUSSION

The results of experiments described in this paper indicate that ETP_H treated with 1.2 mg of lysolecithin per mg protein for 30 minutes at 0° ($lysoETP_H$) was incapable of generating a H^+ and a K^+ gradient, most likely due to the inability of K^+ transport to take place in the absence of intact bilayer structure. Yet $lysoETP_H$ showed complete retention of two coupled functions, namely, uncoupler-stimulated ATPase activity and uncoupler-stimulated NADH oxidase activity. This precludes the possibility that energy coupling in $lysoETP_H$ may be driven by the ion gradients that are too small to be determined accurately by the methods used. $LysoETP_H$ also showed partial retention of ATP-P_i exchange activity and oxidative phosphorylation (see Table I). It cannot be a happenstance that the participation of an uncoupler is a precondition for the demonstration of full retention of coupling in $lysoETP_H$. Electron transfer (or ATP

hydrolysis) results in release of H^+ on the intracristal side and uptake of H^+ on the matrix side of the mitochondrial inner membrane (11). If the intracristal side is not exposed to the external aqueous phase or the release of H^+ is sterically hindered in lysoETP$_H$, the maximum rate of electron transfer (or ATP hydrolysis) would be obtained only in the presence of an uncoupler (a lipophilic anion that can transport H^+). We are suggesting that the mobility of protons within lysoETP$_H$ is low, and that this is the rate-limiting process in oxidative phosphorylation and in ATP-P_i exchange. It is thus not the coupling efficiency as such which is reduced in lysoETP$_H$, but rather the mobility of protons which is reduced in lysoETP$_H$. The fact that the P/O ratio (measured with NADH) for lysoETP$_H$ was not much lower than that of ETP$_H$ is fully compatible with this interpretation.

Since it would appear that the elimination of the capacity to generate a transmembrane ion gradient as in lysoETP$_H$ does not lead to the loss of coupling, it necessarily follows that a transmembrane ion gradient cannot be the driving force in energy coupling. The fundamental postulate of the chemiosmotic hypothesis (1) is that the H^+ gradient is the driving force in energy coupling. This postulate has now been shown to be invalid.

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