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Effect of Phospholipase A on the Structure and Functions of Membrane Vesicles from *Mycobacterium phlei**

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SUMMARY

The phospholipid composition of the electron transport particles and coupling factor-depleted electron transport particles of *Mycobacterium phlei* are the same, but they differ in contents. The accessibility of partially purified phospholipase A to these membrane phospholipids was found to be different. Treatment of membranes of *Mycobacterium phlei* with phospholipase A impairs the rate of oxidation as well as phosphorylation. The inhibition of phosphorylation can be reversed by washing the membranes with defatted bovine serum albumin. The reconstitution of membrane-bound coupling factor-latent ATPase activity to phospholipase A-treated depleted electron transport particles and their capacity to couple phosphorylation to oxidation of substrates remained unaffected after phospholipase A treatment. However, the pH gradient as measured by bromthymol blue was not restored after reconstitution of phospholipase A-treated depleted electron transport particles with membrane-bound coupling factor-latent ATPase. These findings show that the phosphorylation coupled to the oxidation of substrates can take place without a pronounced pH gradient in these membrane vesicles.

The dye 1-anilino-8-naphthalene sulfonic acid (ANS) exhibited low levels of energized and nonenergized fluorescence in phospholipase A-treated membranes. This decrease in the level of ANS fluorescence in phospholipase A-treated membranes was found to be directly related to the amount of phospholipids cleaved. The decrease in the energy-dependent ANS response in phospholipase A-treated electron transport particles, as compared with untreated electron transport particles, was shown to be a result of a change in the apparent K_d of the dye-membrane complex, and of a decrease in the number of irreversible or slowly reversible binding sites, with no change in the relative quantum efficiency of the dye. The decrease in ANS fluorescence in phospholipase A-treated particles appears to be due to a decrease in the hydrophobicity of the membranes.

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Oxidative phosphorylation, electron transport, and active transport of amino acids are all membrane-related phenomena that share common properties. These membrane-related functions demand a high degree of organization within the membranes. Disruption of the organization of membrane components results in a loss of coupled phosphorylation and active transport. Phospholipids constitute a major portion of the total lipids in biological membranes, and it is known that they are essential for various structural and functional roles (1). One way of obtaining information about the interaction between membrane components is to degrade one of the membrane components specifically and to follow the induced alterations in the membranes, and their reconstitution by degraded components. Various phospholipases because of their specific actions have been used as tools in inducing changes in membrane phospholipids (2–8).

Mycobacterium phlei membranes are relatively low in phospholipid contents compared with other mammalian membranes, but in view of the importance of phospholipids in various functions, an attempt is made in the present study to see the involvement of various phospholipids in the structure and function of *M. phlei* membranes. It was found that structural and functional changes induced by phospholipase A action can be correlated to the amount of phospholipids cleaved from the membrane preparations. Furthermore, a correlation was not observed between the pH gradient, as measured by bromthymol blue, and oxidative phosphorylation in phospholipase A-treated membranes.

EXPERIMENTAL PROCEDURE

Mycobacterium phlei cells ATCC 354 were grown and harvested according to the method described previously (9). The electron transport particles capable of coupled phosphorylation were prepared by sonic disruption of washed cells (10).

Phospholipase A Treatment of Membrane Vesicles from M. phlei—Phospholipase A, from *Crotalus terr. terr.* (Calbiochem), was purified on Sephadex G-100 and did not exhibit protease activity (11). The electron transport particles were exposed to phospholipase A (4 μg of phospholipase A/mg of ETP[1] protein) in 0.5 M Tris-HCl (pH 7.4) and 5 mM $CaCl_2$ at 30° for 2 hours. The controls were treated identically but without the enzyme. The reaction was stopped by centrifuging the aliquots at 105,000 × *g* for 60 min, in the presence of 5 mM $MgCl_2$. The resulting pellets were washed twice and spun at the same speed. Finally the pellets were either

[1] The abbreviations used are: ETP, electron transport particles; DETP, electron transport particles depleted of membrane-bound coupling factor; BCF_4, membrane-bound coupling factor-latent ATPase activity; ANS, 1-anilino-8-naphthalene sulfonic acid; E_f, energized fluorescence; NE_f, nonenergized fluorescence.

suspended in 0.15 M KCl or deionized water and kept on ice till used.

Removal of Phospholipase A Hydrolysis Products by Defatted Bovine Serum Albumin—The removal of hydrolysis products of phospholipase A treatment was accomplished by the method of Fiehn and Hasselbach (12). Phospholipase A-treated and untreated membranes (20 mg of protein) were incubated with 2% bovine albumin[2] in 0.1 M Tris-HCl, (pH 7.4) for 10 min at 25°, and then washed three to four times in 0.1 M Tris-HCl buffer, pH 7.4, at 105,000 × *g* for 60 min. The resulting pellets were resuspended in 0.15 M KCl and stored on ice until used.

Extraction of Lipids from Membrane Vesicles—The total lipids of ETP, DETP, and phospholipase A-treated membranes were extracted according to the method of Folch *et al.* (13). Individual phospholipids and their lyso derivatives resulting from the phospholipase A action were separated on thin layer plates of Silica Gel G using different solvent systems: acetone-benzene (4:6, v/v), chloroform-methanol-water (65:25:4, v/v), and chloroform-methanol-acetic acid-water (40:13:8:0.5, v/v). The resolved phospholipids were visualized by exposure to iodine vapors and were identified by comparison with authentic standard phospholipids. The phospholipids identified were scraped from the plate and their phosphorus contents were determined according to Wagner *et al.* (14).

Preparation of Liposomes—The total lipid extracted from ETP was fractionated into neutral lipids and phospholipids by thin layer chromatography-Silica Gel G using a solvent system containing acetone-benzene (4:6, v/v). The phospholipid band was scraped from the plate and extracted thoroughly with chloroform-methanol (2:1, v/v), and the eluates were evaporated under N_2 atmosphere. Phospholipid liposomes were prepared by sonicating the phospholipid (5 mg) in 10 ml of 0.15 M KCl for 15 min using a 10-Kc Raytheon sonicator. The dispersed suspension was centrifuged at 105,000 × *g* for 60 min, and the supernatant was used as substrate for phospholipase A action. The liposome preparation thus obtained was chromatographically pure as detected by thin layer chromatography (15).

Action of Phospholipase A on Liposomes—Incubation of liposomes with phospholipase A was carried out as described for ETP, except that the incubation was for 1 hour. The reaction was terminated by the addition of chloroform-methanol (2:1, v/v), and lipids were extracted and analyzed as described above.

Preparation of Depleted Electron Transport Particles—The membrane-bound coupling factor-latent ATPase was removed from the ETP by washing the ETP two times with water in the absence of Mg^{2+} ions or by sucrose density centrifugation according to the method of Higashi *et al.* (16). The resulting DETP were capable of oxidation but were unable to couple phosphorylation to substrate oxidation (16).

Preparation of Reconstituted ETP—DETP (8.0 mg of protein) was incubated with membrane-bound coupling factor-latent ATPase activity (4.0 mg of protein) in the presence of 10 mM $MgCl_2$ at 30° for 15 min. Centrifugation at 144,000 × *g* for 30 min yielded reconstituted ETP. The reconstituted ETP were suspended in water before use.

Heat Treatment of ETP and DETP—ETP and DETP preparations, suspended in water (15 mg of protein/ml), were heated for 10 min at 50° (17) and then were allowed to cool slowly and kept on ice until used. Heat treatment of ETP has been shown to result in the increased level of phosphorylation and energized ANS fluorescence (18, 19).

Measurement of Oxidation and Phosphorylation—Oxygen uptake was measured by conventional manometric technique with a Gilson differential respirometer at 30°. Following termination of the reaction with 10% trichloroacetic acid, the inorganic orthophosphate was determined by the method of Fiske and SubbaRow (20).

Measurement of Latent ATPase Activity—Latent ATPase activity was determined after trypsin treatment of the ETP or phospholipase A-treated ETP as described earlier (21). The dual wavelength spectrophotometer (American Instrument Co.) was used to measure the steady state levels and rate of reduction of cytochromes. The protein was estimated according to the procedure of Lowry *et al.* (22) after trichloroacetic acid precipitation.

Measurement of ANS Fluorescence—Changes in ANS fluorescence were measured with a Baird-Atomic Fluorispec SF-100 fluorimeter maintained at 25° (18). The excitation wavelength was 368 nm and emission was measured at 480 nm with succinate as an electron donor. NE_f and E_f were used to designate the fluorescence of ANS without substrate and with the substrate, respectively (23).

Dye Binding Determination—ANS binding in phospholipase A-treated and untreated ETP was measured, as described earlier (19). The concentration of protein used in the experiment was low enough to keep the membrane system in the oxidized state during the experiment. The amount of bound and unbound dye in the pellet and supernatant, respectively, was determined in the presence of saturating amount of bovine albumin as reported by Azzi *et al.* (24). The number of binding sites in phospholipase A-treated and untreated ETP, under energized and nonenergized conditions, were obtained by using Scatchard plots (25).

The apparent dissociation constant (K_d) of ETP-ANS complex and phospholipase A-treated-ANS complex, under energized and nonenergized conditions, was determined according to the method of Cheung and Morales (26). The relative quantum efficiencies were determined by plotting a double reciprocal graph of fluorescence *versus* protein concentration (27).

Sulfhydryl Group Determination—The sulfhydryl groups were determined by the method of Ellman using 5,5′-dithiobis(2-nitrobenzoic acid) (28).

Internal and External pH Measurements—The internal pH response of bromthymol blue in treated and untreated membranes were measured as described earlier using a Aminco-Bowman Dual wavelength spectrophotometer (29). The external pH measurements were carried out by using an external electrode. The electrode was inserted carefully in the cuvette so as not to obstruct the light path. Oxygen uptake was measured simultaneously using a platinum vibrating electrode.

RESULTS

Phospholipid Composition of ETP and DETP Membranes—The chemical composition of phospholipids of ETP and DETP membranes is essentially similar but the individual contents are different (Table I). The three major membrane phospholipids present in ETP and DETP are phosphatidylethanolamine, phosphatidylinositol, and cardiolipin, and they are in the order of phosphatidylinositol > phosphatidylethanolamine > cardiolipin. The total phospholipid contents of ETP and DETP membrane are 4.8 and 5.8% of the total lipid, respectively (Table I). About 8 to 16% of the total phospholipid could not be identified and was designated as uncharacterized phospholipids (Table I). The removal of membrane-bound coupling factor-latent ATPase from ETP membranes by sucrose density centrifugation (16) is accompanied by the loss of some lipids, which may possibly explain the observed difference in phospholipid contents between ETP and DETP membranes (Table I).

Action of Phospholipase A on Liposomes—In order to study the specificity of phospholipase A from *Crotalus terr. terr.* venom

TABLE I

Phospholipid composition of ETP and DETP membrane vesicles

Preparation of DETP membrane vesicles was done on sucrose density centrifugation (16). The total lipids were extracted and analyzed from ETP and DETP membranes as described under "Experimental Procedure."

Membranes	Total phospholipid	Individual phospholipids			
		Phosphatidylethanolamine	Cardiolipin	Phosphatidylinositol	Uncharacterized lipid
	% total lipids	*% total phospholipids*			
ETP	4.8	29.4	15.8	47.0	7.8
DETP	5.8	28.8	24.8	30.0	16.4

[2] Bovine albumin is defatted bovine serum albumin.

towards various phospholipids from *Mycobacterium phlei* membrane vesicles, the liposomes of total phospholipids were prepared as described under "Experimental Procedure." The phospholipid liposomes were incubated with the different concentration of phospholipase A (2 to 8 μg of phospholipase A/mg of phospholipid liposomes). It was observed that both the phosphatidylethanolamine and cardiolipin were hydrolyzed by the enzyme, but the contents of phosphatidylinositol remained unchanged (Table II). There was no noticeable difference in the specificity of the enzyme for phosphatidylethanolamine and cardiolipin (4 μg of phospholipase A/mg of phospholipid). However, phosphatidylethanolamine was 95% hydrolyzed at low concentration of phospholipase A (2 μg of phospholipase A/mg of phospholipid) compared with 82% degradation of cardiolipin (Table II).

Hydrolysis of ETP Membrane Phospholipids by Phospholipase A—When ETP were incubated with phospholipase A (4 μg/mg of membrane protein), only 50% of the total membrane phosphatidylethanolamine was hydrolyzed within 2 hours, and further degradation was not observed after prolonged incubation with phospholipase A (Fig. 1). Under the same conditions (2 hours), only 5% of the total cardiolipin was cleaved by the enzyme, and there was no change in phosphatidylinositol content. No further degradation of these ETP membrane phospholipids was achieved even at higher concentration (6 to 8 μg) of phospholipase A (data not shown). The limited degradation of phosphatidylethanolamine and cardiolipin was probably because of the fact that the enzyme had no more access to the membrane phospholipids, or after phospholipase A treatment reorientation of remaining phospholipids in the membrane may have taken place, making more of the phospholipids inaccessible to phospholipase A action.

TABLE II

Hydrolysis of liposomes by phospholipase A

Total phospholipid liposomes from *Mycobacterium phlei* membrane phospholipids (5 to 10 mg of phospholipid) were incubated with the indicated amount of phospholipase A at 30° for 60 min in the presence of 25 mM Tris-HCl (pH 7.4) and 5 mM $CaCl_2$. The amount of individual phospholipid cleaved was determined by quantitative thin layer chromatography of the lipids in the digestion mixture.

Concentration of phospholipase A/mg of phospholipid	Total individual phospholipid hydrolyzed		
	Phosphatidylethanolamine	Cardiolipin	Phosphatidylinositol
	%		
0.002 mg	95	82	0
0.004 mg	100	100	0

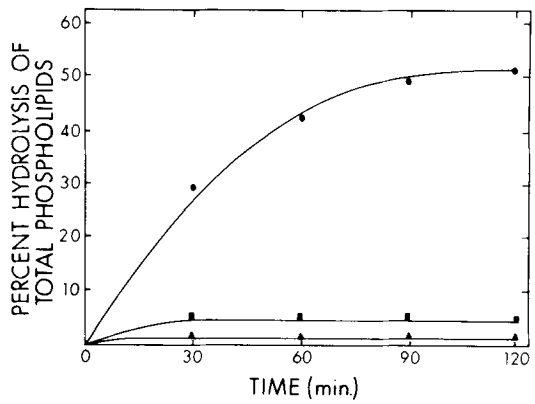

FIG. 1. Hydrolysis of total phospholipids of *Mycobacterium phlei* ETP membrane vesicles by phospholipase A. Suspensions of ETP membrane vesicles (50 mg of protein) were incubated with 0.2 mg of phospholipase A in the presence of 25 mM Tris-HCl (pH 7.4) and 5 mM $CaCl_2$. ●——●, phosphatidyl ethanolamine; ■——■, cardiolipin; ▲——▲, phosphatidylinositol. At the time indicated, aliquots were withdrawn, and the lipids were extracted and analyzed as described under "Experimental Procedure."

Hydrolysis of DETP Membrane Phospholipids by Phospholipase A—The negatively stained electron micrographs of ETP membranes exhibit repeating knoblike structures. When membrane-bound coupling factor-latent ATPase is removed from ETP membranes by washing the membranes with water in the absence of Mg^{2+} ions (16), the electron micrographs revealed the absence of repeating units (30). When such membranes (DETP), which are devoid of membrane-bound coupling factor, were treated with the same concentration of phospholipase A (4 μg/mg of membrane protein) for the same time (2 hours) as ETP, a noticeable amount of cardiolipin (46%) was cleaved along with 66% phosphatidylethanolamine (Fig. 2). A greater amount of cardiolipin and phosphatidylethanolamine is cleaved from DETP when compared to ETP membranes by phospholipase A treatment (Figs. 1 and 2). It is possible that the removal of the membrane-bound coupling factor exposes certain new areas on the membrane that are otherwise inaccessible to phospholipase A in ETP membranes.

Effect of Phospholipase A on the Respiratory Components—Phospholipase A is known to solubilize many respiratory enzymes from mammalian mitochondrial membranes (31–34). However, phospholipase A treatment of ETP or DETP membrane vesicles of *M. phlei* do not seem to affect the enzymatic reduction or contents of cytochrome *b*, *c*, and $a + a_3$ (data not shown). Furthermore, there was no detectable release of respiratory enzymes upon phospholipase A action.

Effect of Phospholipase A on Sulfhydryl Content of Proteins of ETP and DETP Membranes—The total sulfhydryl groups of proteins were determined before and after phospholipase A treatment using Ellman's reagent (28). It was observed that there was no increase in the free 5,5′-dithiobis(2-nitrobenzoic acid)-reactive sulfhydryl groups after phospholipase A treatment of either ETP or DETP membranes (Table III). The total sulfhydryl group contents were increased following denaturation of

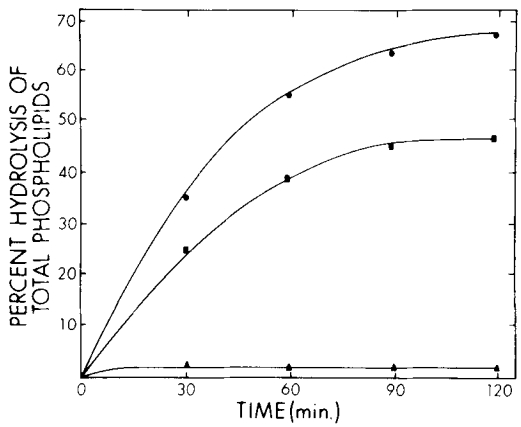

FIG. 2. Hydrolysis of total phospholipids of *Mycobacterium phlei* DETP membrane vesicles by phospholipase A. Experimental conditions were the same as described for Fig. 1, except that DETP membrane vesicles were incubated with phospholipase A. ●——●, phosphatidylethanolamine; ■——■, cardiolipin; ▲——▲, phosphatidylinositol.

membranes by trichloroacetic acid but there was no difference in the sulfhydryl contents between phospholipase A-treated and untreated membranes.

Effect of Phospholipase A on Membrane-bound Latent ATPase Activity of ETP—There was about 45% inhibition of membrane-bound latent ATPase activity after phospholipase A treatment of ETP membranes. This inhibition was due to the presence of free fatty acids released from phospholipase A action, as almost complete restoration of activity was observed when fatty acids were removed following bovine albumin treatment (Table IV). This restoration of activity would indicate that the inhibition observed in membrane-bound latent ATPase activity following phospholipase A treatment is not due to the structural alteration of coupling factor-latent ATPase.

Reconstitution of Membrane-bound Coupling Factor-latent ATPase to DETP Membranes—The effect of phospholipase A treatment upon reconstitution of DETP with membrane-bound coupling factor-latent ATPase was examined. When the same amount of latent ATPase activity was incubated with DETP or phospholipase A-treated DETP, there was less binding of latent ATPase activity (20%) with the phospholipase A-treated membranes. However, complete restoration of latent ATPase activity was observed when free fatty acids were removed from the treated membranes (Table V). Although the removal of membrane-bound coupling factor-latent ATPase did expose certain new areas that were relatively rich in cardiolipin, its hydrolysis did not affect the restoration of latent ATPase activity.

Effect of Phospholipase A Treatment on Oxidation and Phosphorylation in ETP Membranes—When ETP membrane preparations were treated with phospholipase A, a low phosphorous to oxygen ratio was obtained with respect to time of incubation. The lowering in phosphorous to oxygen ratio leveled off after 90 min. Both oxidation and level of phosphorylation were inhibited after phospholipase A treatment. However, washing the phospholipase A-treated membranes with bovine albumin partially restored the level of oxidation and phosphorylation (Table VI). Therefore, the inhibition of oxidation and phosphorylation was due to the accumulation of free fatty acids resulting from phospholipase A action of ETP membrane phospholipids. In mammalian mitochondrial system, Racker and his co-workers (35) have shown also that phospholipase A treatment impairs the rate of oxidation and phosphorylation, which could be prevented to a considerable extent by the presence of bovine albumin.

Relationship of Oxidative Phosphorylation to Proton Gradient in Phospholipase A-treated Membranes—The changes in internal pH of phospholipase A-treated ETP membranes were monitored by the response of the cationic dye, bromthymol blue. The dye has been shown to bind to the inner portions of the membrane in mitochondria, submitochondrial particles, and *M. phlei* mem-

TABLE III

Sulfhydryl content of Mycobacterium phlei membranes before and after phospholipase A treatment

Membranes either untreated or denatured with trichloroacetic acid (10 mg of protein) were stirred with 2.5 ml of water, 2 ml of 0.1 M phosphate buffer (pH 8.0), and 0.5 ml of a solution of 5,5′-dithiobis(2-nitrobenzoic acid) containing 39.6 mg of the reagent in 10 ml of 0.1 M phosphate buffer (pH 7.0). After 5 min, the samples were centrifuged and the intensity of the yellow color in the supernatant solution was measured spectrophotometrically at 465 nm. For direct determinations, treatment with trichloroacetic acid was omitted.

Membrane preparations	Sulfhydryl content	
	Direct	Trichloroacetic acid treatment[a]
		nmol/mg protein
ETP	3.5	19.8
Phospholipase A-treated ETP	3.5	20.0
DETP	3.6	20.2
Phospholipase A-treated DETP	3.6	19.5

[a] Protein sedimented following trichloroacetic acid treatment of different membranes.

TABLE IV

Effect of phospholipase A on membrane-bound latent ATPase activity in ETP membranes

ETP membranes (30 mg of protein) were incubated with phospholipase A for indicated amount of time as described under "Experimental Procedure.)) For the assay of latent ATPase activity, the reaction mixture consisted of trypsin-treated phospholipase A-treated ETP (3 mg of protein), 50 μmol of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-KOH buffer (pH 7.4), 3.0 μmol of $MgCl_2$ and water to a final volume of 1.0 ml. The reaction was started by the addition of 10 μmol of ATP and was run at 30° for 15 min. In case of bovine albumin treatment, both the membrane preparations were washed with bovine albumin before trypsin treatment, followed by latent ATPase activity determination. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and release of inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow (20).

Time of incubation with phospholipase A	Phospholipase A-treated ETP	Bovine albumin-washed phospholipase A-treated ETP
min	*nmol P_i released/min/mg protein*	
0	253	250
30	250	250
60	200	230
90	153	228
120	141	234

TABLE V

Effect of phospholipase A on reconstitution of latent ATPase to DETP membranes

DETP membrane vesicles (devoid of latent ATPase coupling factor activities) were treated with phospholipase A (4 μg/mg of DETP membrane protein) as described under "Experimental Procedure." A control was run under identical conditions without phospholipase A. Both phospholipase A-treated and untreated DETP membranes were incubated with the same amount of latent ATPase activity (240 nmol of P_i released/mg of protein/min), in the presence of 10 mM $MgCl_2$ at 30° for 15 min. The incubation mixture was then centrifuged at 105,000 × *g* for 60 min and the pellets were designated as treated and untreated reconstituted ETP membranes. For the removal of free fatty acids both phospholipase A-treated and untreated membranes were washed with bovine albumin before incubating with latent ATPase activity. The latent ATPase activity in these membrane preparations was assayed as described (21).

Membrane preparations of reconstituted ETP	Before bovine albumin wash	After bovine albumin wash
	nmol P_i released/min/mg protein	
Untreated	237	200
Treated	192	204

TABLE VI

Effect of phospholipase A on oxidation and phosphorylation in electron transport particles of Mycobacterium phlei

ETP membranes were incubated with phospholipase A for indicated time as described under "Experimental Procedure." For oxidative phosphorylation, the reaction mixture consisted of phospholipase A-treated ETP or phospholipase A-treated and bovine albumin-washed ETP (2 mg of protein), 100 μmol of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-KOH buffer, pH 7.4, 50 μmol of glucose, 15 μmol of orthophosphate, 30 μmol of $MgCl_2$, 3.0 mg of hexokinase, 2.5 μmol of ADP, 25 μmol of potassium fluoride, 25 μmol of hydrazine, 0.5 mg of alcohol dehydrogenase, and 1.0 μmol of NAD in a final volume of 2.0 ml. The oxygen uptake was measured for 15 min at 30° using Gilson differential respirometer, and the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. Following centrifugation, an aliquot of the supernatant was used for phosphate determination by the method of Fiske and SubbaRow (20).

Time of incubation with phospholipase A	Phospholipase A-treated ETP			Phospholipase A-treated ETP and bovine albumin-washed		
	O_2	P_i	P/O	O_2	P_i	P/O
min	*μmol*			*μmol*		
0	6.5	6.0	0.92	6.5	6.0	0.92
30	5.0	4.6	0.92	5.5	5.0	0.90
60	4.5	3.5	0.77	5.2	4.8	0.92
90	4.5	3.2	0.71	5.0	4.2	0.84
120	4.3	3.2	0.74	5.0	4.2	0.84

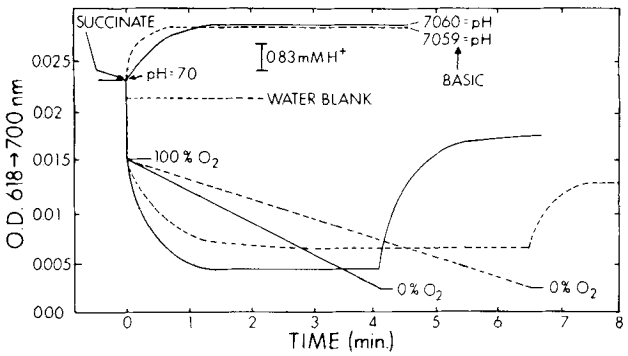

FIG. 3. Bromthymol blue responses in phospholipase A-treated and untreated ETP membranes during substrate oxidation. Reaction mixture consisted of 10 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.0), 3.3 μM bromthymol blue, ETP, or phospholipase A-treated ETP (3.4 mg of protein). The reaction was started by the addition of 17 mM sodium succinate. The external pH measurements were done as described under "Experimental Procedure." The H^+ ion scale was calibrated using a standard HCl solution. The reaction was run at 30°. The rate of substrate oxidation in both phospholipase A-treated and untreated ETP membranes was done separately in the presence of bromthymol blue under identical conditions and was superimposed on the pH measurements. ——, ETP membranes; - - -, phospholipase A-treated ETP membranes.

branes (29, 36, 37). When substrate (succinate) was added to the reaction mixture, there was a rapid decrease in absorbance at 618 nm (Fig. 3), indicating an increase in acidity within the vesicles. When the reaction reached transition to anaerobiosis, the absorbance returned to the original level. On comparison of bromthymol blue response in both ETP and phospholipase A-treated ETP membranes, it was observed that the decrease in absorbance was less in phospholipase A-treated membranes (Fig. 3), and also the time to reach transition to anaerobiosis took a

TABLE VII

Effect of phospholipase A on oxidation and phosphorylation in DETP and its relation to proton gradient as measured by bromthymol blue

Phospholipase A-treated and untreated DETP membranes (3.4 mg of protein) were incubated with latent ATPase coupling factor activity (BCF_4, 1.1 mg of protein) as described for Fig. 4. Oxidative phosphorylation and bromthymol blue response were measured as described under "Experimental Procedure."

Membrane fraction	O_2	P_i	P/O	Decrease in absorbance $A_{618\,nm} - A_{700\,nm}$ (maximum change)
	μatom	*μmol*		*ΔO.D./unit protein*
DETP	6.70	0.0	0.0	0.0028
Treated DETP[a]	1.78	0.0	0.0	0.0019
DETP + BCF_4	6.78	6.2	0.91	0.0132
Treated DETP[a] + BCF_4	4.28	4.2	0.98	0.0033

[a] Phospholipase A-treated DETP.

longer period in phospholipase A-treated membranes. The decrease in absorbance of bromthymol blue in phospholipase A-treated membrane was not due to the presence of free fatty acids, since the washing of phospholipase A-treated ETP with bovine albumin did not alter the observed bromthymol blue response (data not shown). Furthermore, it was observed that the addition of potassium oleate and lysophosphatides, at concentration approximately equivalent to fatty acid and lysophosphatides released during the phospholipase A digestion, to the untreated membrane vesicles or bovine albumin-washed phospholipase A-treated membranes did not affect the bromthymol blue response. In addition, free fatty acids were not detected by chromatographic analysis in membranes washed with bovine albumin following phospholipase A treatment. Therefore the formation of lysophosphatides and free fatty acids by phospholipase A action *per se* is not the basis for the observed decrement in bromthymol blue response.

The external pH gradient was measured in order to determine the net proton flux during oxidation. Fig. 3 shows that in the case of both untreated and phospholipase A-treated ETP membranes the external pH was the same, which became alkaline during substrate oxidation and did not change at anaerobiosis.

DETP membranes devoid of latent ATPase coupling factor activity fail to respond to bromthymol blue (8–10% as compared with ETP) with NADH or succinate as substrates (29). Following phospholipase A treatment of the DETP membranes there was a diminished response (2 to 3%) of bromthymol blue compared to untreated DETP. Furthermore, the addition of phospholipid liposomes to phospholipase A-treated DETP and ETP failed to restore the diminished bromthymol blue response. The proton gradient was restored by the addition of the coupling factor-latent ATPase to untreated DETP (29). However, following phospholipase A treatment of the depleted membranes, the proton gradient as measured by bromthymol blue was not restored on the addition of coupling factor-latent ATPase, nevertheless, restoration of oxidative phosphorylation was achieved (Table VII and Fig. 4). The proton gradient in phospholipase A-treated DETP membranes was also monitored after the removal of free fatty acids from the membranes. It was observed that the removal of free fatty acids did not alter the bromthymol blue response on the addition of coupling factor-latent ATPase activity

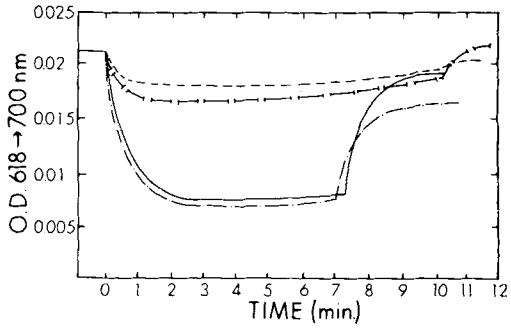

FIG. 4. Bromthymol blue response in phospholipase A-treated and untreated reconstituted ETP. Reaction mixture was the same as described for Fig. 3. Protein (3.5 mg) of either phospholipase A-treated or untreated DETP membranes (bovine albumin-washed or unwashed) were incubated with membrane-bound coupling factor-latent ATPase activity (BCF_4, 1.2 mg of protein) in the presence of 5 mM $MgCl_2$ for 15 min at 30° and then centrifuged at 105,000 × *g* for 60 min. The reconstituted pellet was checked for bromthymol blue response using succinate (17 mM) as an electron donor. ——, DETP + BCF_4; /——/, phospholipase A-DETP + BCF_4; ·——·, DETP (bovine albumin) + BCF_4; – – –, phospholipase-A DETP (bovine albumin) + BCF_4.

TABLE VIII

Effect of phospholipase A on ANS fluorescences in ETP and DETP membrane vesicles

The reaction mixture contained a final volume of 3.0 ml untreated or phospholipase A-treated membranes (0.8 mg of protein), 50 mM Tris-acetate buffer, pH 7.2, and 83.3 μM ANS. The energization of membranes was induced by the addition of 17 mM Tris-succinate (pH 7.2). Other experimental conditions are the same as described under "Experimental Procedure."

Membrane fractions	Change in fluorescence[a]			
	Untreated		Phospholipase A-treated	
	NE_f	E_f	NE_f	E_f
ETP	55	+20	45	+13
DETP	55	+20	45	+5

[a] Arbitrary units.

(Fig. 4). These results show that one can observe oxidative phosphorylation in membrane vesicles of *M. phlei* without a pronounced pH gradient.

ANS Response in Phospholipase A-treated Membranes—The decrease in bromthymol blue response in phospholipase A-treated ETP and reconstituted membranes may be due to some structural alterations in the membrane upon phospholipase A treatment. This was ascertained by using ANS as a probe, which has been widely used to monitor conformational changes in various membranes (2–5, 18, 19). ETP membranes were exposed to phospholipase A, and the fluorescence under E_f and NE_f conditions was monitored. It was observed that the level of E_f of phospholipase A-treated ETP was 65% of that observed in untreated ETP (Table VIII). The NE_f of phospholipase A-treated ETP was also slightly lowered (18.2%) compared with ETP. When DETP membranes, which are devoid of membrane-bound coupling factor, were treated with phospholipase A, it was observed that the E_f in these membranes was 25% of that observed in untreated DETP membranes, while the NE_f was only 18.2% lower (Table VIII). This significant difference in energized fluorescence in phospholipase A-treated ETP and DETP membranes appears

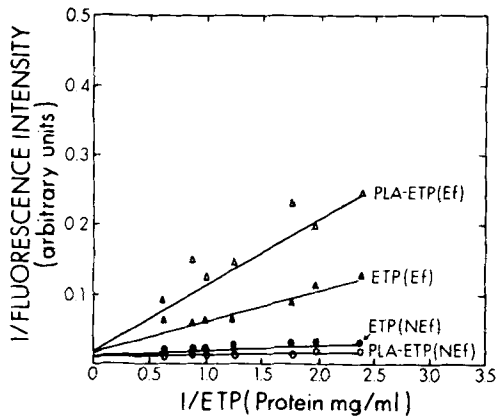

FIG. 5. ANS response under energized and nonenergized conditions at varying concentrations of ETP or phospholipase A (*PLA*)-ETP protein. Reaction mixture contained a final volume of 3 ml, 50 mM Tris-acetate, pH 7.2, 83.3 μM ANS and varying concentrations of ETP or phospholipase A-ETP protein as shown in figure. Energization was induced by the addition of 17 mM Tris-succinate, pH 7.2. The reciprocal of the fluorescence intensity was then plotted *versus* the reciprocal of the ETP or phospholipase A-ETP protein concentration. The resulting data were treated by the method of least squares and plotted as shown.

to be due to the additional cleavage of membrane phospholipids. However, in both cases the energized fluorescence was not affected by the addition of phospholipid liposomes (data not shown).

The decrease in the E_f of ANS fluorescence observed in ETP or DETP membrane vesicles after phospholipase A treatment may result from the accumulation of free fatty acids, which may cause quenching of fluorescence. Therefore, the ANS fluorescence was checked after the removal of free fatty acids from the membranes. It was found that the effect on energized fluorescence of ANS in phospholipase A-treated ETP or DETP membrane vesicles was not due to the accumulation of free fatty acids or lysophosphatides, since their removal by bovine albumin did not change the observed decrease in ANS response. Furthermore, it was observed that the addition of potassium oleate, fatty acids extracted from *M. phlei* lipids, lysophosphatides, or mixture of both at concentrations approximately equivalent to fatty acid and lysophosphatides released during the phospholipase A action, to the untreated ETP or DETP membrane vesicles of *M. phlei* affected the level of E_f only to the extent of 5%. In contrast, the decrease in the level of E_f following phospholipase A treatment of ETP or DETP membranes, was 35 and 75%, respectively. Therefore the changes in the level of E_f of ANS in phospholipase A-treated membranes does not appear to be due to the accumulation of hydrolyzed products resulting from phospholipase A action.

Binding Parameters of ANS to ETP Membrane Vesicles—The observed decrease in ANS fluorescence observed after phospholipase A treatment of ETP may be due to a decrease in the binding of dye molecules to the membrane components, a decreased affinity of the dye to the membrane, or a change in the relative quantum yield. Under both energized and nonenergized conditions, the relative quantum efficiency of ANS bound to ETP or phospholipase A-treated ETP membrane vesicles remained the same (Fig. 5).

In order to determine whether the affinity for ANS was changed following phospholipase A treatment of ETP, the apparent dissociation constant under the energized and nonenergized conditions of the dye-ETP complex was measured. The results show that under energized conditions, the K_d of the un-

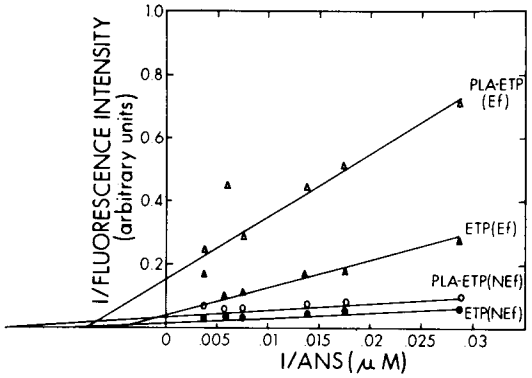

FIG. 6. ANS response at different concentrations of ANS with ETP or phospholipase A (*PLA*)-treated ETP under nonenergized and energized conditions. Reaction mixture was the same as described in Fig. 5, except that the concentration of ANS was varied as shown in the figure. The resulting data were treated by the method of least squares and plotted as shown.

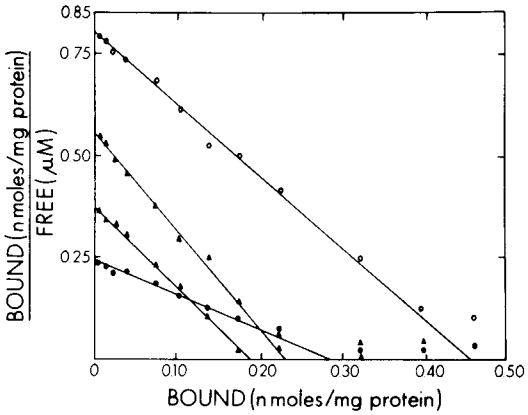

FIG. 7. Scatchard plots of ANS binding to ETP and phospholipase A-treated ETP under nonenergized and energized conditions. Reaction mixtures of 3 ml containing 50 mM Tris-acetate, pH 7.2, 3.3 mM $MgCl_2$, ETP, or phospholipase-treated ETP (1.68 mg of protein) and ANS varying from 19.29 μM to 333.2 μM were incubated at 25° for 5 min in the presence of 17 mM succinate (energized conditions) or 17 mM succinate + 2 mM KCN (nonenergized conditions). After cooling and centrifugation, ANS was determined in supernatants and pellets (19). Nonenergized conditions: △——△, ETP; ▲——▲, phospholipase A-treated ETP. Energized conditions: ○——○, ETP; ●——●, phospholipase A-treated ETP.

treated ETP was 2.2-fold greater than that of phospholipase A-treated ETP. The K_d values under nonenergized conditions of ETP were also 2-fold more than phospholipase A-treated ETP (Fig. 6).

The binding of ANS to untreated ETP and to phospholipase A-treated ETP under nonenergized and energized conditions was measured as described under "Experimental Procedure." Scatchard plots (Fig. 7) based on the measurement of the amount of ANS bound to particles at different concentrations of ANS in both the energized (succinate) and nonenergized (succinate + KCN) states revealed a difference in the binding characteristics between ETP and phospholipase A-treated ETP. In the nonenergized state, the binding capacity was 23 nmol of ANS/mg of ETP protein and 19 nmol of ANS/mg of phospholipase A-treated ETP protein. Energization with succinate as a substrate resulted in a decrease in the binding capacity of ANS for phospholipase A-treated ETP (46 nmol of ANS/mg of ETP protein and 28 nmol of ANS/mg of phospholipase A-treated ETP). However, this method measures only the binding sites that are irreversible or slowly reversible and not the rapidly dissociating sites.

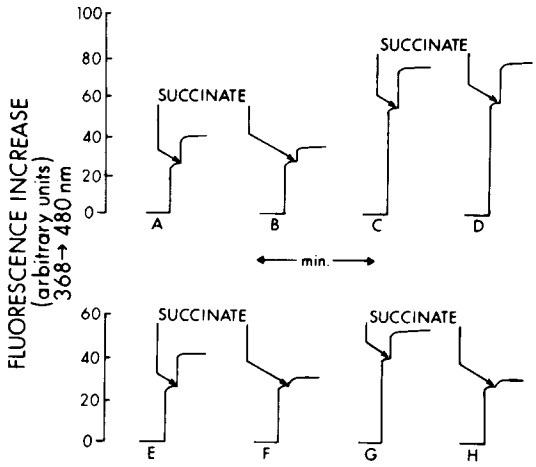

FIG. 8. Effect of phospholipase A on energized and nonenergized fluorescence of ANS after heat treatment of ETP and DETP membranes. Both phospholipase A treated and untreated ETP and DETP membranes were heated to 50° for 10 min as described earlier (17). The reaction mixture for monitoring ANS response in treated and untreated membranes was the same as described for Table VIII. *A*, ETP; *B*, phospholipase A-treated ETP; *C*, ETP heated at 50°; *D*, phospholipase A-treated ETP, heated at 50°; *E*, DETP; *F*, phospholipase A-treated DETP; *G*, DETP heated at 50°; *H*, phospholipase A-treated DETP, heated at 50°.

Effect of Heat Treatment on Phospholipase A-treated ETP and DETP Membrane Vesicles—It was previously demonstrated by Kalra *et al.* (18, 19) that heat treatment of *M. phlei* membrane vesicles at 50° for 10 min resulted in a 2- to 3-fold increase in the level of E_f and also in the increased level of phosphorylation. The effect of heat treatment in the membranes of *M. phlei* appears to be due to structural reorientation of membrane components (18, 19). It was of interest to see whether phospholipase A-treated ETP or DETP exhibited an increased level of E_f following heat treatment. Phospholipase A-treated ETP when heated to 50° for 10 min exhibited the increased level of E_f (Fig. 8, *B* and *D*), while phospholipase A-treated DETP membranes (Fig. 8, *F* and *H*) failed to exhibit an increased level of E_f upon heat treatment. This suggests that a specific orientation of phospholipids in the membranes is required for enhancement of E_f upon heat treatment, and that this orientation is lost in DETP membranes following phospholipase A treatment.

DISCUSSION

Phospholipase A treatment of ETP resulted in 50% hydrolysis of phosphatidylethanolamine and 5% hydrolysis of cardiolipin with no effect on the other phospholipids. Of particular interest was the finding that on removal of the membrane-bound coupling factor-latent ATPase, a small increase (15%) in the hydrolysis of phosphatidylethanolamine was observed; however, cardiolipin hydrolysis increased to 42% of the total amount present. The difference between the ETP and DETP membranes is that the latter do not contain membrane-bound coupling factor-latent ATPase activities that usually occupy certain areas on the surface of the ETP membranes (30). It would appear from the present studies that the phospholipase A had more access to cardiolipin in the DETP membranes. These findings were further substantiated by the fact that both the phospholipids were hydrolyzed almost at the same rate in the liposomes, indicating that the

additional hydrolysis of cardiolipin observed in the DETP membranes is not due to substrate specificity of phospholipase A.

It is well established that phospholipids play a role in respiratory functions of mitochondria (38, 39). Phospholipase A, C, and D have been shown to alter mitochondrial functions (8, 35, 39, 40), and in some instances addition of phospholipids micelles restored these altered functions (39). Exposure of ETP of *M. phlei* to phospholipase A impairs the rate of oxidation as well as phosphorylation. However, the inhibition of phosphorylation was prevented to a considerable extent by washing the phospholipase A-treated membranes with bovine albumin.

Since the removal of membrane-bound coupling factor-latent ATPase from ETP membranes enhanced the accessibility of phospholipase A to cardiolipin, it was of interest to determine the involvement of cardiolipin in the binding of coupling factor to the depleted membranes. It was observed that the increased degradation of phospholipids from phospholipase A-treated DETP did not prevent the binding of coupling factor to these membranes. Thus, the degradation of phospholipids by phospholipase A treatment does not seem to affect the sites required for binding of coupling factor-latent ATPase to DETP membranes. Furthermore, the restoration of membrane-bound latent ATPase activity of reconstituted DETP and the ability of the membranes to couple phosphorylation to oxidation remain unaffected following phospholipase A treatment.

It was shown earlier (29) that membranes that are devoid of coupling factor-latent ATPase do not exhibit proton gradient as measured by bromthymol blue. The proton gradient can be restored by the addition of the coupling factor to the DETP membranes (29). However, following phospholipase A treatment of the DETP membranes, the proton gradient was not restored following the addition of coupling factor; nevertheless, restoration of oxidative phosphorylation was achieved. The possibility that fatty acids released by phospholipase A action may affect the bromthymol blue response was excluded by washing the membranes with bovine albumin. These findings apparently indicate that phosphorylation coupled to the oxidation of substrates can take place without a pronounced pH gradient, but they do not eliminate the contribution of a membrane potential (41, 42). Moreover, it is shown by McCarty (43) that the light-induced pH gradient is not involved in photophosphorylation of subchloroplast particles.

Studies with the fluorescent dye ANS in phospholipase A-treated membrane vesicles revealed that there was a decreased level of E_f of ANS compared with the untreated membranes. This decrease in the level of E_f in phospholipase A-treated membrane-(ETP or DETP) was found to be directly related to the amount of phospholipids cleaved. It was observed in phospholipase A-treated membrane vesicles (ETP) from *M. phlei* that energization resulted in a decrease in the number of binding sites for ANS (from 46 nmol/mg of protein to 28 nmol/mg of protein), a change in the apparent K_d of the dye-ETP complex (from 210 to 95 μM), with no change in the relative quantum yield of ANS. These changes in the binding parameters of the dye-membrane complex in phospholipase A-treated membranes, compared with untreated ETP membranes under energized conditions, can be attributed to a decrease in the hydrophobicity of the membranes, caused by the removal of fatty acids from the membrane phospholipids. According to Hasselbach and Heimberg (3), the fluorescence decrement following the action of phospholipase A on sarcoplasmic reticulum, is due to the displacement of ANS from its normal binding sites by newly formed fatty acids and can be reversed by their extraction with bovine albumin. However, the decrease in ANS fluorescence observed in phospholipase A-treated membranes of *M. phlei* does not appear to be due to the accumulation of free fatty acids, since their removal by bovine albumin does not increase the E_f of ANS.

Membrane vesicles of *M. phlei* when heated to 50° for 10 min exhibited an increase in the level of phosphorylation and also showed 2- to 3-fold increase in the level of E_f. This enhancement of E_f in heat-treated membranes was attributed to the structural alteration of membranes (18, 19). Following phospholipase A treatment of the ETP, heat treatment to 50° for 10 min also exhibited an enhancement in the level of E_f and coupled phosphorylation. However, phospholipase A-treated DETP membranes did not exhibit an enhancement of E_f following heat treatment. Since more phospholipids were cleaved from DETP membranes compared with ETP, and the former failed to exhibit the enhancement of E_f upon heat treatment, it would appear that the heat activation phenomenon in *M. phlei* membranes requires a specific orientation of phospholipids in the membrane.

The over-all conformation of ETP and DETP membrane proteins does not appear to be altered, since there was no increase in 5,5′-dithiobis(2-nitrobenzoic acid)-reactive sulfhydryl groups following phospholipase A action. It is pertinent to mention that Simpkins *et al.* (44) did not observe any change in sulfhydryl residues in phospholipase C-treated enythrocyte ghost and submitochondrial particles using ESR technique. The present studies in membranes of *M. phlei* suggest that phospholipase A treatment produces gross structural changes in the lipid regions of the membrane but probably none in the protein region. It is also possible that a substantial proportion of the lipids is structurally discrete from that of the membrane protein.

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