Effect of Phospholipase A on Active Transport of Amino Acids with Membrane Vesicles of Mycobacterium phlei*

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SUMMARY

Active transport of proline remained unaffected in phospholipase A-treated electron transport particles from Mycobacterium phlei. However, the steady state level of proline was reduced 50 to 60% in phospholipase A-treated depleted electron transport particles that were devoid of membrane-bound coupling factor-latent ATPase activity. The decrease in the uptake of proline in the phospholipase A-treated depleted electron transport particles was not due to a change in the apparent $K_m$ for proline, but it was related to the amount of phospholipid cleaved from the membranes. Restoration in the level of proline transport in phospholipase A-treated depleted electron transport particles was achieved by reconstituting these vesicles with diphosphatidylglycerol and phosphatidylethanolamine liposomes. Diphosphatidylglycerol was found to be most effective in the restoration of proline uptake. In contrast to the effect of phospholipase A treatment on proline transport, similar treatment of the electron transport particles or depleted electron transport particles failed to inhibit the active transport of either glutamine or glutamic acid. Studies with phospholipase A-treated membrane vesicles confirmed earlier findings that a proton gradient is not required for active transport of amino acids.

Active transport is a characteristic of biological membranes and has been studied in bacterial and mammalian systems. Numerous studies have been carried out to delineate the relationship between transport and energy-yielding processes (1–10). Kaback and Milner (3, 4) demonstrated that transport of a wide variety of amino acids and sugars in membrane vesicles proceeds independent of oxidative phosphorylation. Klein and Boyer (5) also showed that aerobic transport of proline in intact cells of Escherichia coli proceeds under conditions where intracellular levels of ATP were low. Studies carried out by Brodie and co-workers (11–13) in membrane vesicles of Mycobacterium phlei have also shown that high energy phosphate, ATP, and high energy phosphorylated intermediates were not involved in the bioenergetics of the transport processes. Nevertheless, these studies suggested that active transport is inextricably associated with oxidation (13). However, studies in membranes of E. coli by Berger (14) indicate that the mode of energy transduction differs for different amino acids. It was shown that proline uptake required the energized membrane state, while glutamine transport was driven directly by phosphate bond energy formed by either oxidative phosphorylation or by glycolysis. These differences in energy transduction mechanisms of transport processes indicate that the active transport of different amino acid proceeds by different molecular mechanisms.

It was shown in the preceding paper (15) that oxidative phosphorylation, pH gradient, and the energy-dependent 1-anilino-8-naphthalene sulfonate response were altered in membranes that were exposed to phospholipase A. These altered functions were found to be directly related to the hydrolysis of membrane phospholipids. Since active transport of amino acid is a membrane-related phenomenon, and relatively little information is available on the effect of various phospholipases on active transport processes, it was of interest to study the involvement of various phospholipids in the active transport of amino acids in membrane vesicles of M. phlei. Kaback and Milner (3, 16) demonstrated that the uptake and efflux of $\alpha$-methyl glucoside in membranes of E. coli were affected by the hydrolysis of membrane phospholipids, particularly phosphatidylethanolamine. The degradation of phosphatidylglycerol did not affect the uptake of $\alpha$-methyl glucoside. There appears to be no documentation that impaired active transport of amino acids in membranes has been restored by the addition of phospholipids.

It is shown in this communication that the active transport of proline is impaired when membranes that are devoid of membrane-bound coupling factor-latent ATPase are subjected to phospholipase A treatment. This impaired transport of proline was found to be directly related to the amount of phospholipids cleaved from the membrane and was restored by the addition of cardiolipin and phosphatidylethanolamine liposomes. However, the active transport of glutamine and glutamic acid was not affected in phospholipase A-treated membranes.

EXPERIMENTAL PROCEDURE

Preparation of Membrane Vesicles—The growth conditions and the procedure for the preparation of electron transport particles and depleted electron transport particles from Mycobacterium phlei whole cells (ATCC 354) have been described in the preceding paper.

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Measurement of Substrate Oxidation—The rate of oxidation of various substrates by phospholipase A-treated and untreated membrane vesicles was measured polarographically at 30° using an oxygen monitor.

Assay of [U-14C]Proline Transport—The method of measuring proline transport was essentially the same as that described in earlier publications (11-13). The 3.0-ml assay system contained 50 mm potassium-N,N'-2-hydroxyethylpiperazine-N,N'-2-ethanesulfonic acid (pH 7.0), 10 mm MgCl₂, 20 mm NaCl, 25 μM l-[U-14C]-proline, and phospholipase A-treated or untreated membrane vesicles (2 mg of protein/ml). The reaction was initiated by the addition of substrate.

Preparation of Phospholipid Liposomes and Reconstitution of Phospholipase A-Treated Membrane Vesicles—Liposomes of cardiolipin and phosphatidylethanolamine were prepared, as described previously (15), for the reconstitution experiments. Phospholipase A-treated and untreated membrane vesicles were incubated with the indicated amount of phospholipid liposomes for 15 min at 37° and were referred to as the reconstituted membranes.

Kinetics of Proline Transport—The Kₘ and Vₘₐₓ for proline uptake in phospholipase A-treated and untreated vesicles were determined by the standard Lineweaver-Burk plot.

Other determinations, like internal pH gradient and protein, were made as described earlier (15).

RESULTS

Effect of Phospholipase A on Proline Transport in Electron Transport Particle Membrane Vesicles—The ETP membrane vesicles were incubated with phospholipase A as described under "Experimental Procedure," and their ability to transport proline was assayed using [U-14C]proline. As shown in Fig. 1, the active transport of proline remained unaffected in phospholipase A-treated ETP membranes with ascorbate-TPD (Fig. 1) as well as with other substrates. Furthermore, the rate of facilitated diffusion of proline remained the same in both the untreated and the phospholipase A-treated membrane vesicles. However, the rate of substrate oxidation in phospholipase A-treated ETP was 35 to 40% lower than for untreated ETP membrane vesicles (15), yet the steady state level of active transport of proline was the same. The removal of free fatty acids from phospholipase A-treated ETP membranes vesicles by defatted bovine serum albumin resulted in the restoration of the reduced oxidation; the steady state level of proline transport, however, remained unaffected.

Effect of Phospholipase A on Proline Transport in DETP Membrane Vesicles—The phospholipids of DETP membranes, which are devoid of membrane-bound coupling factor-lactate ATPase, are more accessible to phospholipase A action than are ETP membrane vesicles (15). The additional cleavage of phosphatidylethanolamine and cardiolipin from DETP membrane vesicles after phospholipase A treatment was found to influence the steady state level of proline uptake. As shown in Fig. 2, the level of proline uptake was 50 to 60% in phospholipase A-treated DETP membrane vesicles, compared with untreated DETP when ascorbate-TPD was used as substrate. With succinate, NADH, or generated NADH the level of proline uptake was 40 to 50% lower in phospholipase A-treated DETP compared with untreated DETP membranes (Fig. 2). The rate of various substrate oxidation was also found to be lower in phospholipase A-treated DETP than in untreated DETP; the impaired oxidation was restored by washing the treated membranes with bovine albumin² (Table I). Of particular interest was the finding that the restoration in oxidation was not accompanied by an increase in the uptake of proline. The possibility that the released products of phospholipase A action, fatty acids, and lysophosphatides may affect the uptake of proline in DETP membrane vesicles was excluded by the finding that the addition of approximately equivalent amounts of hydrolysis products did not affect the steady state level of proline uptake.

Kinetics of Proline Transport in Phospholipase A-Treated DETP Membranes—The decrease in proline transport observed following phospholipase A treatment of DETP membranes can be due to an altered affinity of proline for carrier protein(s) or due to a change in Vₘₐₓ. The apparent Kₘ for proline in DETP

¹ The abbreviations used are: ETP, electron transport particles; TPD, N,N',N⁴,N⁴-tetramethyl-p-phenylenediamine; DETP, electron transport particles depleted of bound coupling factor.

² Bovine albumin is defatted bovine serum albumin.
TABLE I
Rate of oxidation of various substrates in DETP and phospholipase-treated DETP membrane vesicles

<table>
<thead>
<tr>
<th>Substrates</th>
<th>DETP</th>
<th>Treated DETP</th>
<th>Bovine albumin-washed DETP</th>
<th>Bovine albumin-washed Treated DETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generated NADH</td>
<td>245</td>
<td>114</td>
<td>245</td>
<td>200</td>
</tr>
<tr>
<td>NADH</td>
<td>330</td>
<td>200</td>
<td>333</td>
<td>298</td>
</tr>
<tr>
<td>Succinate</td>
<td>212</td>
<td>152</td>
<td>215</td>
<td>175</td>
</tr>
</tbody>
</table>

*Phospholipase A-treated DETP.*

![Fig. 3](http://www.jbc.org/)

**Proline Transport in Phospholipid Reconstituted DETP Membranes**—Since the affinity of proline for carrier protein(s) remained unchanged after phospholipase A treatment, it is therefore possible that phospholipids may be required for the uptake of proline. To test this possibility, the rate of proline transport was followed in phospholipase A-treated DETP membrane vesicles reconstituted with liposomes of chromatographically pure phosphatidylethanolamine and cardiolipin. As a control, liposomes were also added to untreated DETP membranes. It was observed that the steady state level of proline uptake in DETP reconstituted with liposomes following phospholipase A treatment was 50 to 70% higher than phospholipase A-treated DETP in the absence of liposomes (Fig. 4). The optimum concentration of phosphatidylethanolamine and cardiolipin liposomes in reconstitution studies was found to be 0.75 to 0.80 pmol of phospholipid/mg of DETP membrane protein (Fig. 5). Cardiolipin liposomes were more effective (70%) than phosphatidylethanolamine (25 to 30%) in restoring proline uptake with phospholipase A-treated DETP (Fig. 4). However, the addition of an equimolar mixture of liposomes of phosphatidylethanolamine and cardiolipin was not found to increase the steady level of proline more than that observed with cardiolipin alone. It is pertinent to mention that the rate of substrate oxidation was not stimulated by the addition of phospholipid liposomes to phospholipase A-treated DETP membranes. The restoration of proline transport in phospholipase A-treated DETP membranes by liposomes could not be enhanced by the removal of accumulated free fatty acids by bovine albumin treatment. Therefore, the lowered steady state level of proline transport in phospholipase A-treated DETP membranes compared with ETP membranes appears to be the result of increased cleavage of phospholipids from the DETP membrane, particularly cardiolipin, which is otherwise inaccessible in ETP membranes.

**Effect of Phospholipase A on Glutamine and Glutamic Acid Uptake in ETP and DETP Membrane Vesicles**—Glutamine and glutamic acid are two other amino acids, in addition to proline, which are transported actively in ETP membranes of Mycoplasma.
uptake of proline in these reconstituted membranes was followed after 15 min of substrate addition. Generated NADH was used as the substrate. O-O, untreated DETP; △-△, phospholipase A-treated DETP + cardiolipin; A-A, phospholipase A-treated DETP + phosphatidylethanolamine.

**TABLE II**

Effect of phospholipase A treatment on active transport of glutamine and glutamic acid in ETP and DETP membranes

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Steady state level of glutamine</th>
<th>Steady state level of glutamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP</td>
<td>331 pmol/mg protein</td>
<td>361 pmol/mg protein</td>
</tr>
<tr>
<td>Treated ETP</td>
<td>326 pmol/mg protein</td>
<td>366 pmol/mg protein</td>
</tr>
<tr>
<td>DETP</td>
<td>324 pmol/mg protein</td>
<td>358 pmol/mg protein</td>
</tr>
<tr>
<td>Treated DETP</td>
<td>318 pmol/mg protein</td>
<td>353 pmol/mg protein</td>
</tr>
</tbody>
</table>

a Phospholipase A-treated.

**DISCUSSION**

In the preceding paper (15), it was demonstrated that certain membrane functions, e.g. oxidative phosphorylation, proton gradient, and the energy-dependent response of L-anilino-s-naphthalene sulfonate were related to the extent of membrane phospholipids cleaved by phospholipase A action. In the present study, it was observed that the active transport of proline in ETP membranes was not affected by phospholipase A treatment, although the rate of oxidation was impaired. However, DETP membrane vesicles, which are devoid of membrane-bound coupling factor-latent ATPase, showed diminished levels of proline transport with various substrates upon phospholipase A treatment. This decrease in the level of active transport of proline in DETP membranes could be due to the impairment of oxidation of substrates, to an altered affinity for proline, or to the additional cleavage of phospholipids from the membrane that may

<Figure 5>

**FIG. 5.** Effect of phospholipids concentration on active transport of proline in phospholipase A-treated DETP membranes. The indicated amounts of phospholipid liposomes were added to phospholipase A-treated and untreated DETP membranes. The uptake of proline in these reconstituted membranes was followed after 15 min of substrate addition. Generated NADH was used as the substrate. ○-○, untreated DETP; △-△, phospholipase A-treated DETP + cardiolipin; A-A, phospholipase A-treated DETP + phosphatidylethanolamine.

**TABLE III**

Effect of various inhibitors on proline transport in untreated and phospholipase A-treated DETP membranes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentrations</th>
<th>DETP</th>
<th>Treated DETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>810</td>
<td>310</td>
</tr>
<tr>
<td>Dibenzyldiammonium ion</td>
<td>5 mm</td>
<td>576</td>
<td>191</td>
</tr>
<tr>
<td>Tetraphenylboron</td>
<td>50 μM</td>
<td>378</td>
<td>137</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenylhydrazine</td>
<td>50 μM</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>Dicyclohexylcarbodiimide</td>
<td>50 μM</td>
<td>788</td>
<td>292</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>20 μM</td>
<td>185</td>
<td>80</td>
</tr>
</tbody>
</table>

a Phospholipase A-treated.
result in a conformational change in a specific carrier protein for proline.

The decreased level of oxidation in phospholipase A-treated DETP membranes was due to the accumulation of free fatty acids, since washing with bovine albumin restored the impaired oxidation; however, the level of transport remained unaffected. Brodie and his co-workers (11) have also shown that there is no apparent correlation between oxidation rates of various substrates and the ability to transport proline.

The kinetic data revealed that the lowered steady state level of proline uptake in phospholipase A-treated DETP membranes, as compared with untreated DETP membranes, was not due to the change in the apparent $K_m$ for proline. The $V_{max}$, however, was altered. The difference in the accessibility of phospholipids to phospholipase A in ETP and DETP membranes may suggest that the observed decrease in proline uptake is due to the additional cleavage of membrane phospholipids from DETP membranes. The addition of liposomes of phospholipids to the phospholipase A-treated DETP membranes or to bovine albumin-washed membranes restored the level of proline uptake. However, cardiolipin was found to be most effective in restoring this level of uptake of proline. These results indicate that the effect of phospholipase A treatment on active transport of proline in membranes depleted of membrane-bound coupling factor-latent ATPase was due to one of two things. Either there is an alteration of the active conformation of carrier protein(s) for proline, or the phospholipids are required for the uptake of proline in membranes depleted of membrane-bound coupling factor-latent ATPase. Either there is an alteration of the active conformation of carrier protein(s) for proline, or the phospholipids are required for the uptake of proline. In contrast to the proline transport, the steady state levels of glutamine and glutamic acid were not affected following phospholipase A treatment of ETP or DETP membranes. These results may suggest that either the conformation of carrier protein(s) of glutamine and glutamic acid is not altered by phospholipase A treatment, or the phospholipids cleaved from the membrane are not involved in the uptake of glutamine and glutamic acid.

DETP membranes are capable of active transport of proline, but they do not exhibit internal pH gradient as measured by bromothymol blue (17). The addition of membrane-bound coupling factor to DETP membranes restored the pH gradient but did not affect the level of transport of proline. However, the internal pH gradient was not restored by the addition of membrane-bound coupling factor-latent ATPase to phospholipase A-treated DETP membranes (15), and the level of transport remained unaffected. These results substantiate the earlier findings (17) that the proton motive force is not the driving force for active transport of amino acids, though the possibility of electrochemical gradient cannot be excluded and appears to be a more likely candidate for the bioenergetics for active transport of amino acids in M. phlei.

It should be noted from previous studies (19) that uncoupling agents that are specific for intact mitochondria uncouple phosphorylation in ghost preparation of M. phlei, and those that are specific for submitochondrial particles uncouple phosphorylation with ETP. The finding that the active transport in ghost preparation and ETP was inhibited only by agents that inhibit intact mitochondria, and the knowledge that ETP represent a mixture of particles (most oriented inside out) led to the assumption that only the right side out oriented vesicles were capable of active transport (11, 13). However, the finding that the inhibition of transport of proline by phospholipase A treatment required the removal of membrane-bound coupling factor may suggest that both types of particles may be capable of active transport and that the inside out oriented membrane vesicles contain patches or areas within the membrane that differ in orientation.

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