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Complex Lipid Requirements for Detergent-Solubilized Phosphoacetylmuramyl-Pentapeptide Translocase from *Micrococcus luteus*

(Triton X-100/cell wall synthesis/membrane-bound/C_{55}-isoprenyl alcohol/enzyme)

JAY N. UMBREIT AND JACK L. STROMINGER

Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT **Phospho-MurNAc-pentapeptide translocase activity in the membrane of *M. luteus* was lost upon addition of the detergent, Triton X-100, but could be restored by addition of lipid fractions to the assay. By assay in the presence of lipid, the activity of the Triton-solubilized enzyme could be measured. The synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide required C_{55}-isoprenyl-P, and was stimulated by a neutral lipid. The exchange reaction of UDP-MurNAc-pentapeptide with UMP required a polar lipid fraction, but the reaction was not affected by C_{55}-isoprenyl-P or the neutral lipid. Thus, measurement of activity of the detergent-solubilized enzyme requires addition of three lipids, the lipid substrate (C_{55}-isoprenyl-P), the neutral lipid, and a polar lipid.**

Phospho-MurNAc-pentapeptide translocase (1, 2) is the first enzyme of several membrane-bound enzymes involved in biosynthesis of bacterial cell walls. It catalyzes the reaction of UDP-MurNAc-pentapeptide with C_{55}-isoprenyl phosphate to form C_{55}-isoprenyl-P-P-MurNAc-pentapeptide (Eq. 1).

$$\text{UDP-MurNAc-pentapeptide} + C_{55}\text{-isoprenyl-P} \rightleftarrows C_{55}\text{-isoprenyl-pyrophosphate-MurNAc-pentapeptide} + \text{UMP} \quad (1)$$

The solubilization and purification of this enzyme has been hindered by the difficulty of retaining its activity in the presence of detergents (3). In this communication, we show that that inhibition can be overcome by the addition of specific lipid fractions, and that under these assay conditions, the enzyme can be solubilized in a nonionic detergent with retention of activity.

Effect of lipids on the synthesis of C_{55}-isoprenyl-P-P MurNAc-pentapeptide from UDP-MurNAc-[^{14}C]pentapeptide

The addition of Triton X-100 to the particulate enzyme reduced its activity by 30–40%. The addition of a crude chloroform–methanol (2:1) extract not only restored activity, but stimulated it (Table 1), with a concomitant increase in linearity of incorporation over a longer period of time. Saponification of the extract did not decrease its stimulatory effect. The extract was separated on a column of silicic acid into fractions eluted with chloroform, chloroform–methanol 5:1 $CHCl_3$–MeOH 3:1, $CHCl_3$–MeOH 1:1, and methanol. No single fraction restored full stimulatory activity, but a combination of the fraction eluted in chloroform plus that eluted in $CHCl_3$–MeOH 5:1, gave the full stimulatory activity. The chloroform fraction contained very little stimulatory activity by itself, but the $CHCl_3$–MeOH 5:1 fraction alone had about 60% of the total stimulatory activity (Fig. 1).

The $CHCl_3$–MeOH 5:1 fraction contained C_{55}-isoprenyl-P, and could be replaced by synthetic C_{55}-ficaprenyl-P (generously given by Dr. C. Warren, Massachusetts General Hospital, Boston, Mass.). The chloroform fraction stimulated the utilization of C_{55}-ficaprenyl-P. If synthetic all-*trans*-C_{45}-solanesyl phosphate (generously given by Dr. A. Schocher, Hoffman–LaRoche, Basel, Switzerland) was used as a substrate, the chloroform fraction was required for activity (Table 2).

The identity of the compound in the chloroform fraction is still not clear. Neither α-tocopherol, vitamin K_2 (isolated from *Micrococcus luteus*), C_{15}-farnesol, C_{55}-ficaprenol, solanesol, menadione, ubiquinone (Q_6, Q_{10}, or isolated from *M. luteus*) nor polar lipids (phosphatidyl ethanolamine, phosphatidyl glycerol) substituted for the neutral fraction. Vitamin K_1

TABLE 1. *Effect of Triton X-100 and lipid extract on the synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide*

Incubation mixture	cpm Incorporated into C_{55}-isoprenyl-P-P-MurNAC-pentapeptide
Complete	1680
+ 2% Triton X-100	1160
+ 2% Triton X-100, + crude lipid extract*	5000
+ 2% Triton X-100, + saponified lipid extract*	4550

The synthesis of C_{55}-isoprenyl-P-P-pyrophosphate-MurNAc-pentapeptide from UDP-MurNAc-[^{14}C]pentapeptide was measured essentially as described by Anderson *et al.* (2), except in the absence of UDP-GlcNAc. The particulate enzyme was prepared by disruption of freeze-dried cells of *M. luteus* in a Mini-mill (Gifford-Wood Co., Hudson, N.Y.) followed by centrifugation at 5000 rpm in a Sorvall centrifuge (SS34 rotor) to remove debris and at 42,000 rpm (120,000 × *g*) in an International Equipment Co. B-60 ultracentrifuge with an A-211 rotor to collect the membrane fraction. The particulate fraction was suspended in 0.1 M Tris.HCl buffer (pH 8) to a protein concentration of 10 mg/ml.

* The crude lipid extract had no effect on the particulate fraction in the absence of Triton X-100. It was made from 50 g of cells (dry weight) by sonication in 1 liter each of chloroform–methanol 2:1 and methanol, and was extracted by the method of Bligh and Dyer (4). The extract was concentrated to 100 ml, and saponified by the method of Dawson (5). The sample was kept at constant volume to compare the activity before and after saponification. This material (30 μl) was taken to dryness under reduced pressure before addition of the components of the assay mixture.

(Sigma) stimulated the reaction, but only at concentrations greater than 0.5 mg/ml.

Effect of lipids on the exchange reaction

The addition of Triton X-100 completely abolished the exchange of [^{14}C]UMP with UDP-MurNAc-peptide [see Heydanek *et al.* (3)]. This activity could be restored by the addition of a crude chloroform–methanol (2:1) extract. Only some lipid extracts were effective, probably because the active component was present only in small amounts. Purification of the extract by column chromatography on silicic acid resulted in an active fraction eluted from the column with $CHCl_3$–MeOH 9:1. In sharp contrast to the lipid required for synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide, the lipid required for the exchange reaction was sensitive to saponification (Table 3). The lipid was further purified by chromatography on DEAE-cellulose (6) and preparative thin-layer chromatography. The activity resided in a single band, with an R_F = 0.45 in chloroform–methanol–water 60:25:4, and has been tentatively identified as phosphatidyl glycerol. Commercial samples (obtained from Schwartz–Mann, Orangeburg, N.Y.) of phosphatidyl glycerol, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol substituted for the isolated lipid extract (Table 4). Neither the neutral lipid fraction nor ficaprenyl-P had any effect on the exchange reaction.

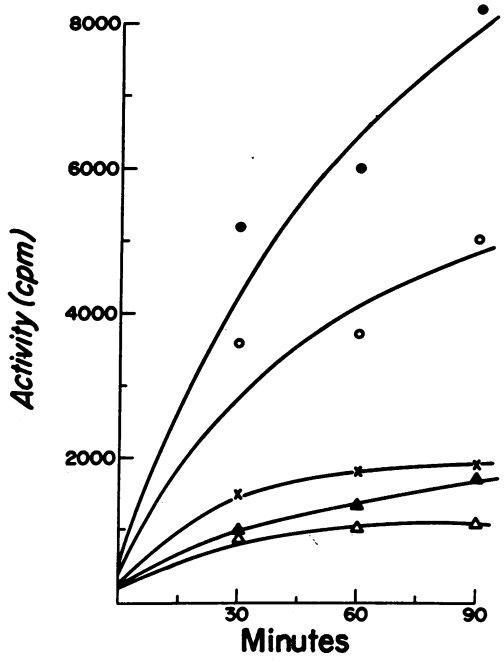

FIG. 1. The effect of crude lipid fractions on the synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide. The lipid extract was made as described in Table 1. 3 μl of lipid extract was added to each tube and taken to dryness. Then, 2 μl of 20% Triton X-100 was added, followed by the remainder of the assay mixture (15 μl). The tubes were then sonicated in a water bath for 5 min, after which the enzyme was added (5 μl of crude membrane preparation with 10 mg/ml of protein) and the mixture was incubated for the indicated time at 25°. ▲——▲, no Triton X-100; △——△, + Triton X-100; ×——×, + Triton X-100 and $CHCl_3$ extract; O——O, + Triton X-100 and $CHCl_3$–MeOH (5:1) extract; ●——●, + Triton X-100, $CHCl_3$ extract, and $CHCl_3$–MeOH (5:1) extract.

TABLE 2. *Effect of the neutral lipid fraction on the synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide*

Incubation mixture	cpm Incorporated into C_{55}-isoprenyl-P-P-MurNAc-pentapeptide
Complete	322
+ Triton X-100	58
+ Triton X-100 + chloroform fraction	78
+ Triton X-100 + solanesol-P	103
+ Triton X-100 + solanesol-P + chloroform fraction	1120
+ Triton X-100 + ficaprenol-P	877
+ Triton X-100 + ficaprenol-P + chloroform fraction	1180

Assays were performed as described by Anderson *et al.* (2). Where indicated, solanesol-P was added, to a final concentration of 2.5 mg/ml, and ficaprenol-P to 0.12 mg/ml. 3 μl of the chloroform extract was added. The lipid fractions were added in chloroform, which was removed under reduced pressure before the addition of the other components of the reaction mixture.

Solubilization of the translocase in Triton X-100

About 50% of the translocase activity was solubilized in 5% Triton X-100. This activity (assayed by the synthetic reaction in the presence of appropriate lipids) was not sedimented

TABLE 3. *Effect of Triton X-100 and lipid extract on the exchange reaction*

Incubation mixture	cpm Incorporated into UDP-MurNAc-pentapeptide
Complete	1360
+ Triton X-100	73
+ Triton X-100, + crude lipid extract*	0
+ Triton X-100, + chloroform–methanol, 9:1 fraction†	2380
+ Triton X-100, + chloroform–methanol, 9:1, fraction saponified‡	0

The exchange between UDP-MurNAc-pentapeptide and [^{14}C]UMP was measured by a modification of the method of Heydanek *et al.* (2). The reaction mixture consisted of 2 μl of 1.5 mM UDP-MurNAc-pentapeptide, 2 μl of [^{14}C]UMP (300 Ci/mol, 10 μCi/ml), 1.5 μl of 1 M Tris·HCl (pH 8.0), and 10 μl of enzyme and water. After incubation at 25° for 30 min, the reaction was terminated by boiling. 0.3 μl of alkaline phosphatase (Sigma, Type IV calf intestine, 5 mg/ml) was added to the reaction mixture, and the mixture was incubated at 37° for 20 min. The reaction was terminated by boiling, and the mixture was spotted on DEAE-paper (Whatman, Balston, England); the paper was washed with water for 20 min, and then with acetone for 5 min. The dried paper was then counted in a toluene-based scintillation fluid.

* The crude lipid extract was prepared as described in Table 1.

† The crude lipid extract (50 ml) was applied to a 2.5 × 30 cm column of silicic acid, which was eluted with 1 liter each of chloroform; chloroform–methanol, 9:1; $CHCl_3$–MeOH, 5:1; $CHCl_3$–MeOH, 3:1; $CHCl_3$–MeOH, 1:1; and methanol. Fractions were concentrated to 30 ml. In the assay, 10 μl of the appropriate fraction was used.

‡ Saponified by the procedure of Dawson (5).

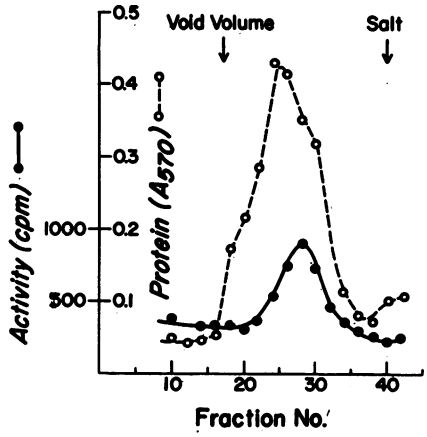

FIG. 2. Gel filtration of the translocase in Triton X-100. 1 ml of crude membrane from *M. luteus* (10 mg/ml protein) was mixed with 50 μl of Triton X-100 at 4° for 30 min and centrifuged in an International Equipment Co. B-60 centrifuge with an A-321 rotor at 45,000 rpm for 60 min. The supernatant solution was decanted and charged to a 1.5 × 26 cm column of Bio-Gel A 5-m equilibrated in a buffer containing 40 mM Tris·HCl (pH 8.0–1 mM 2-mercaptoethanol–1% Triton X-100. 50 Fractions of 1 ml (each) were collected, at a flow rate of 0.25 ml/min. 15 μl of each fraction was assayed for the ability to catalyze synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide in the presence of a crude lipid extract. Protein was determined by the alkaline ninhydrin method of Hirs (7), since Triton X-100 interferes with other methods of protein determination.

at 120,000 × *g* after 60 min (International Equipment Co. centrifuge B-60, A-321 rotor, 45,000 rpm). The activity was included in the column volume of a 6% Agarose column (Bio-Gel A 5-m), indicative of the inclusion of the protein in a detergent micelle (Fig. 2). These data suggest that further purification may now be possible.

DISCUSSION

The initial membrane-associated reaction of cell wall biosynthesis catalyzed by phospho-MurNAc-pentapeptide translocase was stimulated by lipid fractions when the reaction was assayed in the presence of the nonionic detergent Triton X-100. The enzyme can be assayed by measurement of either the synthesis of C_{55}-isoprenyl-P-P-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide and C_{55}-isoprenyl phosphate, or the exchange of UMP into UDP-MurNAc-pentapeptide. The two different assay methods required different lipid fractions for maximum activity. The exchange reaction was not stimulated by C_{55}-isoprenyl-P (see also ref. 1), but a polar lipid that was destroyed by saponification was required for this activity. In contrast, the synthetic reaction not only required the lipid substrate, C_{55}-isoprenyl-P, but was stimulated by a neutral lipid whose activity was not lost upon saponification. The effect of the neutral lipid on the utilization of all-*trans*-C_{45}-solanesyl phosphate, a substrate analogue, was particularly striking. No utilization of this material was observed in the absence of the neutral lipid, but it appeared to be as active as C_{55}-isoprenyl phosphate in its presence. No role for neutral lipids except for their participation in electron transport and photosynthesis has been suggested in bacteria. It should be noted that the particulate enzyme utilizes endogenous C_{55}-isoprenyl-P as its substrate and is not stimulated by addition of C_{55}-ficaprenyl-P in the absence of detergent.

TABLE 4. *Effect of phospholipids on the exchange reaction*

Incubation mixture	cpm Incorporated into UDP-MurNAc-pentapeptide
Complete	3660
+ 2% Triton X-100	0
+ 2% Triton X-100, + chloroform–methanol 9:1 fraction	2210
+ 2% Triton X-100 and:	
+ phosphatidyl serine	2460
+ phosphatidyl ethanolamine	2170
+ phosphatidyl inositol*	2150

Assays were done as described in Table 3. The lipids were obtained from Schwartz–Mann, Orangeburg, N.Y., and were added to a final concentration of 20 mg/ml.

* The effect of phosphatidyl glycerol was examined in a separate experiment, in which 1200 cpm was incorporated in the complete system, 100 cpm on addition of Triton X-100, and 2200 cpm on addition of phosphatidyl glycerol.

Several membrane enzymes require a phospholipid for activity (8), and the requirement of the detergent-solubilized exchange reaction of the translocase for phospholipid adds one more enzyme to this list. Since the exchange reaction is a part of the overall synthetic reaction, it was surprising to find that the polar lipid was required for the exchange reaction, but not for the synthesis. It is conceivable that formation of an enzyme-P-MurNAc-pentapeptide intermediate (which is sufficient to account for an exchange reaction) (1, 3) is so rapid relative to the synthetic reaction that the residual activity in the absence of the polar lipid fraction is sufficient to account for the overall rate of synthesis.

After this manuscript was completed, an abstract by Pless *et al.* appeared that indicated that these workers had also observed the effect of phospholipid on the activity of the translocase (9).

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1. Neuhaus, F. C. (1971) *Accounts Chem. Res.* **4**, 297–303.
2. Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 881–889.
3. Heydanek, M. G., Jr., Struve, W. G. & Neuhaus, F. C. (1969) *Biochemistry* **8**, 1214–1221.
4. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
5. Dawson, R. M. C. (1960) *Biochem. J.* **75**, 45–53.
6. Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C. & Bauman, A. J. (1969) in *Methods in Enzymology*, ed. Lowenstein, J. M. (Academic Press, New York), Vol. XIV, pp. 272–317.
7. Hirs, C. H. (1967) in *Methods in Enzymology*, ed. Hirs, C. H. (Academic Press, New York), Vol. XI, pp. 325–329.
8. Rothfield, L. & Romeo, D. (1971) in *Structure and Function of Biological Membranes*, ed. Rothfield, L. (Academic Press, New York), pp. 251–284.
9. Pless, D. D., Jonah, M. M. & Neuhaus, F. C. (1972) *Fed. Proc.* **31**, 413.