THE ROLE OF CELL ENVELOPE PHOSPHOLIPID IN THE ENZYMATIC SYNTHESIS OF BACTERIAL LIPOPOLYSACCHARIDE: BINDING OF TRANSFERASE ENZYMES TO A LIPOPOLYSACCHARIDE-LIPID COMPLEX*

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The cell envelope of gram-negative enteric bacteria contains lipopolysaccharides which are responsible for the O-antigenic specificity and the endotoxin activity of these organisms. The biosynthesis of the core structure of these lipopolysaccharides proceeds by the sequential transfer of monosaccharide units from nucleotide sugar to the growing polysaccharide chains (Osborn et al., 1962; Nikaido, 1962; Osborn and D'Ari, 1964; Edstrom and Heath, 1964). The transferase enzymes are predominantly localized in the cell envelope, but two of them have also been detected in the soluble fraction of extracts of Salmonella typhimurium (Rothfield et al., 1964a). The present study is concerned with the soluble UDP-galactose:lipopolysaccharide galactosyl transferase, which catalyzes the following reaction: galactose-deficient LPS[1/] + UDP-galactose ⟶ galactosyl-LPS.

We have previously shown that purified lipopolysaccharide does not act as acceptor in the transferase reactions unless the phospholipid of the cell envelope is also present; the inactive lipopolysaccharide is converted into a suitable acceptor by heating and slowly cooling the lipo-

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1/ Abbreviation: lipopolysaccharide, LPS.

polysaccharide-phospholipid mixture (Rothfield and Horecker, 1964). It would appear that this results in formation of a lipopolysaccharide-lipid complex, the nature of which remains to be determined. In the present communication we present evidence that this interaction of phospholipid and lipopolysaccharide is required for binding of the galactosyl transferase enzyme to its lipopolysaccharide substrate, and describe the formation and isolation of a specific enzyme-lipopolysaccharide-phospholipid complex.

Materials and Methods: Glucose-deficient LPS was obtained from a mutant strain of S. typhimurium which lacks UDP-glucose: lipopolysaccharide glucosyl transferase[2/], galactose-deficient LPS was isolated from Strain G-30 which lacks UDP-galactose-4-epimerase, and Rough LPS was obtained from a Rough B strain (Subbaiah and Stocker, 1964). The lipopolysaccharides were extracted by treatment with phenol and purified as previously described (Osborn et al., 1962). The enzyme preparation was the supernatant fraction obtained by centrifugation at 105,000 xg of sonic extracts of S. typhimurium EI-1 as previously described (Rothfield et al., 1964a). The lipid was obtained from E. coli strain AB 312[3/] by extraction of packed cells with 5 volumes of chloroform-methanol (3:1) for 1 hour at room temperature. The extract was washed twice by the method of Folch, Lees and Sloane Stanley (1957), taken to dryness under reduced pressure, dissolved in a minimal amount of chloroform, and precipitated with 10 volumes of cold acetone. Thin layer chromatography on Silica Gel G (Brinkman) with chloroform-methanol-water (65:25:4) indicated that approximately 90% of the material was phosphatidyl ethanolamine, with minor components having the mobilities of fatty acids, lysophosphatidyl ethanolamine and more polar compounds. In several experiments, purified phosphatidyl ethanolamine of E. coli was also tested, with identical results to those

2/ These strains were generous gifts from Drs. B.A.D. Stocker and M. J. Osborn.

3/ This strain was a kind gift from Dr. E. Adelberg.

reported here. Analytic procedures and methods for preparation of other materials have been described elsewhere (Osborn et al., 1962; Rothfield et al., 1964a).

Assay for UDP-galactose lipopolysaccharide galactosyl transferase: Galactose-deficient LPS (0.07 μmoles of heptose) and lipid (0.14 μmoles P in 0.01 ml methanol) were suspended in 0.1 ml of 50 mM Tris-HCl, pH 8.5, and the mixture was heated to 60° for 30 min. and then slowly cooled to 23° in 2 hours. The fraction to be assayed was added, and the mixture adjusted to 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 0.08 mM UDP-galactose-U-^{14}C (1,600 cpm per mμmole), in a total volume of 0.33 ml. After incubation at 37° for 10 min, incorporation of radioactivity into trichloroacetic acid-insoluble material was determined as previously described (1 unit = 1 mμmole of galactose-^{14}C incorporated in 10 min); it has been shown that this procedure measures incorporation of galactose-^{14}C into LPS (Rothfield and Horecker, 1964). These concentrations of LPS and lipid resulted in maximal incorporation of galactose-^{14}C.

Binding of galactosyl transferase: The extent of binding of the soluble galactosyl transferase to the LPS-lipid complex was determined by high speed centrifugation of the mixture under conditions in which the LPS and phospholipid were both completely sedimented (Table I). Sediment and supernatant fractions were then assayed for enzyme activity by adding UDP-galactose-^{14}C and additional LPS-lipid acceptor. When the crude soluble enzyme preparation was centrifuged in the absence of LPS and lipid, the galactosyl transferase activity remained in the supernatant fraction. However, when the same enzyme preparation was first incubated with the LPS-lipid mixture to permit binding to occur, the enzyme activity was recovered in the sediment together with lipid and LPS (last 2 columns, Table I).

More than 95% of the protein of the crude enzyme preparation remained in the supernatant fraction. The exact amount of protein bound

TABLE I

Binding of UDP-galactose lipopolysaccharide galactosyl transferase activity

Additions	Lipopolysaccharide (μmoles heptose)		Lipid (μmoles P)		Protein (mg)		Enzyme Activity (units)	
	Supernatant	Sediment	Supernatant	Sediment	Supernatant	Sediment	Supernatant	Sediment
(1) None	<0.005	<0.005	<0.002	<0.002	1.24	<0.05	2.25	<0.02
(2) Gal-deficient LPS + lipid	0.01	0.25	0.002	0.21	1.24	0.05	0.2	3.15
(3) Gal-deficient LPS	0.01	0.25	<0.002	0.002	1.24	0.08	1.7	0.15
(4) Lipid	<0.005	<0.005	<0.002	0.20	1.23	<0.05	1.8	<0.02
(5) Glu-deficient LPS + lipid	0.02	0.20	<0.002	0.21	1.25	0.06	1.7	0.2
(6) Rough LPS + lipid	<0.01	0.24	<0.002	0.19	1.24	0.09	1.9	0.15
(7) Wild type LPS + lipid	0.08	0.24	0.002	0.17	1.24	0.10	2.1	0.3

The basic reaction mixture (0.5 ml) contained 50 mM Tris-HCl, pH 8.5, LPS (0.28 μmoles heptose) and lipid (0.56 μmoles P in 0.04 ml methanol) as indicated in the table; the mixture was heated to 60° and slowly cooled before use. Enzyme (1.25 mg of protein) was added, and the mixture was adjusted to 50 mM Tris-HCl, pH 8.5, and 5 mM $MgCl_2$, in a total volume of 1 ml. After incubation at 37° for 10 min, the mixture was centrifuged at 105,000 xg for 40 min at 4°. The sediment was suspended in 1 ml of 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, and aliquots (0.2 ml) of supernatant and sediment fractions were assayed for galactosyl transferase activity and were analyzed for LPS, lipid and protein (Lowry et al., 1951). The results are expressed per 1 ml total reaction mixture. When aliquots of each supernatant and sediment fraction were heated to inactivate enzyme, and were added to the complete transferase assay mixture together with fresh enzyme, the added fractions caused no stimulation or inhibition of incorporation of galactose-^{14}C. Galactose-deficient LPS was estimated by determining its ability to accept galactose-^{14}C in the transferase assay after heating to 100° to inactivate any bound enzyme. At these concentrations the rate of galactose incorporation was proportional to concentration of LPS, and the content of LPS in the unknown samples were determined by comparison with assays of known quantities of LPS. Glucose-deficient LPS was determined in an identical manner, except that the glucosyl transferase assay system was used (Rothfield and Horecker, 1964). Lipid was determined by extracting aliquots of supernatant and sediment fractions with 5 volumes of chloroform-methanol (3:1), and determining total extractable phosphate (Ames and Dubin, 1960). A similar extraction of the total reaction mixture before centrifugation

to the sediment was difficult to determine because of the small quantities present and because the LPS itself gives some reaction in the Lowry test.

To demonstrate that the sediment contained the active enzyme-lipopolysaccharide-lipid complex, the sedimented material (Table I, line 2) was resuspended, and 80 mμmoles of UDP-galactose-^{14}C added. There was incorporation of 10.8 mμmoles of galactose-^{14}C into LPS in 10 minutes. Addition of UDP-galactose-^{14}C to the other supernatant and sediment fractions (lines 3-7) under the same conditions resulted in incorporation of less than 0.4 mμmoles of galactose-^{14}C in 10 minutes.

The binding reaction required both components of the LPS-lipid complex. Thus, when the soluble enzyme preparation was incubated with lipid or LPS alone, little or no enzyme activity was recovered in the sediment.

The reaction was highly specific for the appropriate LPS substrate. When galactose-deficient LPS was replaced by lipopolysaccharides which are inactive as substrates for the galactosyl transferase (glucose-deficient LPS and lipopolysaccharides from rough and wild type strains), the enzyme activity remained in the supernatant fraction. The specificity is best illustrated by the observation that no binding of enzyme activity occurred with glucose-deficient LPS which differs from galactose-deficient LPS only in the absence of one glucose residue per two heptose residues (Fraenkel et al., 1963). It has previously been shown that this glucose residue acts as the acceptor site for galactose transfer in the galactosyl transferase reaction (Rosen et al., 1964).

The binding reaction was dependent on the presence of Mg^{++}, and with increasing concentrations of $MgCl_2$ there was a progressive increase in enzyme activity in the sediment with a corresponding disappearance from the supernatant fraction. The optimal concentration of Mg^{++} (4 mM) for binding of galactosyl transferase activity was equal to the concentration necessary for maximal enzyme activity in the assay of the complete system.

Protection against inactivation of galactosyl transferase activity:

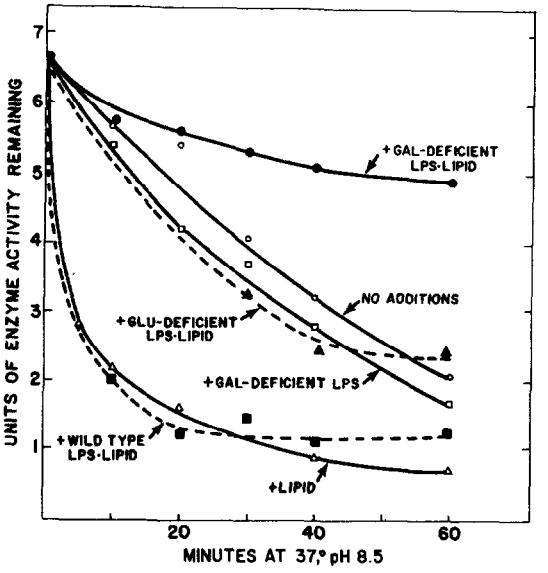

Figure 1. Protection against inactivation of UDP-galactose lipopolysaccharide galactosyl transferase activity: A reaction mixture (1.0 ml) containing LPS (0.88 μmoles heptose), lipid (4 μmoles P in 0.2 ml methanol) and 50 mM Tris-HCl, buffer pH 8.5, was heated to 60° and slowly cooled. Enzyme (2.4 mg protein) was added and the mixture was adjusted to 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, in a final volume of 1.5 ml. Incubation was performed at 37° and 0.15 ml aliquots were removed at intervals and assayed for galactosyl transferase activity. Additional LPS or lipid was added to each assay tube before the final incubation to compensate for the differing amounts added with the samples to be assayed. The results are expressed as units of enzyme activity in the final assay tube. Parallel experiments were performed in which different lipopolysaccharides were used in the initial reaction mixture or in which LPS or lipid or both were omitted.

Binding of transferase enzyme to the LPS-lipid complex was also suggested by the observation that the LPS-lipid mixture provided marked protection against thermal inactivation of enzyme activity (Fig.1). When the soluble enzyme preparation was incubated at 37° there was a progressive disappearance of galactosyl transferase activity, with 70% loss of activity in 60 minutes. The inactivation was largely prevented by the presence of the LPS-lipid mixture; LPS or lipid alone provided no protection. Protection against loss of enzyme activity was highly specific for the substrate LPS. Lipopolysaccharides which are not substrates in the galactosyl transferase reaction (glucose-deficient and wild type lipopolysaccharides) were in-

effective in protecting against enzyme inactivation.

Discussion: The site of binding of galactosyl transferase has not been definitely established, but binding and protection required both components of the LPS-lipid complex, and were highly specific for the galactose-deficient LPS which acts as substrate for the enzyme. These results suggest that the enzyme binds to LPS but some degree of additional binding to lipid is also possible. The mechanism whereby the lipid converts the appropriate LPS into a suitable partner for binding of transferase enzyme is unknown.

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