ABSTRACT An enzyme system catalyzing the synthesis of the β1,2-linked glucon backbone of the membrane-derived oligosaccharides of Escherichia coli from UDP-glucose has an essential requirement for the E. coli acyl carrier protein (ACP). This finding was surprising, because all other characterized functions of ACP involve acyl thioester residues linked to the phosphopantetheine moiety covalently bound to ACP. We now report that the activity of ACP in the synthesis of membrane-derived oligosaccharides is not altered by treatment with the sulfhydryl reagent N-ethylmaleimide nor by complete removal of the phosphopantetheine residue by treatment with a specific phosphodiesterase. The function of ACP in the synthesis of membrane-derived oligosaccharides is thus clearly different from that involved in lipid biosynthesis. We have nevertheless found that the same molecular species of ACP that undergo enzymic acylation with long-chain fatty acid residues also function in the synthesis of membrane-derived oligosaccharides.

Membrane-derived oligosaccharides (MDO) of Escherichia coli are periplasmic glucans containing 6 to 12 glucose units linked by β1,2 and β1,6 bonds and substituted with phosphoglycerol and phosphoethanolamine residues derived from the head groups of membrane phospholipids (1). The synthesis of MDO is a striking example of osmotic adaptation, levels being 16 times higher in cells growing in medium of low osmolarity as compared with the same medium containing 0.4 M NaCl (2).

Weissborn and Kennedy (3) discovered an enzyme system that catalyzes the elongation of β1,2-glucon chains that are the backbone of the MDO structure. In vitro, the enzyme system catalyzes the following overall reaction.

\[ n\text{UDP-glucose} + \text{glucose-O-R} \rightarrow (\text{glucose})_n\text{-glucose-O-R} + n\text{UDP} \]

Here glucose-O-R is the model substrate, octyl β-D-glucopyranoside, that functions as an essential \"primer\" for the reaction. It is thought to function as an analog of a postulated endogenous membrane-bound intermediate in vivo.

The transglycosylation system requires both a membrane component and a cytosolic protein. The latter was isolated by Therisod et al. (4) and identified as the acyl carrier protein (ACP) of E. coli. The discovery that ACP plays an essential role in the biosynthesis of a cell-surface carbohydrate was completely unexpected, because all of its known functions have involved transfer of acyl residues linked to ACP through the phosphopantetheine moiety covalently bound to serine-36 by a phosphodiester linkage. ACP has thus often been regarded as a protein form of coenzyme A.

We now report that the phosphopantetheine moiety of ACP is not needed for ACP to function in the MDO transglycosylation system. The apo form of ACP (apo-ACP), produced by removal of the phosphopantetheine by the action of a specific phosphodiesterase, is fully as active as the intact holo form of ACP (holo-ACP).

We have further found that S-palmitoyl-ACP is also active in the transglycosylation system, providing evidence that the same ACP molecules that are involved in transacylation reactions also have transglycosylation function.

MATERIALS AND METHODS

Assay of Transglycosylase Activity. The conversion of labeled glucose from UDP-[3H]glucose to neutral glucon was assayed as described by Therisod et al. (4). The membrane fraction required for the reaction was prepared from E. coli strain DF214 as described by Weissborn and Kennedy (3). Purified ACP used as standard in this system was prepared from frozen E. coli essentially as described by Therisod et al. (4). A commercial preparation (Sigma) was also used in some experiments.

Gel Electrophoresis. Electrophoresis on nondenaturing gels was carried out by a procedure based on that of Jackowski and Rock (5) with the modifications described by Therisod et al. (4). It has been established (5, 6) that the relative rates of migration in this system are in the following order: acyl-ACP > ACP > apo-ACP.

Preparation of ACP Acylase. The preparation of ACP acylase, an enzyme catalyzing the formation of long-chain acyl thioesters of ACP in an ATP-requiring reaction, was based on the method of Rock and Cronan (7). Membranes from E. coli strain DF214 were extracted with Triton X-100 and heated to 55°C in the presence of 5 mM ATP as described (7). After removal of heat-denatured protein, the preparation was stored at 4°C in the presence of 5 mM ATP and 3 mM sodium azide and used without further purification.

Preparation of ACP Phosphodiesterase. An enzyme catalyzing the hydrolytic cleavage of the phosphopantetheine residue from holo-ACP was prepared by the method of Vagelos and Larrabee (8). The preparation was carried through the stage of ammonium sulfate fractionation, dialyzed, and used without further purification. ACP phosphodiesterase activity was followed by measuring the release of phospho[3H]pantetheine, which is soluble in trichloroacetic acid, from holo-ACP, which is insoluble in trichloroacetic acid.

Abbreviations: MDO, membrane-derived oligosaccharides; ACP, acyl carrier protein.

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acid. The labeled holo-ACP was prepared by growth of strain SJ16 panD in medium containing 3H-labeled β-alanine essentially as described by Rock and Cronan (9).

Preparation of Apo-ACP. Purified holo-ACP was treated with the specific phosphodiesterase described above to cleave the phosphopantetheine moiety from serine-36. The progress of the hydrolysis was monitored in a control experiment by measuring the release of labeled phosphopantetheine from labeled ACP as described above. Conditions were found in which at least 90% of the labeled ACP was cleaved in the control incubation. The non-ACP impurities contributed by the crude phosphodiesterase preparation were removed by fractionation with ammonium sulphate and chromatography on Sephadex G-50 as described by Therisod et al. (4) for ACP purification. The final preparation contained only traces of holo-ACP as judged from gel electrophoresis (Fig. 1A).

*E. coli* strain MP4 acpS, defective in the enzyme catalyzing the transfer of phosphopantetheine from coenzyme A to apo-ACP, has been shown by Polacco and Cronan (10) to contain a mixture of apo-ACP and holo-ACP. An extract of these cells was prepared and partially purified by ammonium sulfate fractionation for use as a standard to localize apo-ACP on nondenaturing gels (Fig. 1).

Preparation of Palmitoyl-ACP. Palmitoyl-ACP was prepared enzymatically by a procedure based on that of Rock and Cronan (7). The incubation mixture contained 0.1 M Tris-HCl (pH 8.0), 0.4 M LiCl, 5 mM ATP, 10 mM MgCl₂, 2 mM dithiothreitol, 80 µM palmitic acid, 4 µM [3H]ACP labeled in the phosphopantetheine moiety as described above; and ACP acylase (10 to 20 µl) of a preparation (see above) containing Triton X-100 (20 mg/ml). The final volume was 40 µl. The high ionic strength of this incubation mixture is needed for optimum activity of the enzyme (7). Incubation was carried out for 1 hr at 37°C.

After the incubation, the reaction mixture was diluted with three volumes of 10 mM Tris-HCl (pH 8.0) to reduce the ionic strength and loaded onto a small (0.5-ml) column of DEAE-cellulose. The adsorbed ACP was then washed successively with a few milliliters of 20 mM Tris-HCl (pH 7.4) to remove Triton X-100; with 80% (vol/vol) propanol to remove fatty acid; and with aqueous buffer to remove the propanol. Palmitoyl-ACP and residual unacylated ACP were finally eluted with 0.5 M KCl/20 mM Tris-HCl, pH 7.4.

Palmitoyl-ACP was separated from unacylated ACP by hydrophobic chromatography on octyl-Sepharose (Fig. 2). Palmitoyl-ACP is retained more strongly than ACP itself, as earlier reported by Rock and Jackowski (6). Electrophoretic analysis of the palmitoyl-ACP on a nondenaturing polyacryl-
phosphopantetheine prosthetic group is not needed for the activity of ACP, we next removed the prosthetic group entirely by treatment with the specific phosphodiesterase. The resulting purified apo-ACP (Fig. 1A) was fully as active as holo-ACP (Fig. 3).

**Activity of Palmitoyl-ACP.** Fatty acyl derivatives of ACP are essential intermediates in lipid synthesis. To determine whether such derivatives may also function in the activation of the transglucosylase, we tested 3-palmitoyl-ACP, prepared as described above. As seen in Fig. 4, the activity of palmitoyl-ACP differs little from that of ACP itself. Interpretation of this result is complicated by the fact that the palmitoyl residue is readily cleaved from the ACP by membrane preparations from *E. coli* as reported by Jackowski *et al.* (12) and confirmed in our study (data not shown), making it difficult to determine the true ratio of the activity of the acyl derivative to that of the unacylated ACP. The significance of the experiment, however, lies in the fact that acylation of ACP causes a striking shift in its elution from octyl-Sepharose (Fig. 2), leading to its ready separation from unacylated species. It is clear that the same molecules of ACP that are acylated are also active in the transglucosylation reaction, whether before or after deacylation.

**DISCUSSION**

A characteristic feature of ACPs throughout nature is the presence of covalently bound phosphopantetheine, which is essential for their role in lipid biosynthesis. Like the ACP of *E. coli*, the ACPs of many other bacteria and plants are small, highly anionic proteins. In contrast, in fatty acid synthase complexes of animal tissues and yeast, ACP represents one domain of a very large multifunctional fatty acid synthase complex.

The discovery of an essential function for the ACP of *E. coli* in a transglucosylase reaction needed for the synthesis of a cell-surface carbohydrate (4) was unexpected because that function bears no apparent relation to the role(s) of ACP in lipid biosynthesis. This disparity is further emphasized by the present result that apo-ACP is fully as active as holo-ACP in the transglucosylation system.

This result raises the question as to whether apo-ACP or holo-ACP is the active factor in vivo. Jackowski and Rock (5) found that apo-ACP is only a very small fraction of the total ACP pool in *E. coli*, making it likely that the holo form is quantitatively more important in living cells.

**Fig. 3.** Activity of apo-ACP and holo-ACP in the transglucosylation system. Amounts of the two forms of ACP in the standard assay system were as indicated. Open circles, holo-ACP; solid circles, apo-ACP.

The presence in *E. coli* of an enzyme, discovered by Vagelos and Larrabee (8), that specifically cleaves the phosphopantetheine residue from holo-ACP with the production of the apo form requires reevaluation. It was thought (5) that this enzyme might regulate the synthesis of fatty acids by controlling the amounts of holo-ACP needed for that process, but this hypothesis seems to be ruled out by Jackowski and Rock (5) who showed that the ratio of holo-ACP to apo-ACP is always very high and cannot be related to rates of lipid biosynthesis. Perhaps the phosphodiesterase functions to assure a small but essential pool of apo-ACP for systems other than lipid biosynthesis pathways.

The possibility was considered that a closely similar protein, other than ACP itself, might be the factor needed for transglucosylation. Such a protein might be a persistent impurity in ACP preparations but would not be substrate for enzymic acylation. The finding in the present study that the same ACP molecules that are enzymatically acylated (which permits them to be chromatographically separated from unacylated ACP as in Fig. 2) also function in the transglucosylation system (Fig. 4) is strong evidence that ACP itself is needed for the transglucosylation that in turn is essential for the biosynthesis of MDO.

Recent work has greatly extended our view of the importance of periplasmic oligosaccharides in the osmotic adaptation of Gram-negative bacteria generally. Cyclic glucans characteristic of *Rhizobium* and *Agrobacterium* like MDO of *E. coli* are localized in the periplasm, and their synthesis is regulated osmotically in the same pattern as in *E. coli* (13). It has also been shown that these cyclic glucans may be substituted with one to three residues of sn-glycerol 1-phosphate per mol (14, 15). These substituents are highly characteristic features of the MDO of *E. coli*. It now seems clear that the MDO of *E. coli* and the cyclic glucans of the *Rhizobiaceae* are closely analogous in structure, in intracellular localization, and in at least some functions.

The cyclic glucans of *Rhizobium* are of particular interest because they are thought to be involved in the signaling between the bacterium and specific plant hosts that is needed for effective nodulation leading to symbiotic nitrogen fixation (16, 17). In the light of the general resemblance of the glucans to MDO of *E. coli*, it seems reasonable to suppose that the biosynthesis of the cyclic glucans may resemble that of MDO in some features at least. For this reason, it is of particular interest that Shearman *et al.* (18) have deduced from the DNA sequence that the nodF gene of *Rhizobium leguminosarum* encodes an acyl carrier protein like that of *E. coli*. Similarly, Horvath *et al.* (19) have reported that the hnsA gene of *Rhizobium meliloti* (which is the analog of the nodF gene of *Rhizobium leguminosarum*) also encodes an *E. coli*
coli-like ACP. These rhizobial gene products are thought to play some as yet unidentified role in the nodulation process. Because the nodF and hsnA genes are not needed for the growth of cells of *R. leguminosarum* and *R. meliloti*, respectively, their gene products cannot be needed for the synthesis of essential lipids. We suggest that the ACP species encoded by such nodulation genes function in the synthesis of some specific cell-surface carbohydrates involved in cell–cell signaling needed for productive nodulation, in analogy to the function of ACP in the synthesis of MDO.

The nodF and hsnA gene products are closely similar to the ACP of *E. coli* in the highly conserved region near serine-36 of the *E. coli* protein, the point of attachment of the phosphopantetheine prosthetic group. Nevertheless, the possibility must now be considered that these “specialized” ACP species may not be substituted with phosphopantetheine, since such substitution is not needed for transglucosylation function in *E. coli*.

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