Domains of *Escherichia coli* Acyl Carrier Protein Important for Membrane-Derived-Oligosaccharide Biosynthesis

LUJIA TANG,† AUDREY C. WEISSBORN,* AND EUGENE P. KENNEDY

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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Acyl carrier protein participates in a number of biosynthetic pathways in *Escherichia coli*: fatty acid biosynthesis, phospholipid biosynthesis, lipopolysaccharide biosynthesis, activation of prohemolysin, and membrane-derived oligosaccharide biosynthesis. The first four pathways require the protein's prosthetic group, phosphpantetheine, to assemble an acyl chain or to transfer an acyl group from the thioester linkage to a specific substrate. By contrast, the phosphopantetheine prosthetic group is not required for membrane-derived oligosaccharide biosynthesis, and the function of acyl carrier protein in this biosynthetic scheme is currently unknown. We have combined biochemical and molecular biological approaches to investigate domains of acyl carrier protein that are important for membrane-derived oligosaccharide biosynthesis. Proteolytic removal of the first 6 amino acids from acyl carrier protein or chemical synthesis of a partial peptide encompassing residues 26 to 50 resulted in losses of secondary and tertiary structure and consequent loss of activity in the membrane glucosyltransferase reaction of membrane-derived oligosaccharide biosynthesis. These peptide fragments, however, inhibited the action of intact acyl carrier protein in the enzymatic reaction. This suggests a role for the loop regions of the *E. coli* acyl carrier protein and the need for at least two regions of the protein for participation in the glucosyltransferase reaction. We have purified acyl carrier protein from eight species of *Proteobacteria* (including representatives from all four subgroups) and characterized the proteins as active or inhibitory in the membrane glucosyltransferase reaction. The complete or partial amino acid sequences of these acyl carrier proteins were determined. The results of site-directed mutagenesis to change amino acids conserved in active, and altered in inactive, acyl carrier proteins suggest the importance of residues Glu-4, Gln-14, Glu-21, and Asp-51. The first 3 of these residues define a face of acyl carrier protein that includes the beginning of the loop region, residues 16 to 36. Additionally, screening for membrane glucosyltransferase activity in membranes from bacterial species that had acyl carrier proteins that were active with *E. coli* membranes revealed the presence of glucosyltransferase activity only in the species most closely related to *E. coli*. Thus, it seems likely that only bacteria from the *Proteobacteria* subgroup γ-3 have periplasmic glucans synthesized by the mechanism found in *E. coli*.

Acyl carrier protein of *Escherichia coli* is a small (77-amino-acid) acidic protein that plays an essential role in several biosynthetic pathways: fatty acid biosynthesis (reviewed in reference 39), phospholipid biosynthesis (reviewed in reference 3), lipopolysaccharide biosynthesis (reviewed in reference 28), and activation of prohemolysin (11). All of these functions require the presence of the phosphopantethein esthetic group on serine 36 of acyl carrier protein. The terminal thiol on this prosthetic group is the site of assembly of the fatty acids, and subsequently, specific acyl chains are transferred to their respective designated substrate: glycerophosphatides, lipid A, or prohemolysin.

In other species of bacteria, specialized acyl carrier proteins function in unique pathways: the *nodF* (*Rhizobium*) gene product for lipochitin oligosaccharide synthesis (reviewed in reference 35), the α-alanyl carrier protein (*Lactobacillus*) for lipoteichoic acid synthesis (8), and acyl carrier proteins specific for polyketide synthesis (reviewed in reference 10). All of these reactions have in common the required use of the phosphopantethein esthetic group for the synthesis or activation of their specific acyl moieties.

In *E. coli*, a role for acyl carrier protein has been identified that does not require the presence of the phosphopantethein esthetic group (37). Acyl carrier protein functions in an unknown way in the biosynthesis of membrane-derived oligosaccharides. A membrane glucosyltransferase has been identified that synthesizes a linear β1→2 linked glucan from UDP-glucose and a β-glucoside primer only when acyl carrier protein is present (38, 42).

Membrane-derived oligosaccharides are a heterogeneous group of branched (β1→6 linkages), substituted (with phosphoglycerol, phosphoethanolamine, and succinate) glucans with a β1→2-linked backbone (41). These molecules are found in the periplasmic space of the bacterium (33). The synthesis of these periplasmic glucans is greatly increased when the bacterium is growing in low-osmolarity environments, and thus, they are thought to help the bacterial cell maintain its osmotic equilibrium in such environmental conditions (15).

Periplasmic glucans that are similar in structure and also are synthesized in response to environments of low osmolarity have been identified in the enteric bacteria that are closely related to *E. coli* (33) and, more recently, in the plant pathogen *Pseudomonas syringae* (36). In *P. syringae*, an open reading frame of the *hrpM* locus that is involved in bacterial pathogenicity and the hypersensitivity response of plants (23) has a deduced protein sequence that bears a striking similarity to the deduced sequence of the protein encoded by the *mdoH* gene of *E. coli* (20). In *E. coli* the *mdoH* gene is necessary for the
expression of the membrane glucosyltransferase that is involved in periplasmic glucan synthesis, although it has not yet been shown to be the structural gene for this enzyme.

Periplasmic glucans that are synthesized in response to environments of low osmolarity have also been found in members of the \textit{Rhizobiaceae} family of bacteria (reviewed in reference 2) and are required for the interaction between these bacteria and their particular plant host. However, the glucans in this family of bacteria are cyclic and are synthesized by a mechanism that does not utilize acyl carrier protein.

The multitude of biosynthetic schemes in which acyl carrier protein participates requires that this protein interact with many enzymes in a specific way. In addition, in those bacterial species that possess more than one acyl carrier protein, the channeling of each acyl carrier protein to its own set of reactions requires further determinants of specificity. It is therefore of special interest to determine the molecular basis of the many functions of the small but highly versatile acyl carrier protein.

The recent publications of Olsen et al. (25) and Woese (45) define the evolutionary relatedness of bacteria based on their 16S rRNA sequences. In the resulting tree, the purple bacteria \textit{(Proteobacteria)} are divided into four subgroups: \(\alpha\), \(\beta\), \(\gamma\), and \(\delta/\varepsilon\). Although periplasmic glucans are found in the \(\alpha\) subgroup (\textit{Rhizobiaceae} family) and \(\gamma\) subgroup (\textit{E. coli} and \textit{P. syringae}), it is currently unknown whether periplasmic glucans are present in members of the \(\beta\) or \(\delta/\varepsilon\) subgroup. The phylogenetic groupings of Olsen et al. (25) and Woese (45) posed the interesting possibility that examples of naturally occurring “mutations” in acyl carrier protein could be found in bacteria that are near relatives of \textit{E. coli} and that these would provide clues as to which residues are important for the function of acyl carrier protein in membrane-derived oligosaccharide biosynthesis in \textit{E. coli}.

Here we have determined the amino acid sequences of acyl carrier proteins from the various subgroups of purple bacteria including marine bacteria \textit{(Oceanospirillum linearum} and \textit{Leurothrix mucor}) from the \(\gamma\) subgroup. Because the marine bacteria would presumably not need periplasmic glucans for adaptation to low-osmolarity environments, any constraint imposed on the structure of acyl carrier protein by the biosynthesis of membrane-derived oligosaccharides would be missing in these strains. We thus identified amino acids that appear to be conserved only in acyl carrier proteins that can substitute for the \textit{E. coli} acyl carrier protein in the glucosyltransferase reaction. We then tested the postulated importance of these amino acids by altering them using site-directed mutagenesis. In addition, we enzymatically and chemically modified the \textit{E. coli} acyl carrier protein to obtain further clues about the regions of acyl carrier protein necessary for participation in the glucosyltransferase reaction.

**Materials and Methods**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. The strains from the American Type Culture Collection were grown with the media and growth temperature recommended in the catalog. \textit{E. coli} DF214 was grown in medium 63 (22) supplemented with 1% Casamino Acids as previously described (42).

**Preparation of membrane glucosyltransferase.** The membrane fraction was prepared essentially as described in Weissborn et al. (44), but the membranes were not washed before storage. Before use in the glucosyltransferase assay, the \textit{E. coli} membrane suspension was incubated with a titrated aliquot of the immunoglobulin fraction of a polyclonal antibody raised against the \textit{E. coli} acyl carrier protein. This treatment inactivated the residual acyl carrier protein that was still bound to the membranes.

**Purification of acyl carrier protein.** Six to 10 liters of the bacterial culture was harvested after growth reached the mid-log phase. All steps of the purification were done at 4°C. The cells were harvested by centrifugation at 4,000 \(\times\) g for 15 min. For resuspension of the cell pellet, 4 ml of 10 mM Tris-HCl, pH 7.8, containing 5 mM dithiothreitol was added for every gram (wet weight) of pellet. The cells were broken in an Aminco French pressure cell at 16,000 lb/in\(^2\). Unbroken cells were removed by centrifugation at 5,000 \(\times\) g for 15 min. The membranes were then removed by centrifugation at 39,000 \(\times\) g for 90 min. Streptomycin sulfate was added until the final weight ratio of streptomycin to protein was 0.6. After the solution was stirred for 20 min, the pH of the solution was adjusted to 5.2 with acetic acid, and the solution was held overnight. The precipitate was removed by centrifugation at 39,000 \(\times\) g for 90 min. The supernatant was then loaded (10 to 15 mg of protein/ml of bed volume) onto a DEAE-cellulose column ((DE52, Whatman) equilibrated in 50 mM sodium acetate, pH 5.2, containing 5 mM dithiothreitol. After the sample was loaded, the column was washed with 1 column volume of the equilibration buffer at a flow rate of 15 ml/h. Fractions of 2 ml were collected. The column was then developed with a gradient of total volume equal to five times the column volume. Equilibration buffer containing 0.1 M NaCl was the starting buffer, and equilibration buffer containing 0.6 M NaCl was the limit buffer. At the end of the gradient, the column was washed with 1 column volume of the limit buffer. The fractions containing acyl carrier protein were detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the glucosyltransferase assay, and/or the acyl carrier protein synthesize assay (30) with modifications described in reference 27]. These fractions were then pooled and concentrated by using a 3,000 molecular-weight cutoff Centrex filter (Schleicher and Schuell). The sample was then loaded onto a Sephadex G-50 Superfine (Pharmacia LKB) column (1.1 by 95 cm) that was equilibrated in 50 mM ammonium acetate containing 5 mM dithiothreitol. The column was eluted at a flow rate of 8 ml/h, and fractions of 1.5 ml were collected. The fractions containing acyl carrier protein were detected as described for the DEAE-cellulose column and then pooled and concentrated as before. The yield of acyl carrier protein from the various bacteria averaged 100 \(\mu\)g per gram of cells (wet weight).

**Protein determination.** Protein concentrations were determined by the Bio-Rad protein assay with ovalbumin as a standard, the micro-BCA (bicinchoninic acid) protein assay (Pierce) with bovine serum albumin as a standard, or amino acid analysis.

**Polyacrylamide gel electrophoresis.** For denaturing gels, the procedure of Laemmli (18) was generally used with a 15% acrylamide separating gel. However, for the acyl carrier proteins that migrated rapidly as expected for their true size, the procedure of Schägger and von Jagow (32) was developed for low-molecular-weight proteins used.

**Membrane glucosyltransferase assay.** The in vitro production of \(\beta\)-1,2-linked glucosyl oligosaccharides from UDP-glucose and a \(\beta\)-glucoside primer was measured by the assay procedure of Weissborn et al. (44) with the following changes in concentration of some components: 1 mM UDP-[\(\beta\]-\(\beta\)]Hglucose at a specific activity of 2,000 cpm/nmol, 2 mM octyl-\(\beta\)-D-glucoside, 10 \(\mu\)g of membrane protein, and acyl carrier protein in the amounts indicated for the individual experiments.

A determination of the \(K_m\) and \(V_{max}\) for each acyl carrier protein was made by determining the velocity of the reaction with four to seven different concentrations of acyl carrier protein both below and above the estimated concentration required for one-half maximal velocity. The results were analyzed by the double-reciprocal plot of Michaelis and Menten, and the best-fitting line for the points was determined by linear regression.

**Synthesis of peptides representing fragments of the \textit{E. coli} acyl carrier protein.** Three peptides that encompassed amino acids 1 to 25, 26 to 50, and 51 to 75 of the intact protein were synthesized in the Biopolymer Facility of our department.

**Clostripain digestion of the \textit{E. coli} acyl carrier protein.** Clostripain (Worthington) at a concentration of 2 mg/ml was activated in 1.0 mM calcium acetate containing 2.5 mM dithiothreitol by incubation at 4°C overnight. Aeryl carrier protein was digested with clostripain for 3 h at 37°C at a ratio of the protein to acyl carrier protein of 1:25, wt/wt. The clostripain was inactivated by the addition of...
a 10-fold molar excess of N-α-tosyl-l-lysine chloromethyl ketone (Sigma). The digested acyl carrier protein was purified on a Sephadex G-50 column and concentrated by centrifugal ultrafiltration as described above.

**Circular dichroism spectra of the E. coli acyl carrier protein.** Circular dichroism measurements were made at 22°C in a cuvette with a 0.1-cm path length. A stock solution of acyl carrier protein in 20 mM potassium phosphate buffer, pH 7, containing 1 mM dithiothreitol was diluted to the various concentrations of protein used for the measurement of the spectra. The protein concentration of the stock solution was determined by amino acid analysis.

**Acetylation of lysines of the E. coli acyl carrier protein.** Acyl carrier protein was acetylated with the reagent succinimidyl acetyl carbonate (Pierce). Five hundred micrograms of acyl carrier protein was mixed with a 50- or 100-fold molar excess of the reagent in 0.1 M sodium borate, pH 8.8. The reaction mixture was incubated for 4 h at 23°C, and then excess ammonium chloride was added to react with unused reagent. The acyl carrier protein was separated from excess reagent on a 10-ml Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 7.2. The extent of acetylation was monitored by assay for available amino groups with the reagent 2,4,6-trinitrobenzenesulfonic acid according to the procedure of Fields (5). 1-Alanine was used as the amino group standard. In a mock acetylation, the reagent and the amine groups were mixed together before the protein was added.

**Amino-terminal amino acid sequences of acyl carrier proteins from Acinetobacter calcoaceticus, Alcaligenes faecalis, and Erthrobacter longus and the complete amino acid sequence of the P. syringae acyl carrier protein.** The partial or complete amino acid sequences of acyl carrier proteins from Acinetobacter calcoaceticus, Alcaligenes faecalis, Erthrobacter longus, and P. syringae were determined in the Biopolymer Facility of the Howard Hughes Medical Institute, Harvard Medical School, Boston, Mass. The complete sequence of the P. syringae acyl carrier protein was assembled from the sequence of peptides resulting from digestion of acyl carrier protein with trypsin or endoproteinase Asp-N.

**Amino acid sequences of acyl carrier proteins from C. testosterone, M. xanthus, O. linum, and L. mucor.** The complete sequences of acyl carrier proteins from Comamonas testosterone, Myxococcus xanthus, O. linum, and L. mucor were determined directly on a Hewlett-Packard HP G1000A protein sequencer in the Microchemistry Facility, Harvard University, Cambridge, Mass. Ambiguities in the sequence of acyl carrier protein from O. linum were resolved by sequencing the peptides that were produced by digestion of this protein with endoproteinase Asp-N.

**Construction of the maltose-binding-protein–acyl carrier protein fusion.** All molecular biological procedures followed protocols described by Sambrook et al. (31). The synthetic E. coli acyl carrier protein gene that was constructed by Cronan et al. (4) and Rawlings and Cronan (29) was removed from its vector plasmid by digestion with EcoRI and HindIII and inserted into the plasmid pMAL-c2 (New England BioLabs) that had been similarly digested. Subsequent, site-directed mutagenesis by the PCR method described by Picard et al. (26) was used to introduce a SalI site into the DNA sequence encoding the amino terminus of acyl carrier protein. The extra five amino acids between the Factor Xa protease cleavage site of the vector and serine 1 of acyl carrier protein were then eliminated by removing the small XmnI-SalI fragment from the plasmid, filling in the recessed 3' end of the SalI-digested end with the Klenow fragment of DNA polymerase I, and finally ligating the two ends with T4 DNA ligase.

**Preparation and purification of the maltose-binding-protein–acyl carrier protein fusion protein.** The maltose-binding protein and subsequent purification of a free acyl carrier protein. The induced expression of the fusion protein by isopropyl-β-D-thiogalactopyranoside was followed by the procedure described elsewhere (24a). After induction, a cell extract was made and processed in the same way as described above for the purification of the protein through the precipitation step. The precipitated protein was then bound to a DEAE-cellulose column prepared as described above. After elution of unwanted proteins with a 0.1 M NaCl wash, the fusion protein was eluted with a 0.35 M NaCl wash. The pooled fractions were applied to a 1.1- by 95-cm Sephadex G-75 Superfine column equilibrated in 50 mM NH₄ acetate containing 5 mM dithiothreitol. This column generates a mother that contains the separated cellular acyl carrier protein from the larger fusion protein. The acyl carrier protein was then cleaved from the fusion protein by digestion with Factor Xa protease for 18 h at 25°C at an enzyme/protein ratio of 1:50 or 1:100 in a reaction mixture containing Tris-Cl, pH 8, and 2 mM CaCl₂. The acyl carrier protein was then purified from uncleaved fusion protein and maltose-binding protein first by rechromatography on the Sephadex G-75 column followed by binding to a DEAE-cellulose column prepared at pH 5.2 as described previously. The maltose-binding protein remaining in the sample was washed off the column with 200 mM NaCl, and acyl carrier protein was eluted as a concentrated sample with 500 mM NaCl. The final acyl carrier protein sample was desalted, and the buffer was changed to 50 mM ammonium acetate containing 5 mM dithiothreitol and then lyophilized.

**RESULTS**

**Purification of acyl carrier protein from selected purple bacteria.** Acyl carrier proteins were isolated from a selection of purple bacteria (Table 1) by essentially the same procedure as that described for the E. coli acyl carrier protein (see Materials and Methods). The final purified proteins were examined by SDS-polyacrylamide gel electrophoresis run with either the Laemmli (18) buffer system (Fig. 1A) or the Schägger and von Jagow (32) (B). The positions of migration of standard proteins are indicated. E. coli, P. syringae, P. syringae, E. coli, P. syringae, P. syringae, M. xanthus, M. xanthus, A. faecalis, C. testosterone.

DNA sequences of the final selected clones were determined to confirm the presence of the encoded acyl carrier protein. The appropriate purification kit from Qiagen was used for the purification of DNA from the PCR reactions, from gels, and from cultures. Sequencing version 2.0 (Amersham) was used for DNA sequencing.

**Sequence accession numbers.** The amino acid sequence data will appear in the Swiss-Prot Protein sequence database under the accession numbers P80916 (Acinetobacter calcoaceticus), P80917 (Alcaligenes faecalis), P80919 (Erthrobacter longus), P80923 (P. syringae), P80918 (C. testosterone), P80921 (M. xanthus), P80922 (O. linum), and P80920 (L. mucor).

**Characterization of the activities of acyl carrier proteins from purple bacteria in the glucosyltransferase reaction.** The purified acyl carrier proteins were substituted for the E. coli acyl carrier protein in the otherwise-standard glucosyltransferase reaction of membrane-derived oligosaccharide biosyn-
The apparent concentration of acyl carrier protein in the membrane glucosyltransferase reaction is about one-half that seen with the wild-type acyl carrier protein. Both the $K_m$ and the $V_{\text{max}}$ values for the acyl carrier protein from one of the tested purple bacteria were similar, however, in the 1- to 2-$\mu$M range. This corresponds to an assay concentration of approximately 0.1 to 0.2 $\mu$M. It is interesting to note that the concentration of acyl carrier protein in the $E.\ coli$ cell is near 100 $\mu$M (calculated from references 3 and 24). The $K_m$ and $V_{\text{max}}$ values for these two acyl carrier proteins and for the other tested acyl carrier proteins were determined from a Michaelis-Menten analysis of the data as described in Materials and Methods. The variation in the absolute value of the maximal activity for $E.\ coli$ acyl carrier protein among the experiments in Fig. 2 (and in later experiments) is a reflection of the age of the membrane preparation. The membrane glucosyltransferase activity gradually declines over a period of several months. Thus, the absolute value of $V_{\text{max}}$ in any given experiment is a function of membrane age. However, the ratio of $V_{\text{max}}$ for the acyl carrier protein from one of the tested purple bacteria to the $V_{\text{max}}$ for the acyl carrier protein from $E.\ coli$ remains the same from experiment to experiment. Thus, the maximal velocity of the reaction for each acyl carrier protein is not used. Instead, the ratio of the calculated $V_{\text{max}}$ of the tested acyl carrier protein to the calculated $V_{\text{max}}$ of the $E.\ coli$ wild-type acyl carrier protein is cited.

Figure 2B compares the activity of the $E.\ coli$ acyl carrier protein to the activities of acyl carrier proteins from the remaining $\gamma$-subgroup bacteria ($L.\ mucor$, $O.\ linum$, and $Achrobacter longus$). The standard glucosyltransferase assay with $E.\ coli$ membranes was used except that the indicated concentrations of acyl carrier protein (ACP) from the indicated bacteria were added. (A and B) $\gamma$ subgroup; (C) $\beta$ subgroup; (D) $\alpha$ and $\delta/e$ subgroups. In each case the activity of the acyl carrier protein was compared to that of the $E.\ coli$ acyl carrier protein. See the legend to Fig. 1 for abbreviations.

![Image](http://jb.asm.org/)

**TABLE 2. Inhibition of glucosyltransferase by inactive acyl carrier proteins**

<table>
<thead>
<tr>
<th>Source of protein</th>
<th>$\mu$g/ml for 50% inhibition</th>
<th>No. of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Different</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>200</td>
<td>69</td>
</tr>
<tr>
<td><em>Leucothrix mucor</em></td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td><em>Mycococcus xanthus</em></td>
<td>50</td>
<td>78</td>
</tr>
<tr>
<td>Synthetic peptide b</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Proteolytic fragment c</td>
<td>40</td>
<td>71</td>
</tr>
</tbody>
</table>

*The standard glucosyltransferase assay was used except that 1 $\mu$g of the $E.\ coli$ acyl carrier protein was added per ml as the control amount of acyl carrier protein, with various amounts of the inhibitory acyl carrier protein added to determine the amount of foreign acyl carrier protein needed for 50% inhibition.*

* Including 1 unidentified residue.

* Residues 26 to 50 of the $E.\ coli$ acyl carrier protein.

* Residues 7 to 77 of the $E.\ coli$ acyl carrier protein.
reaction, three peptides encompassing amino acids 1 to 25, 26 to 50, and 51 to 75 of the **E. coli** acyl carrier protein were synthesized. These peptides were tested singly and in combination for their activating and/or inhibiting properties. Of these three peptides, only the peptide encompassing residues 26 to 50 showed a significant activity, and that was inhibition of the glucoyltransferase reaction. Fifty-percent inhibition of the reaction was observed when 100 µg/ml was added to the reaction mixture. Although this concentration represents a 370 molar excess over intact acyl carrier protein, the observation of inhibition, nevertheless, supports the idea that part or all of the region of amino acids 26 to 50 showed a significant activity, and that was inhibition of 50% inhibition occurring at a 40-fold molar excess of the digested acyl carrier protein.

### Amino acid sequences of acyl carrier proteins from purple bacteria

From the known amino acid sequences of acyl carrier proteins from purple bacteria (**E. coli**, **V. harveyi**, **Rhizobium meliloti**, and **Rhodobacter sphaeroides**), a number of residues could be identified that are always conserved in the acyl carrier proteins that are active in the glucoyltransferase reaction but not in the inactive proteins. These are Glu-4, Ile-11, Gin-14, Glu-21, Asn-24, Val-29, Leu-46, Asp-51, Thr-52, Glu-57, Glu-58, Lys-61, Thr-63, Gin-66, Ala-67, Ile-69, Asp-70, Tyr-71, and Asn-73 (Fig. 4). To investigate further the importance of these residues, we determined the complete amino acid sequences of acyl carrier proteins from the bacteria **P. syringae** (active), **O. linum** (active), **C. testosteroni** (active), **M. vulgaris** (inactive), and **M. xanthus** (inactive). In addition, the sequence of the first 25 amino acids was determined for three species: **Alcaligenes faecalis** (active), **Acinetobacter calcoaceticus** (inactive), and **Erythrobacter longus** (inactive). These sequences are shown in Fig. 4. The amino acid sequence determinations revealed the following residue identities with the **E. coli** acyl carrier protein: **P. syringae**, 69 of 77; **O. linum**, 64 of 76; **C. testosteroni**, 59 of 77; **M. vulgaris**, 60 of 76; **M. xanthus**, 27 of 78 (including 1 unidentified residue). The asterisk at the position of serine 36 of **E. coli**, the site of phosphopantetheine attachment, indicates that no residue was identified at that position during automated sequencing. This is the expected result if the serine is substituted with phosphopantetheine. All five of these acyl carrier proteins can act as substrates for the **E. coli** enzyme acyl-acyl carrier protein synthetase and can be monitored during the course of purification by assay with this enzyme. The accuracy (Fig. 3B versus 3C). This unfolded 71-amino-acid fragment of acyl carrier protein could still function to inhibit the glucoyltransferase reaction (Table 2), with 50% inhibition occurring at a 40-fold molar excess of the digested acyl carrier protein.

![FIG. 3. Circular dichroism spectra of the **E. coli** acyl carrier protein. (A) Spectra of untreated acyl carrier protein at concentrations of 100 µM (closed circles) and 10 µM (open circles); (B) spectrum of the clostripain digest of the **E. coli** acyl carrier protein; (C) spectrum of untreated acyl carrier protein (10 µM) in 8 M urea.](http://jb.asm.org/)

![FIG. 4. Complete and partial amino acid sequences of acyl carrier proteins from purple bacteria. The acyl carrier proteins that are active in the glucoyltransferase reaction are listed first, followed by the inactive acyl carrier proteins. The residues are numbered as in the **E. coli** protein. The amino acids that are identical to the corresponding residues in the **E. coli** protein are shaded. The **E. coli** sequence is from articles by Vanaman et al. (40), Jackowski and Rock (12), and Rawlings and Cronan (29); the **V. harveyi** sequence is from the article by Shen and Byers (34); the **Rhizobium meliloti** sequence is from the article by Platt et al. (27); and the **Rhodobacter sphaeroides** sequence is as cited by Platt et al. (27). * and ? no residue identified in that cycle of the automated sequencer. The asterisks are at the position expected for the serine to which the phosphopantetheine is attached.](http://jb.asm.org/)
Acetylation of the lysines of the *E. coli* acyl carrier protein. Previous studies had shown that when its lysine residues were acetylated, acyl carrier protein retained its ability to participate in fatty acid biosynthesis (21). The importance of the lysine residues for the participation of acyl carrier protein in membrane-derived oligosaccharide biosynthesis was of some interest because of the reported importance of lysine residues in enzymes that utilize nucleotide sugars as a substrate (13, 14). We acetylated the lysines as described in Materials and Methods and subsequently analyzed the acetylated protein for the presence of any remaining amino groups. This analysis showed that, with a 50- or 100-fold excess of acetylation reagent, only 0.6 or 0.4 mol, respectively, of amino group equivalents remained of the original 5 mol in the protein. This value is consistent with the essentially complete acetylation of the four lysines and the amino-terminal amino group. *E. coli* acyl carrier protein acetylated with a 50- or 100-fold molar excess of the reagent essentially retained its full activity in the glucosyltransferase reaction. Lys-61 is therefore not an important residue for the activity of acyl carrier protein in the glucosyltransferase reaction.

Site-directed mutagenesis of the *E. coli* acyl carrier protein. Now that invariant amino acid residues had been identified, the candidate residues for site-directed mutagenesis could be chosen. Replacement residues were chosen by picking an amino acid that appeared in an inactive acyl carrier protein at the position of interest. The strategy for constructing a plasmid that contained the acyl carrier protein gene centered around the issue of being able to separate the mutagenized acyl carrier protein from the cellular, wild-type acyl carrier protein. To that end the synthetic gene for acyl carrier protein constructed by Cronan and colleagues (4, 29) was joined to the maltose-binding protein as described in Materials and Methods. The substantially larger size of the fusion protein relative to acyl carrier protein itself allowed for easy elimination of the cellular acyl carrier protein during gel filtration chromatography. The mutations were then created with mutagenic oligonucleotides as described in Materials and Methods. Table 3 lists the sites and the residue changes that were made. The final purified mutant acyl carrier proteins all had essentially the same mobility as the wild-type *E. coli* acyl carrier protein when analyzed by SDS-polyacrylamide gel electrophoresis (data not shown).

Characterization of the activity of the mutagenized *E. coli* acyl carrier protein in the glucosyltransferase reaction. The mutant acyl carrier proteins prepared above were all tested in the membrane glucosyltransferase reaction of *E. coli*. The $K_m$ and the ratio $V_{max}/E. coli$ $V_{max}$ (determined as described in Materials and Methods and as discussed above) of each mutant acyl carrier protein in the glucosyltransferase reaction are listed in Table 3. Of the 10 amino acid substitutions made, the changes E4A, Q14H, and E21K resulted in a significant increase in the $K_m$ and the change D51G resulted in significant decrease in the $V_{max}/E. coli$ $V_{max}$ ratio.

**Assay of membrane glucosyltransferase in bacteria possessing transferase-active acyl carrier protein.** Membrane-derived oligosaccharides have so far been found only in members of the γ subgroup that are very closely related to *E. coli* (33, 36). Are periplasmic glucans that are similar to membrane-derived oligosaccharides found in less closely related purple bacteria? Perhaps the presence of an acyl carrier protein that can substitute for the *E. coli* acyl carrier protein in the glucosyltransferase reaction of membrane-derived oligosaccharides will provide an initial screen for bacteria with periplasmic glucans synthesized by the mechanism utilized by *E. coli*. The membrane fraction was prepared from *P. syringae*, *O. linum*, *C. testosteroni*, and *Alcaligenes faecalis* as described in Materials and Methods. Glucosyltransferase activity was present in *P. syringae* as expected from the presence of the mdoGH-like gene cluster (the hrpM locus) and genetic complementation of an *E. coli* strain defective in the mdoH gene with the corresponding gene from *P. syringae* (19, 20). The membranes from *P. syringae* exhibit, however, a low response to added acyl carrier protein. The addition of *P. syringae* acyl carrier protein to *P. syringae* membranes resulted in just over a twofold increase in product formation (Fig. 5). The *E. coli* acyl carrier protein was more active with the *P. syringae* membranes, resulting in an almost fourfold increase in product formation (Fig. 5). The $K_m$ of the membranes from *P. syringae* for acyl carrier protein from either *E. coli* or *P. syringae* was calculated as described in Materials and Methods and after the product that was formed in the absence of any added acyl carrier protein was subtracted from each velocity measurement. The resulting $K_m$ was more than 10 times higher, in the range 25 to 35 µg/ml, than the $K_m$ of the *E. coli* membranes for the two acyl carrier proteins (1 to 2 µg/ml).

During their preparation, membranes from *E. coli* retain acyl carrier protein, which can be rendered inactive by preincubation with antibody prepared against the protein (Fig. 6). After such pretreatment, the *E. coli* membranes synthesize only a very small amount of specific glucan product, 0.1 nmol. In contrast, antibody against the *E. coli* acyl carrier protein did not cause any reduction in the membrane-associated activity from *P. syringae* (Fig. 6). *P. syringae* membranes synthesize...
As an indicator of the presence of periplasmic glucans synthesized glucosyltransferase-active acyl carrier protein cannot be used protein gave the same negative result. Thus, the finding of a ever, were completely inactive in the glucosyltransferase reaction. add to the antibody before the membranes were added to an otherwise-standard reaction. One-hundred-percent activity for the E. coli membranes (closed circles) is 1.0 nmol of product, and for the P. syringae membranes (open circles) it is 0.7 nmol of product. IgG, immunoglobulin G.

Membranes from C. testosteroni or Alcaligenes faecalis, however, were completely inactive in the glucosyltransferase reaction. Addition of either E. coli or species-specific acyl carrier protein gave the same negative result. Thus, the finding of a glucosyltransferase-active acyl carrier protein cannot be used as an indicator of the presence of periplasmic glucans synthesized by a mechanism like that for the membrane-derived oligosaccharides of E. coli.

**DISCUSSION**

Acyl carrier protein appears to have at least two domains that interact with the membrane enzyme. At least one of the domains is likely to include the loop region(s) of the protein’s structure. Another domain of acyl carrier protein that interacts with the membrane enzyme likely includes the side of helix 1 that faces the middle of the residue 16 to 36 loop (Fig. 7). In addition, Asp-51 plays an important role either indirectly by maintaining the proper tertiary structure or directly by participating in the interaction of acyl carrier protein with the membrane enzyme.

The inhibition of glucosyltransferase activity both by an unfolded protein missing only the first 6 amino acids and by a synthetic peptide encompassing the middle third of the protein suggested that a single binding site alone was not sufficient for the observed participation in the enzymatic reaction. It also suggested that portions of the loop regions of acyl carrier protein (residues 16 to 36 and 52 to 64 according to reference [17]) were candidates for the proposed interaction site. Short stretches of amino acids in these regions would likely be minimally affected by loss of helical structure. The acyl carrier protein from L. mucor is a strong inhibitor, and it has only five residue changes in the first loop and no residue changes in the second. The acyl carrier protein from M. xanthus shows modest inhibition, despite substantial sequence divergence. This suggests that a portion of one interacting site remains intact. If the amino acid residues in the M. xanthus acyl carrier protein that correspond to the two loop regions of the E. coli protein are considered, then the largest groupings of amino acids identical to those in the E. coli protein are found here (residues 27 to 36 and 54 to 58). In contrast, the acyl carrier protein from Rhodobacter sphaeroides is a poor inhibitor despite substantial sequence identity in the two loop regions, 61% (21 of 34 residues). However, this acyl carrier protein is only 69 amino acids long, and this shortening removes about one-half of the residues that would be present in the final helix of the E. coli acyl carrier protein. It seems plausible that the Rhodobacter sphaeroides acyl carrier protein has a tertiary structure in which the amino acid residues in the helices are significantly different from those of the E. coli protein. Perhaps if this protein were unfolded, it also would be a good inhibitor.

The acyl carrier protein of O. linum was found to be a poor activator. Five of the 12 amino acids that are changed from the E. coli protein occur in the two loop regions. Two of the residues that were introduced into the E. coli protein by site-directed mutagenesis in this study (A26S and A67D) were identical (A26S) or similar (A67E) to changes found in the O. linum protein. These substitutions were found, at least singly, to have no effect on the E. coli protein. Future mutagenesis of the E. coli acyl carrier protein at the remaining 10 sites that are altered in O. linum (E. coli to O. linum: I11V, G12S, Q19E, V22I, N25A, E30D, D51E, I72V, N73V, and G74S) would perhaps reveal amino acids that are important to the efficient participation of acyl carrier protein in the glucosyltransferase reaction.

Three amino acid substitutions that showed significant alterations in the ability of acyl carrier protein to participate in the biosynthetic reaction all lie on one face of acyl carrier protein: E4A, Q14H, and E21K. This aspect of the protein is the side of helix 1 (residues 3 to 15) that faces the middle of the loop region, residues 16 to 36. Figure 7 depicts this aspect of the E. coli acyl carrier protein with the side chains of residues 4, 14, and 21 depicted as their space-filling structures. Further studies that alter additional amino acids that are exposed on this face of acyl carrier protein would be of considerable interest in...
regard to detailing the role of acyl carrier protein in membrane-derived oligosaccharide biosynthesis.

Conversely, the face of acyl carrier protein that includes Ser-36 or the amino terminus of the protein is unlikely to be the locus of interaction with the membrane enzyme. The presence or absence of the phosphopantetheine moiety at Ser-36 has no effect on the ability of acyl carrier protein to participate in the glucosyltransferase reaction (37). Likewise, the presence of the maltose-binding protein fused to the amino terminus of the protein does not alter its participation in the glucosyltransferase reaction (43).

It seems likely that residue 51 (Asp) plays an important role for the participation of acyl carrier protein in the biosynthesis of membrane-derived oligosaccharides. The change from Asp to Gly in the E. coli acyl carrier protein results in a reduction in the relative maximal velocity of the reaction to 0.3 of that of wild-type acyl carrier protein. All the inactive acyl carrier proteins have a glycine at this position. Although the acyl carrier protein of C. testosteroni also has a glycine at this position, it should be noted that, of the active acyl carrier proteins, this is the one with the most amino acid changes compared to E. coli, with 18 residues that are different. One could speculate that a compensatory change, from the point of view of the biosynthesis of membrane-derived oligosaccharides, had also occurred. In contrast, L. mucor has only 16 changed residues and a glycine at position 51, yet it is unable to substitute for the E. coli acyl carrier protein in the biosynthetic reaction. Whether this residue change, D51G, affects the tertiary structure of acyl carrier protein, the cation binding site proposed by Frederick et al. (6), or a direct interaction with the membrane enzyme is an interesting subject for future investigation.

Although the role of acyl carrier protein in the biosynthesis of membrane-derived oligosaccharides remains unknown, the possibilities for the contribution this protein makes to the glucosyltransferase reaction could be summarized as follows. Either both acyl carrier protein and the membrane glucosyltransferase contribute amino acid residues for the catalytic activity or all the residues for catalytic activity reside in the membrane enzyme. In the latter case, the binding of acyl carrier protein to the membrane enzyme could perhaps produce a conformational change in the glucosyltransferase to enhance its catalytic effectiveness.

If the amino acid residues in acyl carrier protein are directly involved in the catalytic process, then one possible role could be to bind the substrate, UDP-glucose. However, the acetylation of the lysyl residues of acyl carrier protein had no effect on the participation in the glucosyltransferase reaction. The alternative is that all the catalytic residues are found in the membrane enzyme. This possibility is suggested by the limited responsiveness of the glucosyltransferase from P. syringae to added acyl carrier protein. This result could be explained in at least two ways. For example, the acyl carrier protein from P. syringae could be bound very strongly by the P. syringae membranes and thus cannot be inactivated by binding to anti-acyl carrier protein antibody, which is a weaker interaction. Furthermore, there are few unoccupied sites left on the membrane enzyme for acyl carrier protein to bind, thus resulting in limited response to the acyl carrier protein that is added. However, the idea of increased strength of binding is not supported by the finding that high concentrations of P. syringae acyl carrier protein give only a limited increment of activity (Fig. 5). Alternatively, the membranes from P. syringae could have gained some measure of independence from acyl carrier protein. The low responsiveness is due, in this case, to the poor binding of acyl carrier protein by the glucosyltransferase from P. syringae. The latter idea suggests that changes could readily occur in the membrane enzyme to compensate for the loss of acyl carrier protein participation. Such changes would most likely improve upon an activity already possessed by the membrane enzyme rather than create an entirely new function. If the mdoH gene does encode the membrane glucosyltransferase, it is interesting to note that the deduced amino acid sequence of the open reading frame of the hprM locus that can complement an mdoH defect in E. coli encodes a gene product that is only 70% identical to that of the E. coli mdoH gene product (20) whereas the acyl carrier proteins of the two species show 90% identity. The variation in the mdoH-like gene product would allow room for the proposed alterations in the membrane enzyme.

As a final point, this study provides further evidence that periplasmic glucans synthesized by the same mechanism as that found in E. coli are likely found only in those species of bacteria that are the closest relatives of E. coli. Of the species of bacteria examined in this report that had active acyl carrier proteins, only P. syringae was found to have a membrane glucosyltransferase by direct assay. Indeed, the genetic and product analysis of Bohin and colleagues (20, 36) had already anticipated this result. In the evolutionary grouping of Olsen et al. (25), P. syringae is found in the branch most closely connected to that of E. coli (the γ-3 subgroup). The question as to whether the other subgroups of purple bacteria have periplasmic glucans with an as yet undetermined structure will remain open for a future study.

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