JOURNAL OF BACTERIOLOGY, Sept. 1990, p. 5440–5444
0021-9193/90/095440-05$02.00/0

Vol. 172, No. 9

Isolation and Characterization of the Constitutive Acyl Carrier Protein from *Rhizobium meliloti*

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Received 6 March 1990/Accepted 28 June 1990

Rhizobium **species produce an inducible acyl carrier protein (ACP), encoded by the *nodF* gene, that somehow functions in an exchange of cell signals between bacteria and specific plant hosts, leading to nodulation of plant roots and symbiotic nitrogen fixation, as well as a constitutive ACP needed for the synthesis of essential cell lipids. The periplasmic cyclic glucans of *Rhizobium* spp. are also involved in specific rhizobium-plant interaction. These glucans are strongly similar to the periplasmic membrane-derived oligosaccharides (MDO) of *Escherichia coli*. *E. coli* ACP is an essential component of a membrane-bound transglucosylase needed for the biosynthesis of MDO, raising the possibility that either or both of the rhizobial ACPs might have a similar function. We have now isolated the constitutive ACP of *R. meliloti* and determined its primary structure. We have also examined its function, together with those of ACPs from *E. coli*, *Rhodobacter sphaeroides*, and spinach, in the MDO transglucosylase system and as substrate for the *E. coli* ACP acylase enzyme. All four ACPs act as acceptors of acyl residues, but only the *E. coli* ACP functions in the transglucosylase system.**

Rapid progress has recently been made in the genetic analysis of symbiotic nitrogen fixation by *Rhizobium* species in association with specific plant hosts, but it has been more difficult to obtain much-needed information at the biochemical level.

Symbiotic nitrogen fixation takes place in nodular structures on the plant root. The formation of such nodules requires a complex interchange of signals between rhizobia and specific plant hosts and is dependent on the function of rhizobial *nod* (nodulation) genes (8).

Shearman et al. (15) and Debelle and Sharma (3) have reported that the *nodF* gene of *Rhizobium leguminosarum* and of *Rhizobium meliloti* encodes a protein with a structure strongly suggestive of an acyl carrier protein (ACP). The ACPs of bacteria and of plants that possess fatty acid synthases of type II are small, anionic proteins, each with a phosphopantetheine prosthetic group covalently linked to a serine residue via a phosphodiester bond. Because the *nodF* gene is inducible (15), so that its product is synthesized only when cells are grown in the presence of root exudate or other inducers, and because *nodF* deletions have no effect on viability, the NodF protein cannot be needed for the biosynthesis of essential lipids, which therefore must be the function of another, constitutive ACP.

The periplasmic cyclic 1,2-glucans of *Rhizobium* species are also thought to be involved in the cell signaling that leads to nodulation. These carbohydrates are now recognized to be very similar to the periplasmic membrane-derived oligosaccharides (MDO) of *Escherichia coli* (7, 12, 13). Recent work in this laboratory (16, 17) has led to the discovery of an entirely novel function of the ACP of *E. coli* as an essential component of a UDP-glucose-linked transglucosylation system (18) that is needed for the biosynthesis of MDO. This unexpected finding raised the possibility that the constitutive ACP of *Rhizobium* species or the *nodF* gene product, or both, may function in the synthesis of cyclic glucan or of some other cell surface carbohydrate.

Clearly, the presence of rhizobia of two distinct forms of ACP, one constitutive and the other inducible, offers an unusual opportunity, not only for understanding the possible role of these proteins in the nodulation process but also for investigation of the structure-function relationships of the family of ACPs. In this study, we have isolated the constitutive ACP of *R. meliloti* 1021 and determined its amino acid sequence. To obtain some insight into the domains of ACP that function in the MDO-linked transglucosylase on the one hand and in lipid acyl transfer reactions on the other, we have tested the purified constitutive *R. meliloti* ACP, as well as ACP from *Rhodobacter sphaeroides* and from spinach, for activity in both the *E. coli* transglucosylase and ACP acylation enzyme reactions. Despite the strong similarity of these four proteins, all of which function as substrates for the ACP acylase, only the *E. coli* ACP has detectable activity in the transglucosylase system.

MATERIALS AND METHODS

R. meliloti 1021 and Dek5 (a derivative of *R. meliloti* 1021 with a *nodF* deletion) were kindly provided by F. Ausubel. These strains were routinely grown on TY (1) medium at 30°C and maintained on slants of the same medium at 5°C.

Frozen cells of *E. coli* were obtained from Grain Processing Corp., Muscatine, Iowa. ACP was prepared from these frozen cells by a procedure based on that of Therisod et al. (17). *E. coli* ACP labeled with tritium in the β-alanine moiety of the phosphopantetheine residue was the kind gift of T. Fischl of this laboratory, who prepared it from cells of the β-alanine auxotroph SJ16 by a procedure based on that of

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Therisod et al. (17). ACP from *Rhodobacter sphaeroides* was the gift of Donald Lueking of Michigan Technological University, to whom we are also grateful for information on its sequence prior to publication. We are similarly indebted to John Ohlrogge for a gift of pure spinach ACP-1, the sequence of which was previously determined (9).

[9,10-^{3}H]palmitic acid was purchased from Dupont, NEN Research Products, Boston, Mass.

Assay of ACP acylase. The procedure of Rock and Cronan (14) was used to partially purify ACP acylase of *E. coli*, an enzyme that catalyzes the following reaction: ATP + RCOOH + ACP-SH → RCO-S-ACP + ADP. The procedure was carried through the stages of Triton extraction of *E. coli* membranes and subsequent heat treatment.

The principle of the assay was the separation of the substrate, [^{3}H]palmitate, from the product, [^{3}H]palmityl-S-ACP, on a small (0.5-ml) column of DEAE-cellulose. The incubation mixture contained 0.1 M Tris buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM ATP, 60 μM [^{3}H]palmitate, 0.4 M LiCl, 25 mU of ACP acylase, and 0 to 4.5 μg of ACP. The total volume was 80 μl. After 60 min of incubation at 37°C, the reaction was terminated by the addition of an equal volume of methanol, followed by 5 volumes of 10 mM Tris buffer (pH 7.4) containing 5 mg of Triton X-100 per ml (buffer A). This mixture was applied to a column (0.5 ml) of DEAE-cellulose in a Pasteur pipette. The labeled palmitate was removed from the column by the following successive washes: 1.5 ml of 0.2 M KCl in buffer A; 1.5 ml of 80% (vol/vol) 1-propanol containing 5 mg of Triton X-100 per ml; and a second wash with 1.5 ml of 0.2 M KCl in buffer A. The labeled palmityl ACP, which was retained on the column, was then eluted with 1.5 ml of 0.5 M KCl in buffer A, and a portion was counted.

Assay of MDO transglucosylase. The transfer of labeled glucose from UDP-glucose to the model acceptor octyl-β-D-glucoside in an ACP-requiring reaction catalyzed by membrane preparations from *E. coli* was measured essentially as described by Therisod et al. (17).

Polyacrylamide gel electrophoresis. Electrophoresis in 15% polyacrylamide gels containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) was carried out according to the method of Laemmli (10). Electrophoresis in nondenaturing gels was performed as described by Jackowski and Rock (5).

Amino acid sequence of constitutive ACP of *R. meliloti*. Tryptic and chymotryptic peptides of the purified ACP were separated by narrow-bore reverse-phase high-performance liquid chromatography on a Hewlett-Packard 1090 apparatus. For sequence analysis, samples were applied directly to a Polybrene precycled glass fiber filter and placed in the reaction cartridge of an ABI model 477A protein sequencer. The samples were subjected to automated Edman degradation, using a slightly modified version of the program NORMAL-1. The resultant phenylthiohydantoin amino acid derivatives were subsequently identified with the use an on-line ABI model 12A high-performance liquid chromatograph. This part of the work was carried out by one of us (W.L.) in the Harvard Microchemistry Facility.

RESULTS

Isolation of constitutive ACP from *R. meliloti* 1021. Because of the presumed resemblance of the constitutive ACP of *R. meliloti* to the *nodF* gene product, which might make separation of the two proteins difficult, we used *R. meliloti* Dek5, a derivative of *R. meliloti* 1021 with a deletion of the *nodF* gene, as the source of the constitutive ACP.

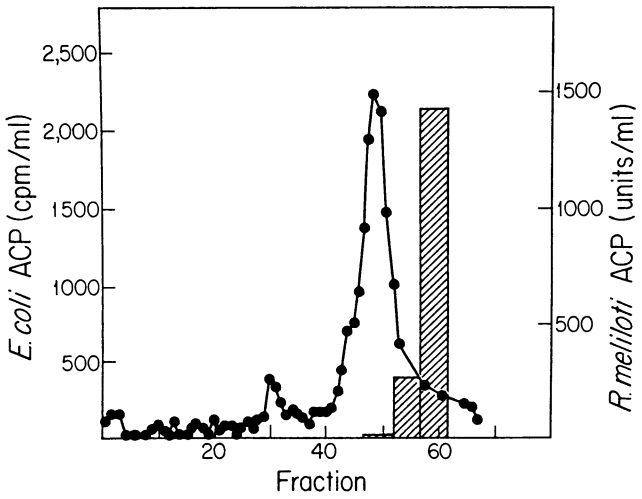

FIG. 1. Chromatography of ACP on DEAE-cellulose. Shown are the radioactivity of labeled *E. coli* ACP mixed with the extract from *R. meliloti* cells as described in the test (●) and the level of *R. meliloti* ACP in arbitrary units, as measured by the ACP acylation assay described in the text (▨).

Cells of *R. meliloti* Dek5 were grown for 24 h in a New Brunswick Magnaferm fermentor in TY medium at 30°C. The cells, with an A_{650} of 0.8, were harvested by centrifugation and suspended in disruption buffer (210 ml) containing 50 mM morpholinepropanesulfonic acid (MOPS; pH 7.4), 6 mM $MgSO_4$, and 5 mM dithiothreitol. The cells were broken by two passages through a French press at 1,100 lb/in^2.

Subsequent operations were carried out at 4°C. Cell debris and unbroken cells were removed by centrifugation at 5,000 × *g* for 15 min. The membrane fraction was then removed by centrifugation at 100,000 × *g* for 1 h. At this point, a small amount of ACP from *E. coli*, containing a total of 100,000 cpm of tritium in the β-alanine moiety and a negligible amount of protein, was added to the preparation. It was anticipated that it would be a useful marker for the presence of the closely similar constitutive, rhizobial ACP, which indeed proved to be the case.

The 100,000 × *g* supernatant fraction (ca. 190 ml), after the addition of labeled *E. coli* ACP, was treated with streptomycin sulfate (0.2 mg/mg of protein), and the suspension was stirred for 1 h to precipitate nucleic acids. The pH of the suspension was then adjusted to 5.2 by the addition of 2 N acetic acid to remove proteins insoluble at this pH. The suspension was held overnight at 4°C to complete precipitation, after which the precipitate was removed by centrifugation.

The supernatant solution (187 ml containing 16 mg of protein per ml) was applied to a DEAE-cellulose column (15 cm in length and 2.5 cm in diameter) previously equilibrated at pH 7.4. The column was washed with two column volumes of 50 mM acetate buffer (pH 5.2) containing 6 mM 2-mercaptoethanol. The column was then eluted with the same buffer in a linear gradient from 0.1 to 0.5 M NaCl in a total volume of 500 ml. Fractions (4.7 ml) were collected and assayed for protein content and for radioactivity.

Most of the radioactivity (representing *E. coli* ACP) emerged with a peak at fraction 48, corresponding to ca. 0.25 M NaCl (Fig. 1). Pooled fractions in this region were tested for their content of ACP by gel electrophoresis and by assaying the conversion of labeled palmitate to palmityl-S-ACP by the *E. coli* acylase. A peak of *R. meliloti* ACP was

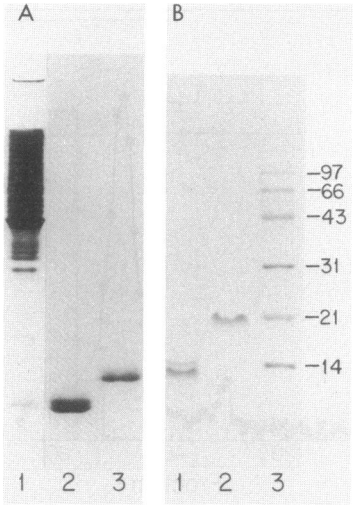

FIG. 2. Gel electrophoresis. Proteins were stained with Coomassie blue. (A) Nondenaturing gel (6). Lanes: 1, unfractionated extract of *R. meliloti*; 2, *R. meliloti* ACP purified through the stage of Sephadex G-50 fractionation; 3, ACP from *E. coli*. (B) SDS gel. Lanes: 1, *R. meliloti* ACP; 2, *E. coli* ACP; 3, a mixture of standard proteins. The sizes (in kilodaltons) of the latter are indicated on the right: rabbit muscle phosphorylase (97), bovine serum albumin (66), hen ovalbumin (43), bovine carbonic anhydrase (31), soybean trypsin inhibitor (21), and hen egg white lysozyme (14).

detected immediately after the labeled *E. coli* ACP as a band stained strongly with Coomassie blue, moving slightly more rapidly than *E. coli* ACP in nondenaturing gels. The identity of this band as rhizobial ACP was confirmed by assay with the ACP acylating enzyme (Fig. 1, right-hand ordinate).

Fractions containing rhizobial ACP were pooled and dialyzed overnight against 50 volumes of 25 mM MOPS buffer (pH 7.4) containing 6 mM 2-mercaptoethanol and then concentrated by lyophilization. The rhizobial ACP was further purified by chromatography on a column (37 cm in length and 1.1 cm in diameter) of Sephadex G-50 eluted with MOPS–2-mercaptoethanol buffer. The ACP was located by the acylation assay as described above.

ACP-containing fractions were pooled and concentrated with the use of an Amicon microconcentrator. The purity of the *R. meliloti* ACP was evaluated by electrophoresis (Fig. 2). In the nondenaturing gel (Fig. 2A), the rhizobial ACP ran as a doublet band, distinctly ahead of the purified *E. coli* ACP run as a marker. In the SDS-containing gel (Fig. 2B), the *R. meliloti* ACP again ran as a doublet, slightly faster than the lysozyme marker and distinctly faster than the *E. coli* ACP. The resolution into a doublet band may be related to the fact that about one-third of the *R. meliloti* ACP molecules have retained the N-terminal methionine, as discussed below.

Amino acid sequence of the constitutive ACP from *R. meliloti*. The amino acid sequence of the *R. meliloti* constitutive ACP isolated as described above is shown in Fig. 3, together with those of three other species studied in this work. About one-third of the *R. meliloti* ACP molecules appear to have retained the amino-terminal methionine.

Function of *R. meliloti* ACP and *Rhodobacter sphaeroides* ACP as substrates for *E. coli* ACP acylase. ACP from *R. meliloti* was compared with *E. coli* ACP as acceptor for labeled palmityl residues in the reaction catalyzed by the ATP-requiring acylase (14) of *E. coli*. The ACP from *R. meliloti* functioned as an effective acceptor, at about half the activity of similar concentrations of the *E. coli* ACP (Fig. 4). Approximately the same activity was shown by the ACP from *Rhodobacter sphaeroides* and by ACP from spinach (data not shown).

	10	20	30	40
1)	STIEERVKKI	IGEQLGVKQE	EVTNNASFVE	DLGADSLDTV
2)	(M)SDIAERVKKI	VIDHLGVDAE	KVSEGASFID	DLGADSLDTV
3)	SDIADRVKKI	VVEHLGVEEE	KVTETTSFID	DLGADSLDTV
4)	AKKETIDKVSDI	VKEKLALGAD	VVVTADSEFS	KLGADSLDTV

	50	60	70	
1)	ELIMALEEEF	DTEIPDEEAE	KITTVQAAID	YINGHQA
2)	ELVMAFEEEF	GVEIPDDAAD	SILTVGDAVK	FIEKAQA
3)	ELVMAFEEEF	GIEIPDDAAE	TIQTFGDAP	
4)	EIVMNLEEEF	GINVDEDKAQ	DISTIQQAAD	VIEGLLEKKA

FIG. 3. Amino acid sequence of ACP from four sources. 1, *E. coli* K-12 (6); 2, constitutive ACP of *R. meliloti* (this study); 3, *Rhodobacter sphaeroides* (personal communication from Donald Lueking); 4, spinach ACP-1 (9). The numbering is that of the *E. coli* ACP.

Tests of ACPs from *R. meliloti*, *Rhodobacter sphaeroides*, and spinach for transglucosylation function. When ACP from *R. meliloti* was tested in the UDP-glucose-requiring transglucosylation system that is needed for the synthesis of the β-1,2-glucan backbone structure of membrane-derived oligosaccharides, no activity could be detected over a wide range of concentration under conditions in which maximum stimulation by *E. coli* ACP was found (Fig. 5).

When the ACPs from *Rhodobacter sphaeroides* and from spinach were similarly tested, they also were without significant activity. Activities expressed as nanomoles of UDP-glucose converted to glucan with no ACP added and with ACP from *E. coli*, *R. meliloti*, *Rhodobacter sphaeroides*, and spinach were 0.28, 1.50, 0.24, 0.23, and 0.22, respectively.

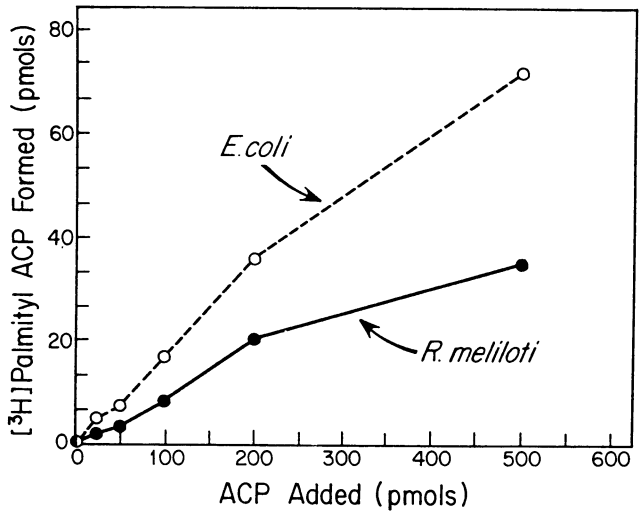

FIG. 4. Activity of constitutive ACP of *R. meliloti* in the ACP acylation reaction. The amounts of ACP added to the acylation system (see Materials and Methods) were varied as shown on the abscissa. The ordinate indicates the amount of [^{3}H]palmityl ACP formed.

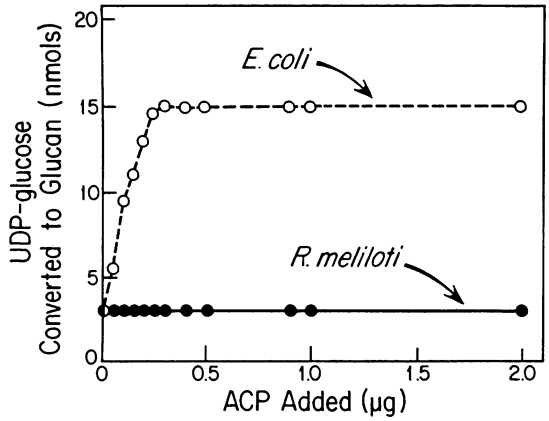

FIG. 5. Test of *E. coli* ACP and *R. meliloti* constitutive ACP in the MDO transglucosylase assay. The amounts of each type of ACP were varied as shown. The ordinate indicates the amount of labeled UDP-glucose converted to neutral glucan product.

DISCUSSION

The primary structure of the constitutive ACP of *R. meliloti*, determined in this study, resembles the ACP of *E. coli* much more closely than it does the *R. meliloti nodF* gene product, as deduced from the DNA sequence by Debelle and Sharma (3). The constitutive ACP from *R. meliloti* is identical with the *E. coli* ACP at 46 of 77 residues (Fig. 3) and with the NodF product (3) at only 19 of 77.

Because the *nodF* gene product is not essential for the growth of *R. meliloti*, it is obviously not required for the biosynthesis of essential cell lipids. Presumably it is required for the synthesis of some cell-signaling substance that functions in the nodulation of specific plant hosts. From analogy with the functions of ACP in *E. coli*, such a substance could be either a specific lipid (perhaps a modified lipopolysaccharide) or a cell surface carbohydrate. Because the production of periplasmic cyclic glucans does not depend on induction of the *nod* genes, the NodF protein cannot play an essential role in their biosynthesis, although the possibility must be considered that it is required for the formation of some specifically modified cyclic glucan. A recent study by Bibb et al. (2) suggests that the rhizobial NodF protein may resemble an ACP that is apparently needed for the production of polyketide antibiotics in *Streptomyces glaucescens*.

Clearly, a definitive understanding of the biochemical functions of the NodF protein requires its isolation and direct study. Because it is obviously important to distinguish the NodF protein from the constitutive ACP, this study is an essential step toward this goal. Work on the isolation of the NodF ACP is now under way in this laboratory.

The amino acid sequences of the four different ACPs studied in this work are shown in Fig. 3. Their close similarity is striking, especially that of the three bacterial species, each from a gram-negative bacterium. It is nevertheless the case that only the ACP from *E. coli* functions in the MDO transglucosylase system.

The three bacterial ACPs, although very closely related, do differ rather strikingly at the carboxyl terminus. Four of the last nine residues of the constitutive ACP of *R. meliloti* represent nonconservative changes from the *E. coli* protein, whereas this region is missing altogether from the *Rhodobacter sphaeroides* protein. Holak et al. (4) have carried out a study of the three-dimensional structure of *E. coli* ACP by nuclear magnetic resonance methods. According to these workers, the principal structural motif is composed of three helices that line a hydrophobic cavity. One of these helices is made up of residues 65 to 75, at the carboxyl terminus, suggesting the possibility that this region is important not only for the structure but also perhaps for the transglucosylase activity of the *E. coli* ACP. A direct approach to study of the domains that are needed for the transglucosylase function of *E. coli* ACP is that of site-directed mutagenesis. Our results, although obviously not definitive, suggest that the carboxyl terminus might be an interesting starting point for such studies.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants GM19822 and GM22057 from the National Institute of General Medical Sciences.

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