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Abstract The nodulation protein NodF of *Rhizobium* shows 25% identity to acyl carrier protein (ACP) from *Escherichia coli* (encoded by the gene *acpP*). However, NodF cannot be functionally replaced by AcpP. We have investigated whether NodF is a substrate for various *E. coli* enzymes which are involved in the synthesis of fatty acids. NodF is a substrate for the addition of the 4′-phosphopantetheine prosthetic group by holo-ACP synthase. The K_m value for NodF is 61 μM, as compared to 2 μM for AcpP. The resulting holo-NodF serves as a substrate for coupling of malonate by malonyl-CoA:ACP transacylase (MCAT) and for coupling of palmitic acid by acyl-ACP synthetase. NodF is not a substrate for β-keto-acyl ACP synthase III (KASIII), which catalyses the initial condensation reaction in fatty acid biosynthesis. A chimeric gene was constructed comprising part of the *E.coli acpP* gene and part of the *nodF* gene. Circular dichroism studies of the chimeric AcpP-NodF (residues 1–33 of AcpP fused to amino acids 43–93 of NodF) protein encoded by this gene indicate a similar folding pattern to that of the parental proteins. Enzymatic analysis shows that AcpP-NodF is a substrate for the enzymes holo-ACP synthase, MCAT and acyl-ACP synthetase. Biological complementation studies show that the chimeric AcpP-NodF gene is able functionally to replace NodF in the root nodulation process in *Vicia sativa*. We therefore conclude that NodF is a specialized acyl carrier protein whose specific features are encoded in the C-terminal region of the protein. The ability to exchange domains between such distantly related proteins without affecting conformation opens exciting possibilities for further mapping of the functional domains of acyl carrier proteins (i. e., their recognition sites for many enzymes).

Key words Nodulation · *Rhizobium* · Lipo-chitin oligosaccharide · NodF · Fatty acid biosynthesis

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Introduction

Bacteria of the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium*, collectively called rhizobia, are able to interact with leguminous plants. This interaction can result in the formation of root nodules, inside which a differentiated form of the rhizobia, called bacteroids, is present, which is capable of fixing atmospheric nitrogen to form ammonia.

The process of nodulation involves an exchange of signals between the bacterium and the plant. Plants secrete flavonoids that are recognized by the bacteria. This leads to the production of bacterial signals called Nod factors. These Nod factors are synthesized by proteins encoded by *nod*, *nol* or *noe* genes (Dénarié et al. 1996).

The Nod factors have been identified as β–1,4 linked oligomers of *N*-acetyl glucosamine with a fatty acid attached to the nitrogen of the non-reducing terminal saccharide residue. Hence these molecules are designated lipo-chitin oligosaccharides (LCOs). Modifications of this basic structure are very important for host specificity in the nodulation process. In the case of *Rhizobium leguminosarum* and *R. meliloti* these modifications include the presence of an unusual *N*-linked fatty acid that has *trans* double bonds conjugated to the carbonyl group (Lerouge et al. 1990; Spaink et al. 1991; Spaink et al. 1995; van der Drift et al. 1996).

The *nod* genes *nodF* and *nodE* are the only specialized genes required for the production of these unusual polyunsaturated fatty acids. NodF and NodE are homologous to acyl carrier proteins and β-keto-acyl-ACP synthase (KAS) enzymes, respectively (Shearman et al. 1986; Bibb et al. 1989). KAS enzymes are also known as condensing enzymes, since they couple malonyl units to the growing acyl chain, releasing one CO_2 molecule per unit added. In *Escherichia coli* three KAS enzymes have been described. KASI and KASII, encoded by *fabB* and *fabF*, respectively, both use acyl-acyl carrier protein (ACP) and malonyl-ACP for condensation and they differ only in their substrate specificity for unsaturated fatty acids. KASIII, encoded by *fabH*, performs the first condensation reaction between acetyl-CoA and malonyl-ACP. This is the only condensation step in which CoA is used to donate substrates. In all other steps only fatty acids donated by ACP are used. Therefore ACPs are central proteins in fatty acid biosynthesis as they carry the malonyl elongation units and the acyl chain throughout biosynthesis (for a review see Magnuson et al. 1993). ACPs bear a prosthetic 4′-phosphopantetheine group coupled to a serine residue, to which the fatty acid is attached. The prosthetic 4′-phosphopantetheine group in NodF is coupled to serine 45. In addition to showing 25% sequence identity to *E. coli* AcpP, NodF has three α-helices in positions that are equivalent to those of the three α-helices in AcpP (Geiger et al. 1991; Ghose et al. 1996).

Deletion of *nodF* from *R. leguminosarum* biovar *viciae* leads to the biosynthesis of LCOs that do not carry the polyunsaturated fatty acids. The presence or absence of multiply unsaturated fatty acids can be sensitively monitored in a nodulation assay using a recently developed genetic test system (Ritsema et al. 1994; Bloemberg et al. 1995). Using this system we have been able to show that an acyl carrier protein involved in the biosynthesis of housekeeping fatty acids is not able to replace NodF for nodulation. In this paper we investigate the recognition of NodF by the *E. coli* enzymes holo-ACP synthase, malonyl-CoA:ACP transacylase (MCAT), acyl-ACP synthetase and KASIII (encoded by *fabH*). The study shows that NodF can be used by all of these enzymes except for KASIII.

A chimeric gene was constructed which comprises part of the *acpP* gene and part of the *nodF* gene. The chimera, which consists of residues 1–33 of AcpP and amino acids 43–93 of NodF is able to replace NodF in nodulation. We conclude that NodF is a specialized acyl carrier protein whose specific features are encoded in the C-terminal region of the protein.

Materials and methods

Bacterial strains and growth conditions

Rhizobial strains were grown at 28° C on YMB medium (Hooykaas et al. 1977) solidified with 1.8% agar.

The rifampicin-resistant strain *Rhizobium leguminosarum* biovar *viciae* RBL5560, which induces wild-type nodulation on *Vicia*, harbors the symbiosis (Sym) plasmid pRL1JI that contains a Tn*5* insertion in the gene for medium bacteriocin production (Zaat et al. 1987). Strain RBL5900 harbors a pRL1JI derivative that bears deletion A69 (Downie and Surin 1990; Ritsema et al. 1994). In RBL5900 the only *nod* genes present are *nodABCIJ* and *nodD*. As a source of the *nodL* and *nodE* genes of *R. leguminosarum* bv. *viciae*, the plasmids pMP2109 and pMP258, respectively, were used. pMP2109 is an IncW vector containing the *nodL* gene under control of the *nodA* promoter. It also encodes spectinomycin and streptomycin resistances. pMP258 (Bloemberg et al. 1994) is an IncP vector which contains the *nodE* gene under the control of the *nodA* promotor and encodes a tetracycline resistance. The genes encoding NodF, AcpP from *E. coli,* and the chimera composed of parts of each are present on an IncQ vector which encodes chloramphenicol and streptomycin resistances (Fig. 1A). Plasmid pMP2368 encodes NodF (Ritsema et al. 1994), pMP2395 encodes AcpP, and pMP2527 encodes AcpP-NodF (Fig. 1A).

Plasmids were mobilized from *E. coli* into *Rhizobium* as described previously, using pRK2013 as the helper plasmid (Ditta et al. 1980). Rifampicin (20 mg/l) was used to select against *E. coli*. Rhizobial strains harboring plasmids were grown on media containing the appropriate antibiotics. Concentrations used were: spectinomycin, 100 mg/l (IncW vectors); tetracycline, 2 mg/l (IncP vectors); chloramphenicol, 10 mg/l; streptomycin, 500 mg/l (IncQ vectors). In *E. coli* the same concentrations were used, except for tetracycline, which was added at a concentration of 20 mg/l. Kanamycin (50 mg/l) and carbenicillin (100 mg/l) were used to select for pET-derived vectors in *E. coli* (Studier et al. 1990).

Construction of plasmids

Recombinant DNA techniques were performed as described by Sambrook et al. (1989) and Innes et al. (1990). DNA restriction, polymerization and ligation enzymes were obtained from Pharmacia LKB (Uppsala, Sweden). The construction of plasmids is outlined in Fig. 1A. The primers used for the polymerase chain reaction (PCR) in which the DNA-fragment encoding residues 1–33 of AcpP was obtained were oMP102(5'-TAAGCATATGAGCACTATCGAAGAACGC-3') and oMP103(5'-AAAAGGATCCAAGAGAATCGATGCCCAGGTCTTC-3') (Eurogentec, Seraing, Belgium). In order to clone the *acpP* fragment a *Cla*I site is present in oMP103 just in front of the codon for the active site serine residue 36. This alters the codon for alanine 34 to that for isoleucine, the amino acid that is present at the homologous position 43 in NodF. The *acpP* fragment obtained was used to replace the corresponding *nodF* sequences.

For expression in *E. coli*, the genes were cloned into pET vectors. The *nodF* and *acpP* genes were inserted into pET9a, which confers resistance to kanamycin. Plasmid pMP2301 harbors *nodF* (Ritsema et al. 1994); pMP2390 harbors *acpP* cloned from pET2000 into pET9a using *Xba*I and *Eco*RI restriction sites. pMP2390 was cloned into pMP190 to yield construct pMP2395. The chimeric gene was cloned into pMP2500, a modified pET3a which confers resistance to carbenicillin. For the construction of pMP2500, the *Cla*I site from pET3a was removed by standard methods (Sambrook et al. 1989). For use in *Rhizobium*, the pET

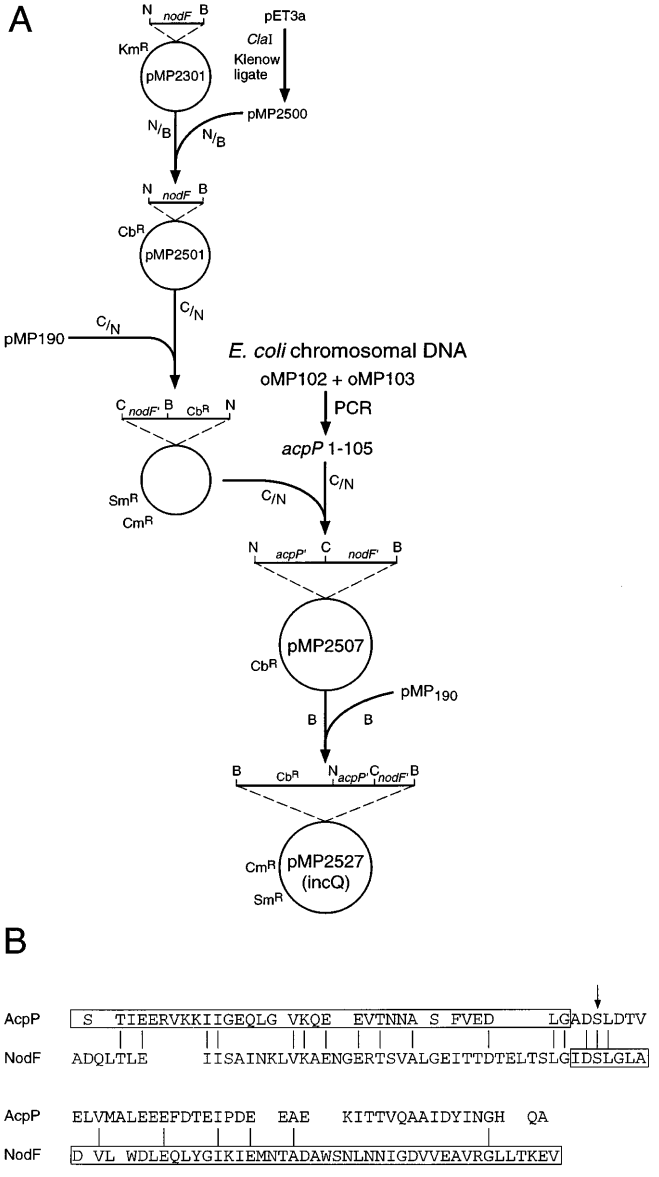

Fig. 1A Construction of plasmids. The *nodF* gene was obtained from the pET9a derivative pMP2301 (Ritsema et al. 1994), which contains, in addition to the *Cla*I site in *nodF*, two other *Cla*I sites, one of which is present in the kanamycin resistance marker. The *Cla*I site of pET3a was removed and *nodF* was recloned into the modified pET3a vector pMP2500. To exchange the 5' portion of the *nodF* sequence by the corresponding section of *acpP*, an intermediate was first constructed consisting of part of *nodF* and the pET3a vector cloned in pMP190. The missing part of *nodF* was replaced by the corresponding segment of *acpP*, and pMP190 was removed. For use in *Rhizobium* the pET-derived plasmids were cloned into the broad-host-range vector pMP190. Abbreviations: Km, kanamycin; Cb, carbenicillin; Cm, chloramphenicol; Sm, streptomycin; B, *Bam*HI; C, *Cla*I; N, *Nde*I. **B** Amino acid sequences of *E. coli* AcpP, NodF and the chimeric AcpP-NodF. The sequence of the chimeric AcpP-NodF is *boxed*. The *arrow* indicates the active site serine

vector was cloned into an IncQ plasmid. The genes were under the control of a T7 promoter, which expresses the genes constitutively in *Rhizobium* (Ritsema et al. 1994). Both the T7 promoter and the Shine-Dalgarno sequence are derived from the pET vector (Fig. 1A).

Protein production and purification

For the production of proteins, derivatives of *E. coli* strain JM101 harboring the constructs pMP2301(NodF), pMP2390(AcpP) or pMP2507(AcpP-NodF) were grown in LC (containing 10 g/l bactotryptone, 5 g/l yeast extract, 8 g/l NaCl, 2.45 g/l $MgSO_4 \cdot 7H_2O$ pH 6.6) to an OD_{620nm} of 0.2. Protein production was induced by the addition of phage mGP1-2 (Tabor and Richardson 1987) with a titer of 10^9 particles per ml, and cells were allowed to produce protein for 5 h. Cells were harvested and frozen at −80° C overnight and thawed at room temperature while gently shaking in 10 mM Bis-TRIS pH 6.5 (Morris et al. 1993). Proteins in the supernatant fluid were fractionated by electrophoresis on a 17.5% native polyacrylamide gel (PAGE) and protein bands were visualized using Coomassie Brilliant Blue.

Proteins were purified to homogeneity from 0.5-l cultures of *E.coli* cells. After the freeze-thaw step, the cells were removed by centrifugation, and the supernatant fluid was applied to a Prep-Cell (Bio-Rad Laboratories, Hercules, Calif., USA) containing a cylindrical 17.5% native polyacrylamide gel. Fractions were collected and aliquots were analysed on a 17.5% native slab gel to detect the protein-containing fractions.

Circular dichroism spectra were recorded at room temperature on a Jasco-600 spectropolarimeter from 190 nm to 260 nm, using 0.2-mm path length cells with a scan speed of 10 nm/min, a time-constant of 1 s, and a bandwidth of 1 nm. Four spectra were accumulated and, after spectral subtraction of a blank sample, quantitative deconvolution of CD spectra in terms of predefined reference spectra was performed by computer using the least-squares method, as described previously (Keller et al. 1992).

Enzymatic assays

Purified NodF and AcpP-NodF were converted to their holo forms using *E. coli* holo-ACP synthase. The protein to be modified was dissolved at 1 mg/ml in the following incubation mixture: 1 mM CoASH, 10 mM $MgCl_2$, 12.5 mM DTT, and 50 mM TRIS-HCl pH 8.8. Holo-ACP synthase was added (1 μM) and the mixture was incubated at 37° C for several hours. The apo to holo conversion was shown by electrospray mass spectrometry. The acyl carrier proteins were dissolved in acetonitrile/water/formic acid (47.5/47.5/5, v/v) to a final concentration of approximately 1 μg/μl. Positive-mode electrospray mass spectra were obtained on a VG Platform II single quadrupole mass spectrometer. Aliquots of 10 μl of the samples were infused into a mobile phase of acetonitrile/water (50/50, v/v) and introduced into the electrospray source at a flow rate of 5 μl/min. Spectra were scanned at a speed of 10 s^{-1} for m/z 700–1700, with a cone voltage of 60 V, and recorded and processed using the MassLynx software, version 2.0. Mass calibration was performed by multiple-ion monitoring of horse-heart myoglobin signals.

K_m and k_{cat} values for the transfer of 4′-phosphopantetheine from CoASH to NodF and AcpP-NodF were determined by radioassay (Lambalot et al. 1995). Various concentrations of each substrate were incubated with 75 mM TRIS-HCl pH 8.8, 10 mM $MgCl_2$, 25 mM DTT, 200 μM [^{3}H]-(*pantetheinyl*)CoASH and holo-ACP synthase in a final volume of 100 μl at 37° C. The reactions were quenched after a specified time with 10% trichloracetic acid (TCA) and 375 μg of bovine serum albumin was added as carrier. The precipitated protein was recovered by centrifugation and washed three times with 10% TCA. The amount of [^{3}H]phosphopantetheine incorporated into the substrate protein was quantified by liquid scintillation counting.

Proteins were malonylated with MCAT and [^{14}C]malonyl-CoA. MCAT was obtained as described by Serre et al. (1994). The holo form of AcpP (40 μg), NodF (15 μg) or AcpP-NodF (15 μg) was treated with an excess of MCAT in 0.1 M sodium phosphate (pH 7) and 1 mM malonyl-CoA in a total volume of 40 μl. After incubation at 32° C for 5 min, the reaction was terminated by the addition of glycerol and urea to final concentrations of 15% and 0.25 M, respectively. Samples were analysed on an 18% confor-

mation-sensitive polyacrylamide gel containing 0.5 M urea as described by Post-Beittenmiller et al. (1991).

Condensation of malonyl-AcpP or malonyl-NodF with [^{14}C]acetyl-CoA was performed with KASIII. KASIII was purified as described by Verwoert et al. (1995). 1 mM acetyl-CoA and an excess of KASIII was added to the reaction mixture as described above for malonyl coupling. Incubation, termination and analysis of the samples was carried out as described above for the reaction with MCAT.

Acylated AcpP, NodF, and AcpP-NodF were obtained by incubating 50-μg samples of the holo forms of the proteins with 3.4 μg of acyl-ACP synthetase (Sigma, St Louis, Mo.) and 1 nmol [^{3}H]palmitate (NEN Du Pont, Dreiereich, Germany). The incubation mixture contained 75 mM TRIS-HCl (pH 8), 4 mM ATP, 7 mM $MgCl_2$, 1.5 mM DTT, 75 mM LiCl and 1.5% Triton X-100 in a total reaction volume of 75 μl. The mixture was incubated at room temperature overnight with shaking. AcpP, NodF, and the chimeric protein were purified essentially as described by Rock et al (1981). Acyl-ACP synthetase was removed using Blue Sepharose CL-6B (Pharmacia LKB, Uppsala, Sweden) and acylated proteins were recovered from a DEAE-Cellulose column (DE-52, Whatman Scientific, Maidstone, England) using 0.7 M NaCl.

Nodulation assays

Vicia sativa ssp. *nigra* seedlings were inoculated with rhizobia (final concentration of 10^3 bacteria per ml) in liquid Jensen medium. The roots were shielded from the light in brown glass containers (van Brussel et al. 1992). Nodulation was scored after 14 days.

Results

In vitro assays for acyl carrier functions of NodF

NodF was purified from *E. coli* using the two-step procedure described in Materials and methods. Some 20 mg of purified protein was obtained per liter of *E. coli* cells. The protein eluted from the preparative gel as a single peak. On an analytical native polyacrylamide gel the protein appeared, however, as several bands (see also Geiger et al. 1991). The two major bands were collected separately and electrospray mass spectrometric analysis of both revealed that the two bands both correspond to NodF, have the same molecular weights and do not contain a prosthetic 4′-phosphopantetheine group (Table 1). We conclude that NodF can exist in two conformations, resulting in separation of two bands on a native gel. Comparison of these experimental data with the calculated average masses shows a difference of 132 Da. This can be attributed to the expected absence of the N-terminal methionine (131 Da), a common observation for proteins produced by bacterial or yeast systems (Adams 1968; Miller 1987).

The enzyme holo-ACP synthase of *E. coli* (encoded by the gene *acpS*) is able to convert apo-ACP to the holo-protein (Gehring et al. 1997). This is achieved by covalent transfer of 4′-phosphopantetheine from Co-ASH to ACP. Purified apo-NodF was tested as a substrate for pure holo-ACP synthase. Analysis of the holo-ACP synthase-treated NodF using electrospray mass spectrometry revealed a mass increase of 338.4 ± 3.6 Da (Table 1), corresponding to the incorporation of a 4′-phosphopantetheine group (mass increasement 340 Da) to form holo-NodF. K_m determinations with holo-ACP synthase using radiolabeled CoASH yielded a K_m for NodF of 61 μM and a k_{cat} of 2.8/min (Table 2). Using holo-ACP synthase, we converted 7 mg of apo-NodF into holo-NodF, which was used for further biochemical studies.

Malonate is used as a building block in each elongation cycle in fatty acid biosynthesis. Therefore, an essential function of ACP is its ability to carry malonate. With the enzyme MCAT, the transfer of malonate to NodF was investigated. We used MCAT, purified from *E. coli,* and radioactive [^{14}C]malonyl-CoA for this assay and analysed the products by conformation-sensitive PAGE. The results show that transfer of the radioactive malonyl group to holo-NodF indeed occurred (Fig. 2A, lane 5). We therefore conclude that holo-NodF is recognized by the *E. coli* MCAT enzyme.

The first condensation step in *E. coli* fatty acid biosynthesis is the condensation of acetyl-CoA and malonyl-ACP by KASIII. Purified KASIII from *E. coli* was used to test the transfer of the [^{14}C]acetate group from [^{14}C]acetyl-CoA to malonyl-NodF. In this experiment holo-NodF, MCAT, and KASIII were added together with the substrates malonyl-CoA and acetyl-CoA, one of which was radioactively labelled, and the results were analysed by PAGE (Fig. 2A). When [^{14}C]malonyl-CoA was used, the bands that appeared had the same Rf values as those from the assay in which KASIII was not present (Fig. 2A, lane 6). When [^{14}C]acetyl-CoA was used no radioactive band was detected. When the same experiment was performed with holo-ACP, bands with a different Rf value appeared, and these were labelled by both [^{14}C]malonyl-CoA and [^{14}C]acetyl-CoA (Fig. 2A, lanes 2 and 3, respectively). As indicated by the arrows

Table 1 Masses of the proteins determined using electrospray mass spectrometry

Protein	M (exp)[a]	M (calc)[a]	M minus N-terminal methionine (calc)
Apo-NodF	9813.9 ± 1.2	9945.3	9814.1
Holo-NodF	10152.5 ± 2.4	10285.6	10154.4
Apo-AcpP-NodF	9158.0 ± 0.9	9290.6	9159.4
Holo-AcpP-NodF	9496.8 ± 2.1	9630.9	9499.7

[a]Experimental and calculated masses, together with the mass calculated for the protein lacking the N-terminal methionine are given. All masses are given as average values

Table 2 Steady-state kinetic parameters for phosphopantetheine transfer catalyzed by holo-AcpP synthase

Protein	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM.min^{-1})
Apo-AcpP	2	80–100	50
Apo-NodF	61	2.8	0.05
Apo-AcpP-NodF	70	0.9	0.01

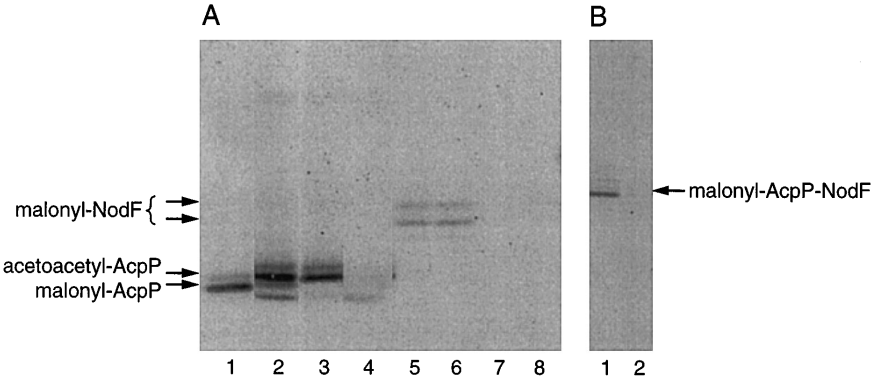

Fig. 2A, B In vitro assays with MCAT and KASIII using various acyl carrier proteins as substrates. **A** AcpP from *E. coli* (lanes 1, 2, 3 and 4) and NodF (lanes 5–8) were tested. We used MCAT and [^{14}C]malonyl-CoA (lanes 1 and 5); MCAT and KASIII with [^{14}C]malonyl-CoA and acetyl-CoA (lanes 2 and 6); MCAT and KASIII with malonyl-CoA and [^{14}C]acetyl-CoA (lanes 3 and 7); and KASIII with [^{14}C]acetyl-CoA (lanes 4 and 8) as a control for transacetylase activity of KASIII. **B** AcpP-NodF tests. We used MCAT and KASIII with [^{14}C]malonyl-CoA and acetyl-CoA (lane 1); MCAT and KASIII with malonyl-CoA and [^{14}C]acetyl-CoA (lane 2). NodF can exist in several conformational forms, which explains why multiple bands are observed by PAGE. The putative identities of the reaction products are indicated

in Fig. 2, one of the two slower moving reaction products in lanes 2 and 3 is suggested to be acetoacetyl-AcpP. The results show that KASIII of *E. coli* is active with malonyl-AcpP and not with malonyl-NodF.

Construction of a chimeric AcpP-NodF and studies on conformation and ACP function

A chimeric gene comprising parts of *acpP* from *E. coli* and *nodF* was constructed using the PCR technique. The *acpP* fragment obtained was used to replace the corresponding *nodF* sequence, resulting in the plasmids pMP2507 and pMP2527, encoding AcpP residues 1–33 and NodF residues 43–93 (Fig. 1).

The protein encoded by the chimeric gene was purified from *E. coli* using the same procedure as was used for NodF. The protein eluted as a single peak and no other proteins were detected on native gels. On analysis by electrospray mass spectrometry, only AcpP-NodF protein lacking both the N-terminal methionine and a prosthetic 4′-phosphopantetheine group was detected (Table 1).

AcpP from *E. coli* and NodF have been shown to have a high α-helical content (Holak et al. 1988; Ghose et al. 1996) and are therefore suitable proteins for circular dichroism (CD) studies. The CD spectra for AcpP, NodF and the AcpP-NodF chimera are almost identical, confirming the high α-helix content (Fig. 3). For both AcpP and NodF 38% α-helix and 62% random coil is predicted. For the AcpP-NodF chimera, spectral analysis predicts 32% α-helix and 68% random coil.

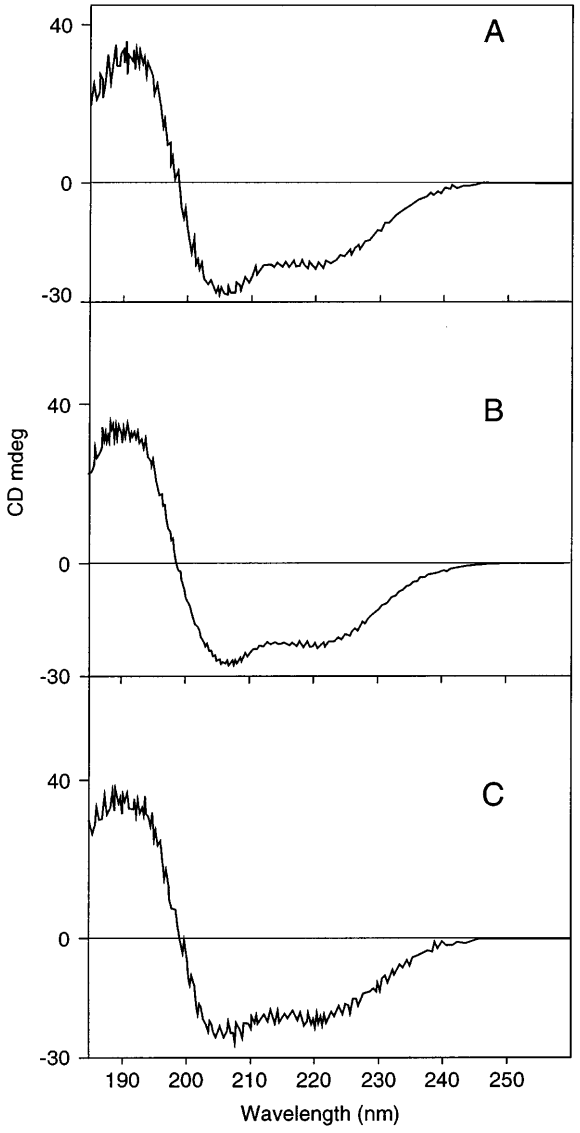

Fig. 3A–C Circular dichroism spectra of AcpP, NodF and the AcpP-NodF chimera. Purified apo-proteins were measured as described in Materials and methods. **A** NodF. **B** AcpP. **C** AcpP-NodF

Purified holo-ACP synthase was tested for its activity with the apo-form of the chimeric AcpP-NodF protein as substrate. Electrospray mass spectrometric analysis of

the products shows that the protein is converted by holo-ACP synthase to the holo-form (Table 1). In the samples containing the holo-form of the protein, minor quantities of the corresponding apo-form were also detected.

The K_m value for holo-ACP synthase was determined, yielding a value of 70 μM for the chimeric protein AcpP-NodF (Table 2). If this value is compared with the K_m values of AcpP (2 μM) and NodF (60 μM), it can be concluded that the chimeric protein is a less efficient substrate than AcpP, and almost as efficient as NodF. If maximal turnover rates (k_{cat}) are compared, a similar picture emerges. The k_{cat} values for NodF (2.8/min) and the AcpP-NodF chimera (0.9/min) are much lower than the maximal turnover number for AcpP (100/min) (Table 2). Based on catalytic efficiency (k_{cat}/K_m) it was calculated that holo-ACP synthase has the highest catalytic activity with AcpP as a substrate, while this is one thousand times lower with NodF and with the AcpP-NodF chimera (Table 2). Further details of the enzymatic characteristics of the AcpS protein are discussed by Gehring et al (1997).

The differences in kinetics between the chimeric protein and the progenitor enzymes did not pose any problems for large-scale production of its holo form: 3 mg of holo-AcpP-NodF was obtained for further biochemical studies. Incubation of the holo-form of the chimeric protein with MCAT, KASIII, [^{14}C]malonyl-CoA, and acetyl-CoA resulted in the labeling of the chimeric protein as detected by conformation-sensitive PAGE (Fig. 2B, lane 1). When [^{14}C]acetyl-CoA and malonyl-CoA were used, no radioactive protein was detected (Fig. 2B, lane 2). These results demonstrate that the chimeric protein is a substrate for malonylation by MCAT, whereas it is not recognized by KASIII.

Enzymatic coupling of [^{3}H]palmitate to holo-NodF and holo-AcpP-NodF was tested using acyl-ACP synthetase. Based on the incorporation of radioactivity into the purified protein (Table 3) we conclude that NodF and the chimera are both recognized by acyl-ACP synthetase and therefore are both able to carry a long-chain fatty acid. Duplicate experiments (data not shown) show that the low level of acylation of holo-NodF is significant and can be used to obtain acyl-NodF protein for future experiments.

Table 3 In vitro labeling of acyl carrier proteins with [^{3}H]palmitate using acyl-ACP synthetase

Protein[a]	cpm
None	6×10^1
NodF	3×10^2
AcpP-NodF	1×10^3
AcpP	8×10^3

[a]As a negative control the reaction was performed without an acyl carrier protein present. An aliquot containing approximately 0.25 μg purified protein was counted

Genetic complementation of *nodF* by *acpP* and the chimeric gene

In order to test the chimeric gene in *Rhizobium* bacteria, the gene was recloned into a broad-host-range vector of the IncQ incompatibility group. This resulted in pMP2527, derived from pMP2507(AcpP-NodF) (Fig. 1A). As a control the NodF-encoding plasmid pMP2368 and the AcpP-encoding plasmid pMP2395 were used (see Materials and methods). These plasmids were introduced into the *Rhizobium* strain RBL5900.pMP2109.pMP258, which lacks *nodF,* and the resulting strains were tested for their nodulation ability.

Inoculation experiments on *V. sativa* ssp. *nigra* show that the *Rhizobium* strains harboring either pMP2368(NodF) or pMP2527(AcpP-NodF) are able to nodulate *Vicia*, whereas a strain harboring pMP2395(AcpP) is not (Table 4). A delay of 1 day in the appearance of nodules was observed with the strain harboring pMP2527(AcpP-NodF). We conclude from these results that the N-terminal part of NodF can be replaced by the corresponding segment of a housekeeping AcpP without loss of functionality, showing that the C-terminal part of NodF is important for its specific function.

Discussion

In this paper we describe an investigation of structure-function relationships of the specialized acyl carrier protein NodF from *Rhizobium*. Previous results have shown that NodF is essential for the synthesis of LCOs with C18:4 fatty acids and for nodulation of *Vicia*

Table 4 Nodulation of *Rhizobium leguminosarum* biovar *viciae* strains harboring different acyl carrier proteins on *Vicia sativa* ssp. *nigra*

Strain	Protein	Percentage nodulated plants[a]	Number of nodules per plant (±SD)[a]
RBL5900.pMP2109.pMP258.pMP2368	NodF	100	4.8 (±1.7)
RBL5900.pMP2109.pMP258.pMP2395	AcpP	0	0
RBL5900.pMP2109.pMP258.pMP2527	AcpP-NodF	92	3.2 (±1.8)

[a]Nodules were counted after 14 days, 24 plants were scored

(Ritsema et al. 1994). Here we show that the C-terminal domain of NodF is specialized for nodulation of *Vicia* and we conclude from this observation that this region of NodF is important for the synthesis of LCOs with C18:4 fatty acids. Although the exchange of the N-terminal domain of *nodF* results in a protein in which 28% of the original amino acids have been substituted (Fig. 1B), this domain is apparently structurally and functionally very well conserved. The ability to exchange domains of such distantly related proteins without affecting the basic conformation offers exciting possibilities for mapping of the multiple enzyme recognition sites characteristic of acyl carrier proteins.

The structures of both *E. coli* AcpP and NodF have been investigated using nuclear magnetic resonance (NMR) techniques. Following extensive study seven different structures were proposed for *E. coli* AcpP (Holak et al. 1988). A two-state model could however also explain the NMR data for this very flexible protein (Kim et al. 1989). An NMR investigation of NodF revealed the positions of three α-helices: the first helix extends from residues 6 to 16, the second one is at 46–58, and the third at 77–86 (Ghose et al. 1996). From NMR data a total α-helix content of 37% can be calculated, almost identical to the 38% predicted from the CD spectrum (Fig. 3). Although the primary structures of NodF and AcpP shows only 25% overall identity, AcpP has three helices in similar positions to those in NodF. One helix is positioned at the N-terminus, one at the C-terminus and the third one starts just beyond the active site serine residue. It has been proposed that these three helices together form a hydrophobic cleft in which the fatty acid is (partially) shielded from the environment (Jones et al. 1987; Holak et al. 1988). The remainder of the AcpP and NodF proteins is less structured, possibly accounting for the flexibility of the protein.

In addition to their structural similarity, we have gathered evidence for a functional similarity of NodF and AcpP. Previously we have shown that serine residue 45 of NodF is important for nodulation, since only a NodF with this amino acid can carry a prosthetic 4′-phosphopantheteine group (Geiger et al. 1991; Ritsema et al. 1994). In this paper we have described the results of several general assays for acyl carrier protein functions of NodF. This was achieved by performing assays with *E. coli* enzymes that produce the holo-form of ACP (holo-ACP synthase), malonate holo-ACP (MCAT) and palmitoylate holo-ACP (acyl-ACP synthetase). The results show that these key enzymes can use NodF as a substrate. We therefore conclude that (i) NodF can act as an acyl carrier protein and (ii) holo-ACP synthase, MCAT and acyl-ACP synthetase can use heterologous substrates. We also show that a chimeric protein consisting of parts of *E. coli* AcpP and NodF is recognized by these enzymes.

Besides the general ACP-like features of NodF, this protein has specialized features for the synthesis of C18:4 fatty acids. There are two possible explanations for the specialization of NodF. One possibility is a specific recognition by NodE. Based on sequence similarity, NodE is proposed to be a specialized condensing enzyme responsible for elongation of the polyunsaturated fatty acids (Bibb et al. 1989). The *trans* double bonds are introduced into the fatty acid during the last three elongation rounds. The nodulation results obtained with the chimeric protein may indicate that the specific recognition of NodF by NodE is encoded in the C-terminal half of the NodF protein. We have found that NodF is not recognized by KASIII of *E. coli*, the condensing enzyme that performs the initiation reaction in fatty acid biosynthesis. Although they need to be confirmed using the rhizobial KASIII protein, these results suggest that NodF is unlikely to be involved in this initiation step. The inability of KASIII to use NodF supports the model proposed previously (Spaink 1992), in which NodF and NodE take over fatty acid biosynthesis from their housekeeping homologues during the last three elongation rounds. The chimeric protein is also not recognized by KASIII, suggesting that at least one recognition site for this enzyme is located in the C-terminal region.

Another possible explanation for the specialization of NodF is the need to avoid recognition by enoyl-ACP reductase. During general fatty acid biosynthesis, *trans*-unsaturated double bonds are formed that are reduced by enoyl-ACP reductase to form saturated bonds. The presence of the three *trans* double bonds at the carboxyl end of the poly-unsaturated C18:4 fatty acid is easily explained by a lack of enoyl reduction during the last three elongation cycles. The data presented in this paper can be explained if a specific recognition site for enoyl-ACP reductase is located in the C-terminal domain of the AcpP sequence but not in the C-terminal domain of NodF: thus, enoyl-ACP reductase cannot recognize the latter protein.

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