Rhizobium NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase

(nitrogen fixation/lipo-oligosaccharide/inclusion bodies/protein refolding)

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ABSTRACT The common nodulation genes nodABC are conserved in all rhizobia and are involved in synthesis of a lipo-oligosaccharide signal molecule. This bacterial signal consists of a chitooligosaccharide backbone, which carries at the nonreducing end a fatty acyl chain. The modified chitooligosaccharide molecule triggers development of nodules on the roots of the leguminous host plant. To elucidate the specific role of the NodB protein in nodulation factor synthesis, we have purified recombinant NodB and determined its biochemical role by direct assays. Our data show that the NodB protein of Rhizobium meliloti deacylates the nonreducing N-acetylgluco- 

/lacosamine residue of chitooligosaccharides. The monosaccha-

dride N-acetylgluco-samine is not deacylated by NodB. In the pathway of Nod factor synthesis, deacylation at the nonreducing end of the oligosaccharide backbone may be a necessary requirement for attachment of the fatty acyl chain.

Soil bacteria of the genus Rhizobium symbiotically interact with leguminous plants, resulting in formation of root nodules in which the bacteria fix nitrogen (1). The development of nodular structures on roots is elicited by host-specific lipo-oligosaccharide signal molecules released by the bacteria (2-7). Basically, these molecules consist of a chitooligosaccharide backbone of different chain length that carries at the nonreducing end a long-chain unsaturated fatty acid (2-5).

Using Rhizobium strains with mutations in the highly conserved nodABC genes (8, 9) and Escherichia coli strains expressing cloned nod genes, it has been shown that the corresponding NodABC proteins produce extracellular factors that cause root hair deformation and branching (10-12), formation of thick short roots (13), and cortical plant cell division (14). Recent findings have indicated that no other symbiotic genes in addition to nodABC are required to synthesize core acylated glucosamine oligosaccharide signals, which induce these plant responses (4). The cytosolic proteins NodA (15) and NodB (16) are sufficient to produce compounds that stimulate mitosis in protoplasts derived from both leguminous and nonleguminous plants (16). Furthermore, transgenic tobacco plants transformed with nodA and nodB exhibit significant growth abnormalities, indicating that the products of these genes are somehow involved in production or modulation of growth signals in plants (ref. 17; J. Schmidt et al., unpublished data). To explain the effects caused by these genes in nonlegume transgenic plants, and to elucidate their specific role in the pathway of Nod signal synthesis, we have purified the NodB protein and determined its biochemical function.

MATERIALS AND METHODS

Overproduction and Purification of Recombinant NodB from E. coli. Recombinant DNA techniques were carried out essentially as described (18). The T7 promoter vector pET-3c (19) was used for production of intact NodB protein. To construct pJS3030, a 0.7-kb Rsa I fragment containing the NodB coding region of Rhizobium meliloti (16) was subcloned into the BamHI site of pUC18. The resulting BamHI nodB fragment was cut at the Alu I site immediately preceding the ATG by partial digestion. Insertion of the 670-bp Alu I/BamHI nodB fragment into the BamHI and filled-in Nde I sites of pET-3c yielded plasmid pJS3030, which was used to transform E. coli BL21(DE3) (19). E. coli cultures were grown in M9 salts (20) supplemented with 0.2% Casamino acids/0.4% glucose. Transformed cells were induced with 1 mM isopropyl-β-d-thiogalactoside, collected by centrifugation, and resuspended in 20% (wt/vol) sucrose containing 3 mM EDTA (pH 7.3). Cells were sonicated for 3 min on ice. Inclusion bodies were collected by centrifugation and were purified as described (15). For refolding of active NodB from insoluble and inactive protein aggregates, the inclusion bodies were dissolved in 50 mM Tris-HCl (pH 8.0) containing 5 M guanidine hydrochloride and 0.005% Tween 80 at 4°C. To promote correct disulfide bond formation, the clear solution was diluted 1:5 with refolding buffer containing 50 mM Tris-HCl (pH 8.0), 0.005% Tween 80, 2.4 mM reduced glutathione, and 0.024 mM oxidized glutathione. The procedure of refolding the NodB protein was essentially the same as described for urokinase (21). After dialysis and concentration by ultrafiltration, the refolded protein was applied to a Bio-Gel A-5.0m (Bio-Rad) gel chromatography column (2.6 × 120 cm) equilibrated and eluted with 20 mM Mops, pH 7.2/150 mM NaCl/0.1 mM dithiothreitol. Eluted and pooled fractions were dialyzed for testing against 20 mM Mops (pH 7.2) and were concentrated before storage at −20°C. The samples from each step in the purification were analyzed by electrophoresis on a SDS/12% polyacrylamide gel (22). Proteins were visualized by Coomassie blue staining (see Fig. 1) or immunostaining with anti-NodB antibodies (ref. 16; data not shown). Protein concentrations were determined by the method of Bradford (23) with bovine immunoglobulin as the standard.

Enzyme Assays and TLC of Products. Standard assay mixtures contained the following components in a final vol of 100 μl: 20 mM Mops (pH 7.2), 1 mg of substrate (GlcNAc or chitooligosaccharide; Sigma), and 50 μg of NodB protein from peak 2 (see Fig. 1C). After incubating at 30°C for 20 h, the reaction was terminated by heat inactivation (5 min at 95°C) followed by freezing. Control incubations lacking NodB were also carried out. To test whether anti-NodB antibodies can block the NodB-catalyzed deacetylation of chitooligosaccharides, monospecific polyclonal antibodies (20 μg) to NodB (16) and to NodA (15) as control were preincubated with 10 μg of NodB protein in 20 mM Mops (pH 7.2) on ice for 1 h. After centrifugation at 20,000 × g for 20 min, the supernatant was used for incubation with 100 μg of

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diacetylchitobiose at 30°C for 16 h. For hydrolysis of the NodB reaction product triacetylchitotetraose with chitinase from *Serratia marcescens*, the recombinant chitinase was purified by hydroxyapatite chromatography (24) from the culture supernatant of an *E. coli* strain overproducing this enzyme (25). Incubation was carried out in 100 μl of 25 mM sodium phosphate (pH 6.5) containing 100 μg of triacetylchitotetraose and 65 μg of recombinant chitinase. The reaction was maintained at 37°C for 16 h and stopped by heating; the products were analyzed by TLC. Re-N-acetylation of the NodB reaction products with acetic anhydride was carried out as described (26). Carbohydrates were separated by TLC on silica gel 60 plates (Merck) using 1-butanol/ethanol/water (5:3:2) as solvent. Sugars were detected on the TLC plates by spraying with the diphenylamine/aniline/orthophosphoric acid reagent (27). The deacetylated NodB reaction products were scraped from the TLC plates, extracted from silica with water, and finally purified by HPLC on a Supelcosil LC-SAX column (Supelco) eluted with water. Column effluent was monitored at 220 nm, and the breakthrough peak was collected and then used for structural analysis.

The pH optimum of deacetylase was determined by incubation of the enzyme (40 μg) with diacetylchitobiose (1 mg) in buffer solutions of various pH values for 4 h at 30°C. The buffers used were 0.1 M Tris-HCl (pH 7.0–9.5) and 0.1 M Pipes (pH 6.0–7.5). The amount of acetate released was determined enzymatically by using the test combination from Boehringer Mannheim.

**End Labeling of NodB-Treated Chitotetraose and Subsequent Enzymatic Degradation.** NodB-treated chitotetraose was isolated from preparative TLC plates and was purified by HPLC as described above. Re-N-acetylation of the tetrasaccharide with acetic [1-14C]diacetylchitobiose (Amersham) was carried out as described (26). Oligosaccharide aldolts were prepared by reduction with NaBH₄ essentially as described (28). Conditions for cleavage of the tetrasaccharide with chitinase from *S. marcescens* are given above. Cleavage of [14C]diacetylchitobiose with β-N-acetylglucosaminidase from beef kidney (Boehringer Mannheim) was performed in 200 μl of 50 mM sodium phosphate buffer (pH 5.0) with 4 units of enzyme. Incubation was carried out at 37°C for 16 h and was terminated by heat inactivation. HPLC of chitooligosaccharides and oligosaccharide aldolts was performed with two Aminex HPX-42 columns (Bio-Rad) with water as eluent (27). The elution of radioactive compounds was determined by collecting fractions and assaying suitable aliquots.

**RESULTS**

**Overproduction and Purification of NodB.** The *nodB* gene was expressed in *E. coli* under the control of the phase T7 promoter (ref. 19; Fig. 1A). Induction of cells with isopropyl β-D-thiogalactoside resulted in synthesis of high levels of NodB protein (Fig. 1B, lane 1), which accumulated in the form of an inclusion body (25). The inclusion body, readily pelletted by centrifugation through a 20% sucrose solution. Under these conditions, the inclusion bodies sedimented more rapidly than the cell debris, and purification to near homogeneity was readily achieved (lane 2). After denaturation in guanidine hydrochloride, NodB was refolded in a glutathione redox buffer (21) yielding a biologically active protein. Gel chromatography resulted in separation of the renatured NodB into three active fractions with different relative molecular masses, which may represent oligomeric forms of this protein (Fig. 1C, peaks 1–3). SDS/PAGE analysis of these fractions revealed that the relative molecular mass of 28.5 kDa was consistent with the expected size of the NodB polypeptide chain (30). Small amounts of NodB protein were also purified by immunoaffinity chromatography (data not shown). These preparations showed the same biological activity as described below for the refolded enzyme.

**NodB Deacetylases Chitooligosaccharides.** Purified NodB was incubated with N-acetylglucosamine and various chitooligosaccharides. TLC analysis of the reaction mixtures showed that chitooligosaccharides were almost completely converted into products that migrated on the silica plate (Fig. 2A, lanes 6, 8, and 10) with a slow rate comparable to that of glucosamine (lane 2). The monosaccharide N-acetylglucosamine was not modified (lane 4). The reaction of NodB with chitooligosaccharides was specifically blocked by the addition of anti-NodB antibodies to the incubation mixture (Fig. 2B, lanes 4 and 5). The fact that chitobiose was not fully converted in the control assay (lane 4) was due to the relatively high amount of buffer salts added with the anti-NodA antibodies. We observed that the NodB protein released acetate from chitooligosaccharides (Table 1), which indicated that the β-1,4-linked N-acetylglucosamine-containing oligosaccharides were deacetylated by NodB. Since every mol of substrate released 1 mol of acetate (Table 1), we concluded that only one N-acetylglucosamine residue of the oligosaccharide chain was deacetylated. We could also...
The chitooligosaccharide deacetylase had an optimum between pH 7.0 and 8.0, and a substantial loss of activity occurred below pH 6.0 and above pH 8.5. NodB was most active at 30°C, and the activity decreased rapidly above 40°C. The enzyme was strongly inhibited by heavy metal ions (at 1 mM). NodB Deacetylates Chitooligosaccharides at the Nonreducing N-Acetylglucosamine Residue. To identify the N-acetylaminogroup that is deacetylated in chitooligosaccharides, NodB-treated tetraacetylchitotetraose was re-N-acetylated with acetic [14C]anhydride and was subsequently converted into an alditol by borohydride treatment of the reducing end. This end-labeled tetraacetylchitotetraitol was sequentially digested with chitinase (Fig. 3A) and the exoglycosidase β-N-acetylglucosaminidase (Fig. 3B). Hydrolysis of the labeled tetrasaccharide by chitinase, followed by HPLC analysis of the products, yielded a nonradioactive disaccharide alditol (Fig. 3A, peak 2) and a radiolabeled diacetyldichitobiose (peak 1). The radiolabeled disaccharide deriving from the nonreducing end of the original substrate molecule was converted into an alditol and cleaved with the exoglycosidase (Fig. 3B). The products were N-acetylglucosaminitol (Fig. 3B, peak 3) and radiolabeled N-acetylglucosaminitol (peak 4), which indicates that the 14C label has been introduced at the nonreducing end of the NodB-treated tetrasaccharide.

**DISCUSSION**

It has been shown recently that the common nodABC genes are sufficient to synthesize basic signal molecules consisting of N-acetylated glucosamine oligosaccharides (4). The biosynthetic pathway of these lipid-linked oligosaccharides is not yet understood in detail. The first step in the assembly of the lipooligosaccharide is probably formation of the chitooligosaccharide backbone. A possible candidate for the synthesis of the carbohydrate backbone could be NodC, which shows homology to chitin and cellulose synthases (33). It is reasonable to assume that prior to attachment of the fatty acyl chain to the nonreducing N-acetylgulosamine residue of the chitooligosaccharide backbone, the corresponding N-acetylaminogroup must be deacetylated. For this reason, purified recombinant NodB was tested for deacetylase activity.

Several lines of evidence support our conclusion that NodB deacetylates the nonreducing N-acetylgulosamine residue of chitooligosaccharides. First, the NodB-treated chitooligosaccharides could be re-N-acetylated with acetic anhydride (Fig. 2B). We also observed that NodB released acetate from chitooligosaccharides. Evidence that the enzyme deacetylates only one N-acetylgulosamine residue of the chitooligosaccharide chain is provided by the release of equimolar amounts of acetate independent from the chain length of the substrate molecule (Table 1). This finding is supported by the observation that the NodB-treated tetrasaccharide could be enzymatically cleaved into equal amounts of nondeacetylated and deacetylated disaccharides (Fig. 2C), which demonstrates that NodB deacetylates both enzyme groups only at one end of the oligosaccharide chain. The site of deacetylation was determined by labeling NodB-treated chitotetraose with acetic [1-14C]anhydride at the nonreducing end (Fig. 3).

Little is known about the action of deacetylases on oligosaccharides. A deacetylase with a mode of action similar to that of NodB has been partially purified from Clostridium tertium (34). This enzyme deacetylates the terminal nonreducing N-acetylgalactosamine residue in blood group A substance, which leads to a loss of serological activity. A computer search revealed no significant homology of NodB to other proteins. Our data suggest that during lipoooligosaccharide synthesis NodB removes the acetyl group from the terminal nonreducing N-acetylgulosamine residue in a chitooligosaccharide precursor molecule. The free amino group

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**Table 1. Release of acetate from chitooligosaccharides by NodB**

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<tr>
<th>Substrate</th>
<th>Acetate released, µmol</th>
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<tr>
<td>N-Acetylglucosamine</td>
<td>0</td>
</tr>
<tr>
<td>N,N'-Diacetyldichitobiose</td>
<td>0.24</td>
</tr>
<tr>
<td>N,N',N'-Triacetyldichitriose</td>
<td>0.23</td>
</tr>
<tr>
<td>N,N',N',N'-Tetraacetyldichitotetraose</td>
<td>0.23</td>
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Duplicate enzyme incubations were carried out with the following components in a final volume of 100 µl: 0.24 µmol of substrates, 20 mM Mops buffer (pH 7.2), and 100 µg of NodB protein. The reaction was performed at 30°C for 20 h and was stopped by heat inactivation (5 min at 95°C). Completeness of the reaction was examined by TLC. Amount of acetate released was determined enzymatically (32).
at the nonreducing terminus could then be acylated with a specific fatty acyl chain.

When NodB is synthesized in transgenic tobacco plants, this deacetylase causes characteristic morphological alterations (17), indicating that tobacco must contain substrates that can be modified by NodB to form plant growth signals. We want to use this enzyme as a tool to isolate and label the N-acetylglucosamine-containing oligosaccharide signals from plants, which hopefully will lead to a better understanding of plant growth and development in general.

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