Dictyostelium discoideum Fatty-acyl Amidase II Has Deacylase Activity on Rhizobium Nodulation Factors*

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Dictyostelium discoideum (Amoeboidea) secretes cell-lysing enzymes: esterases, amidases, and glycosylases, many of which degrade soil bacteria to provide a source of nutrients. Two of these enzymes, fatty-acyl amidases FAA I and FAA II, act sequentially on the N-linked long chain acyl groups of lipid A, the lipid anchor of Gram-negative bacterial lipopolysaccharide. FAA I selectively hydrolyzes the 3-hydroxyxymyristoyl group N-linked to the proximal glucosamine residue of de-O-acylated lipid A. Substrate specificity for FAA II is less selective, but does require prior de-N-acylation of the proximal sugar, i.e. bis-N-acylated lipid A is not a substrate. We have synthesized a 14C-labeled substrate analog for FAA II and used this in a novel assay to monitor its purification. Inhibitory studies indicate that FAA II is not a serine protease, but may have a catalytic mechanism similar to metalloprotein de-N-acylases such as LpxC. Interestingly, rhizobial Nod factor signal oligosaccharides that induce root nodules on leguminous plants have many of the structural requirements for substrate recognition by FAA II. In vitro evidence indicates that Rhizobium fredii Nod factors are selectively de-N-acylated by purified FAA II, suggesting that the enzyme may reduce the N2-fixing efficiency of Rhizobium-legume symbioses. In contrast, N-methylated Nod factors from transgenic R. fredii carrying the rhizobial nodS gene were resistant to FAA II, suggesting a mechanism by which Nod factors may be protected from enzymatic de-N-acylation. Since FAA II and Nod factors are both secreted, and Nod factors that lack the N-acetyl group are unable to induce nodules, dictyostelial FAA II may decrease the efficiency of symbiotic nitrogen fixation in the environment by reducing the available biologically active nodule inducer signal.

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¶ The abbreviations used are: FAA, fatty-acyl amidase; GlcN-C18, N-palmitoyl[1-14C]glucosamine; HPLC, high performance liquid chromatography; RMM, rhizobial minimal medium.
leguminous plants. Interestingly, Nod factors from a transconjugate of USDA257 expressing the nodS N-methyltransferase gene from Rhizobium sp. NGR234 are found to be more resistant to FAA II, suggesting that N-methylation may protect Nod factors against deacetylation by Dictyostelium in soil.

EXPERIMENTAL PROCEDURES

Materials—D. discoideum strain Ax-3 (ATCC 28368) was obtained from American Type Culture Collection (Rockville, MD). The naga-deleted strain (HL101) was obtained from William Loomis (University of California, San Diego, CA). Rhizobium strains were obtained from Steve Pueppke (University of Missouri, Columbia, MO). Radiolabeled [1-14C]glucosamine (specific activity of 45–60 mCi/mmol) and sodium [2-14C]acetate (540 mCi/mmol) were obtained from NEN Life Science Products. Serine protease inhibitors were obtained from Sigma, as were buffers, chemicals, and chromatography supplies. Kodak X-OMat AR-5 was purchased from S & W X-Ray (Syracuse, NY). Buffer A contained 20 mM Tris-HCl (pH 7.8) plus 0.1 mM EDTA, and Buffer B contained 5 mM sodium acetate (pH 4.0).

Synthesis of the Radiolabeled Substrate N-Palmitoyl[1-14C]glucosamine—[1-14C]Glucosamine (0.5 μCi, 50 nCi) was spotted at the origin on a normal-phase Silica Gel 60 thin-layer chromatography plate, dried under a stream of warm air, and overspotted with palmitoyl chloride (2 μl). After an 18-h reaction at room temperature, the plate was developed with chloroform, methanol, and aqueous ammonium hydroxide (50:40:5 by volume). Untreated [1-14C]glucosamine was run in a control lane. The plate was dried until free of solvent and autoradiographed for 5 days.

Isolation and Purification of FAA II—Culture supernatant (14 liters) from D. discoideum strain Ax-3 was concentrated by passage through a 30-kDa cutoff Amicon filter, and the concentrated supernatant (1 liter) was stirred overnight with DEAE-cellulose (50 g, 4 °C). The protein pellet was washed with Buffer B (5 bed volumes) and eluted with Buffer B (30 ml) containing 150 mM sodium chloride in cold Buffer A (125 ml), and eluted with Buffer C (1 ml) containing 0.2% Triton X-100 in Buffer B at 30 °C.

Preparation of Metabolically 14C-Labeled Nod Factors—Rhizobia were grown aerobically at 30 °C on rhizobial minimal medium (RMM) in the presence of succinate (12 mM) and glutamate (6 mM) as described previously (11). Cultures (10 ml) were radiolabeled by the inclusion of [1-14C]glucosamine (40–60 mCi/mmol, 1 μCi/ml) at the start of the growth period. After 4 h of growth (A550 = 0.3), the nod genes were induced by the addition of the species-specific flavonoid apigenin (5 × 10−4 M). After 18 h, the bacteria were removed by centrifugation (10,000 × g, 4 °C), and radiolabeled Nod factors were recovered from the culture supernatant by solid-phase extraction with C18-functionalized silica (Waters) (18). After washing with water (5 ml), radiolabeled metabolites were eluted with methanol and evaporated to dryness.

RESULTS

Substrate Specificity and Development of an Enzyme Assay—We prepared and purified, by thin-layer chromatography with autoradiographic detection (Fig. 2), a radiolabeled synthetic substrate for FAA II, N-palmitoyl[1-14C]glucosamine (Fig. 3). Conditions were optimized for two-phase partitioning of N-palmitoyl[1-14C]glucosamine from the free sugar [1-14C]glucosamine, making use of ethyl acetate/hexane (90:10, v/v) and aqueous sodium chloride (10%, w/v). This allowed selective recovery of the radiolabeled monosaccharide in the aqueous layer. Thus, treatment of N-palmitoyl[1-14C]glucosamine with FAA II in aqueous Buffer B and the addition of sodium chloride (to 10%, w/v), selective counting of the aqueous and organic phases is an indication of the reaction progress. The salt also served the dual purpose of stopping the reaction.

Solvent extraction of radiolabeled GlcN-C16 was optimized such that 98% of the radioactivity from the enzyme-free control partitioned into the solvent phase (Table I). Following treatment with FAA II (10 mg/ml, 18 h, 30 °C), 35.4% of the counts remained in the aqueous buffer as deacylated [1-14C]glucosamine. The degree of activity in secreted dictyostelial exo-
bial Nod factors were radiolabeled metabolically by culturing radiolabeled \([1-14C]\)glucosamine (0.1 mM) at a final concentration of 50 mM. As Table I indicates, these Table I. EDTA was prepared in Buffer B (292 mg/ml) and used an ethanol or acetonitrile and used at the concentrations shown in methylsulfonyl fluoride were prepared at 50 mM in either methanol, evaporated to dryness, and stored at \(-20^\circ C\).

extracts was monitored, allowing for complete purification and a mechanistic study of FAA II.

**Purification and Mechanistic Studies of FAA II**—Purification was undertaken on a secreted fraction from the culture supernatant of *Dictyostelium* strain Ax-3 after passage through a 30-kDa cutoff filter. Activity was monitored by the two-phase partitioning assay. Enzyme assays were typically run at 10 mg/ml total protein, giving a 56.3% decrease in counts in the organic phase relative to controls after a 18-h reaction time. A concomitant increase in counts was recorded in the aqueous extraction buffer. Lower concentrations of FAA II or shorter reaction times resulted in decreased activity. Final purity of the enzyme was ascertained by SDS-polyacrylamide gel electrophoresis and reverse-phase HPLC (Fig. 4). Verret et al. (2) were unable to fully purify FAA II free from acetylhexosaminidase A (NagA), but estimated its relative mass to be in the range of 60–80 kDa. Here the molecular mass of FAA II was determined by gel electrophoresis and gel-filtration HPLC (Fig. 4). The protein eluted on gels with a molecular mass of 66,000 (Fig. 4). By gel filtration, the same purified fraction eluted as a single peak, but in this case, the relative molecular mass was estimated at 32,985 Da.

FAA II may be mechanistically similar to serine proteases or metalloproteases in that amide bonds are being hydrolyzed. To examine this, we assayed purified FAA II in the presence of two known protease inhibitors (Table I). Stock solutions of phenylmethylsulfonyl fluoride (PMSF) and EDTA (50 mM) were prepared in buffer B (292 mg/ml) and used at a final concentration of 50 mM. As Table I indicates, these inhibitors had no significant effect on the activity of FAA II under the conditions tested, and results were within \( \pm 4.1\%\) of the non-inhibited FAA II reaction.

**FAA II Selectively Deacylates Rhizobial Nod Factors**—Rhizobial Nod factors were radiolabeled metabolically by cultivating rhizobial strains on minimal medium in the presence of precursor \([1-14C]\)glucosamine and apigenin, an appropriate flavonoid nod gene inducer (18). Alternatively, optimal incorporation of radiolabel from acetate was attained by incubation with sodium \([2-14C]\)acetate as the sole carbon source prior to chasing with succinate (12 mM) and glutamate (6 mM). The secreted hydrophobic fractions containing the Nod factors were separated from excess radiolabeled precursor and other labeled polar metabolites on a reverse-phase cartridge. The induction of Nod factors, as assessed by TLC/autoradiography, was clearly dependent on the presence of apigenin (Fig. 5). Induc-
Purification of fatty-acyl amidase II. FAA II was purified from *D. discoideum* culture exudate by 1) passage through a 30-kDa cutoff filter, 2) DEAE ion-exchange chromatography (pH 7.8), 3) salting out with ammonium sulfate (70%), and 4) DEAE ion-exchange chromatography (pH 4.0). Fractions were assayed for protein using the BCA assay and for FAA II activity as described under “Experimental Procedures.” Active fractions were stored at −20 °C prior to purification by high performance liquid chromatography. A, SDS-polyacrylamide gel stained with Coomassie Blue. Left lane, FAA II (indicated by an arrow); right lane, molecular mass markers (66 and 48.5 kDa indicated by arrows). B, reverse-phase HPLC trace of FAA II after separation on a Spheri-5 C₈ analytical column with a linear gradient (10–100% acetonitrile and 0.05% trifluoroacetic acid). Detection was by absorbance at 220 nm and by BCA protein assay.

Activity of FAA II on metabolically radiolabeled Nod factors (lipo-oligosaccharides) from *R. fredii*. Nod factors were isolated from *R. fredii* USDA257 grown on RMM medium with appropriate radiolabeled precursors. The Nod factor fractions were isolated on a C₁₈ reverse-phase cartridge, washed free of excess radiolabel, and eluted with methanol. Amidase reactions were started by the addition of FAA II in Buffer B (10 mg/ml, 100 μl) plus Triton X-100 (0.2%). Following incubation (18 h, 30 °C), an equivalent volume of methanol was added to stop the reaction, and samples were centrifuged (10,000 × g, 2 min) and spotted onto Silica Gel 60 TLC plates. Plates were eluted with butanol/ethanol/water (5:3:2 by volume), air-dried, and autoradiographed for 5 days. A and B, [1-¹⁴C]acetate-labeled; C, [1-¹⁴C]glucosamine-labeled. *Lanes 3* and *4*, cultures induced with 10 mg/ml protein, 18 h, 30 °C; *lanes 1* and *2*, enzyme-free controls. *Lanes 1* and *2* (as shown in Fig. 5) are reproduced for comparison. Chromatography and autoradiography were carried out as described for Fig. 5.

The FAA II assay was modified so that activity on Nod factors could be monitored by TLC/autoradiography. Hydrophobic extracts of the conditioned culture medium from apigenin-induced or -uninduced *R. fredii* cultures were treated in parallel with FAA II prior to chromatographic separation (Fig. 5). Comparable reactions lacking the FAA II enzyme served as controls. Treatment of [¹⁴C]acetate-labeled Nod factors with FAA II for 18 h resulted in an attenuation of the labeling (Fig. 5, A and B), suggesting that either the hydrolyzed acyl group or the unhydrolyzed Nod factor was sequestered with the amidase. This was confirmed by the presence of radioactivity in the protein pellet. Extracts from uninduced cultures were unaffected by this treatment. Similar experiments on [¹⁴C]glucosamine-labeled Nod factors resulted in a chromatographic mobility shift, but without attenuation of intensity (Fig. 5C). These data are consistent with metabolic incorporation of [¹⁴C]acetate into the Nod factor acyl chain and of [¹⁴C]glucosamine into the lipo-oligosaccharide backbone. Following deacylation, radiolabel is lost from [¹⁴C]acetate-labeled Nod factors or retained but chromatographically shifted for [¹⁴C]glucosamine-labeled Nod factors.

Wild-type *R. fredii* USDA257 produces fucosylated and N-vaccenyloxy-labeled Nod factors which are inducible by apigenin and by the flavonoid-inducible incorporation of metabolic label from [methyl-¹³C]methionine.² Apigenin-inducible, [¹⁴C]glucosamine-labeled Nod factors were obtained from this strain as observed by TLC/autoradiography (Fig. 6). However, unlike the wild-type *R. fredii* Nod factor, the majority of the N-methylated factor was unaffected by the FAA II treatment.

² A. E. Tobin and N. P. J. Price, manuscript in preparation.
and was observed on plates to co-migrate with the untreated control (Fig. 6).

**DISCUSSION**

Fatty-acyl amidases play a central role in the deacylation of bacterial lipid A (Fig. 1) by selective removal of the N-linked fatty acid chains. The presumed natural substrate for FAA II is generated by degradation of de-O-acylated lipid IVβ by FAA I to form 4-phosphoryl-N-β-hydroxyxymristoyl-d-glucosaminyl-β,1,6-glucosamine 1-phosphate. Removal of the O-acyl chains and the 2-deoxyketooctulosonic acid residues is presumed to occur prior to this due to the action of other secreted hydrolases (3). Interestingly, neither amidase requires the C-1 or C-4 phosphate group for substrate recognition, and although FAA II requires a deacylated proximal sugar, the positive charge on the zinc atom is buried within the protein (15). FAA II is an extracellular protein that most probably requires additional stability to maintain a folded conformation. It too may have an inaccessible buried metallo-center so that enzymatic activity is retained in soil.

We have shown that Nod factors are selectively de-N-acylated by the action of the fatty-acyl amidase FAA II. Since non-acylated Nod factors lack nodule-promoting biological activity, deacetylation may well have an effect upon the ability of rhizobia to nodulate host plants. Moreover, we propose that Nod factors that are modified by the presence of conjugated double bonds (such as those from *Rhizobium meliloti* and *Rhizobium leguminosarum*) or by N-methylation of the nonreducing residue (such as, for example, *Rhizobium NGR234*) may be altered in their sensitivity to enzymatic de-N-acylation by FAA II. Consequently, these modifications may protect Nod factors against degradation by *D. dissoideum* in soil. Truchet et al. (23) have shown that nodulation efficiency is concentration-dependent and that nodule induction does not occur below a threshold Nod factor concentration of ~10⁻⁷ M, even though a legume root hair deformation response is observed at far lower concentrations. FAA II de-N-acylation of Nod factors may therefore reduce the efficiency of legume nodulation by reducing the concentration of available biologically active Nod factors. Since Nod factors and FAA II are both secreted by soil-dwelling *Rhizobium* and *Dictyostelium* species, respectively, Nod factor degradation may also occur in soils by this mechanism, decreasing the efficiency of nitrogen-fixing *Rhizobium*-legume symbioses.

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