Enzymatic radiolabelling to a high specific activity of legume lipo-oligosaccharidic nodulation factors from *Rhizobium meliloti*

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In this paper we describe the two-step coupled ^{35}S-radiolabelling of the lipo-oligosaccharidic nodulation (Nod) factors of the bacterium *Rhizobium meliloti* to a specific radioactivity of 800 Ci/mmol. These radiolabelled Nod factors bind to a particulate fraction from roots of the bacterium's symbiotic host, *Medicago truncatula*, with an equilibrium dissociation constant (K_D) of 117 nM, similar to that observed with a synthetic tritiated ligand. The first step of the ^{35}S-labelling involves the synthesis of 3′-phosphoadenosine 5′-phospho[^{35}S]sulphate ([^{35}S]PAPS) from ATP and [^{35}S]sulphate using yeast enzymes. The second step exploits the sulphotransferase activity of the *R. meliloti* NodH protein, which has been expressed in *Escherichia coli*, to transfer the labelled sulphate group from PAPS to non-sulphated Nod factors. This enzyme was found to be active in *E. coli* cultured at 18 °C but not 37 °C. NodH could also transfer the sulphate group from PAPS to a model substrate, tetra-*N*-acetyl chitotetraose, with apparent K_m values of 56 and 70 μM respectively, and exhibited an apparent K_m value for non-sulphated Nod factors of 28 μM. Coupling the two steps of the radiolabelling resulted in an efficiency of ^{35}S incorporation from inorganic sulphate to the Nod factors of ~ 10%. These labelled factors will be a valuable tool in the search for high-affinity receptors for the lipo-oligosaccharidic nodulation factors.

INTRODUCTION

In order to establish the important nitrogen-fixing, legume–rhizobia symbiosis, the two partners exchange chemical signals (for reviews, see [1,2]). Firstly, the plant exudes flavonoids or other chemicals which induce the expression of the rhizobial *nod* genes, a set of genes shown genetically to be responsible for initiating the production of nodules on the roots of the host plant, in which the bacteria fix nitrogen. The majority of the *nod* genes encode enzymes involved in the synthesis of an excreted signal, termed the nodulation (Nod) factors. These factors have been shown to be lipo-oligosaccharides consisting of 3–6 glucosamine residues, N-acetylated for most of the sugars (hence resembling chitin oligomers) but N-acylated on the non-reducing sugar. Other decorations may be present, depending on the rhizobial species. The major Nod factor of *Rhizobium meliloti* (NodRm factor), for example, consists of four glucosamine residues, which are sulphated on C6 of the reducing sugar, *O*-acetylated on C6 of the non-reducing sugar, and contain a $C_{16:2}$ fatty acid (this factor is termed NodRm-IV(Ac,S,C16:2); see Scheme 1). In addition, *R. meliloti* produces a number of other variant Nod factors based on this lipo-oligosaccharidic theme [3–5].

Purified Nod factors have been shown to elicit a number of biological responses on the host-plant roots including root-hair deformations, cortical-cell divisions, depolarization of root-hair membranes, expression of specific genes and induction of nodules [3,4,6–10]. These responses occur at pico–nanomolar Nod factor concentrations and some of them are highly dependent on the decorations on the molecule. For example, for the *R. meliloti* factors, the sulphate group is of particular importance [11] but the presence of the O-acetate, the structure of the acyl group, the number of glucosamine residues and the integrity of the reducing sugar have all been shown to effect some of the biological responses.

These observations suggest that the Nod factors may be perceived by specific receptors located on the roots of the host plant. As a first step to identifying such receptors it is useful to radiolabel the putative ligand to a high specific activity. Recently NodRm-IV(Ac,S,C16:2) has been synthesized chemically [12] and a tritiated derivative has been used to demonstrate the presence of a Nod-factor-binding site in root extracts of both a legume and a non-legume plant which is not dependent on the sulphate group [13]. However, it is possible that other binding sites that are specific for the symbiosis have not been detected because of the relatively low specific activity of tritium. In an attempt to radiolabel the Nod factors with ^{125}I, the reducing or the non-reducing sugar was opened up and derivatized with phenolic compounds. However, these modifications and subsequent iodination led to at least a 100-fold reduction in biological activity, as measured by the root-hair deformation bioassay (P. Lerouge, J.-J. Bono and J. V. Cullimore, unpublished work). In this paper we describe an enzymatic method for radiolabelling non-sulphated NodRm factors with ^{35}S to yield a highly labelled, biologically active ligand.

In *R. meliloti*, mutations in the *nodH* gene lead to the production of non-sulphated Nod factors. As the sequence of *nodH* shares low but significant identity with those of sulphotransferases, it is reasonable to believe that this gene encodes an enzyme involved in the sulphation of the Nod factors [11]. The

Abbreviations used: APS, adenosine 5′-phosphosulphate; IPTG, isopropyl β-D-thiogalactopyranoside; NFBS, Nod-factor-binding site; Nod, nodulation; NodRm, Nod factor of *Rhizobium meliloti*; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; PEI, polyethyleneimine; PVDI, polyvinyl imidazole.

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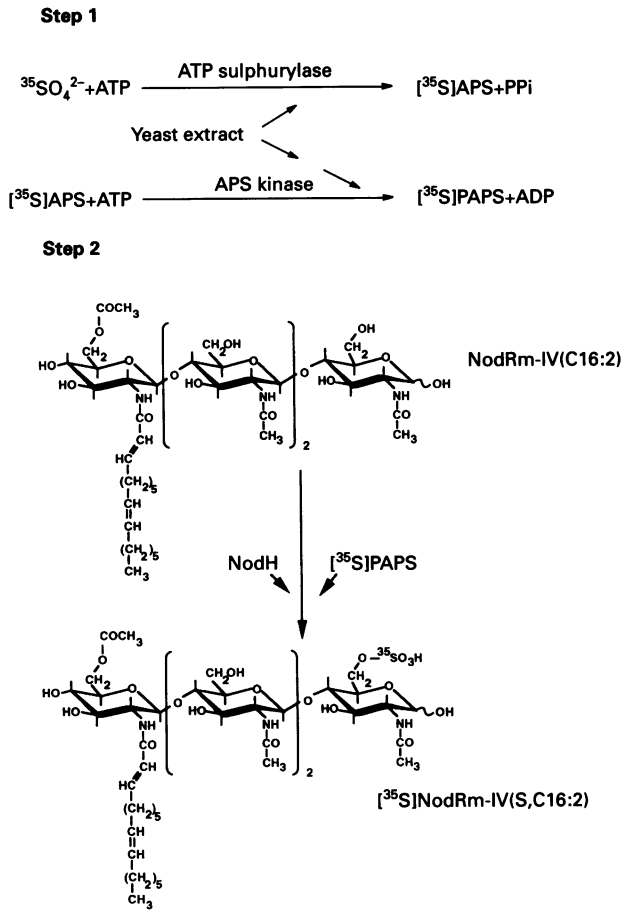

Scheme 1 Enzymatic two-step labelling of NodRm factors with [^{35}S]sulphate

The first step is the synthesis of [^{35}S]PAPS by yeast enzymes using [^{35}S]sulphate, and the second step is the sulphation of non-sulphated NodRm factors by the enzyme NodH using the [^{35}S]PAPS.

nodPQ genes are also important for sulphation of NodRm factors and encode enzymes involved in creating an activated form of sulphate [adenosine 5′-phosphosulphate (APS) and possibly PAPS] [14]. As PAPS acts as a sulphate donor in other systems, it is probable that PAPS is the sulphate donor for a NodH sulphotransferase [14]. In this paper we have prepared a PAPS-synthesizing extract from yeast and have expressed *R. meliloti* NodH in *Escherichia coli* and partially characterized its activity. Using these two sets of enzymes we describe a two-step coupled method of labelling NodRm factors using ^{35}S (Scheme 1).

EXPERIMENTAL

Materials and reagents

Reagents

Baker's yeast (*Saccharomyces cerevisiae*), brand L'Hirondelle, was purchased from Lessaffre (Paris, France). ATP, isopropyl β-D-thiogalactopyranoside (IPTG), PAPS, rifampicin, ultra pure Trizma base (sulphate < 0.0005%, w/w), and yeast hexokinase were products of Sigma (St. Louis, MO, U.S.A.). Radiolabelled carrier-free $Na_2{}^{35}SO_4$ (1500 Ci/mmol), [^{35}S]methionine, and En^{3}hance, an autoradiography enhancer, were from Dupont De Nemours (Wilmington, DE, U.S.A.). Methionine assay medium was a product of Difco (Detroit, MI, U.S.A.). Tetra-*N*-acetyl chitotetraose was from Yaizu Suisan Kagaku (Tokyo, Japan). Luteolin was a product of Extrasynthèse (Genay, France). Analytical grade water (sulphate < 0.00001%, w/v) and $MgCl_2$,$6H_2O$ (sulphate < 0.002%, w/w) were from Merck (Darmstadt, Germany).

Equipment

H.p.l.c. apparatus was from Hewlett–Packard. Ion-exchange column polyvinyl imidazole (PVDI) was from Shandon-Société Française de Chromatographie sur Colonne (France) and the C18 Spherisorb ODS2 column used in this work was a product of Chromatosud (France). C18 Bond-elut was a product of Analytichem International (Harbor City, CA, U.S.A.). Silica and polyethylene imine (PEI) t.l.c. plates were from Merck.

Bacterial strains and plasmids

The *R. meliloti* strains used were: GMI357 (carries a pSym deletion: *nodD1ABC*) [15], and GMI6372 (*nodH*::Tn*5*#2313 / pGMI149*nodH*::Tn*5*#2313) (pGMI149 contains a 30 kb insert from Rm pSym*a* carrying the *nod* region) [11]. The *E. coli* strains used were: K12 strains TG1 (*Δlac-pro supE thi hsdS* / F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZDM15*) [16], BL21 (λ lysogen containing the T7 RNA polymerase gene under control of the *lac* promoter) [17] and HB101 (*pro leu thi lacY hsd20 endA recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*) [18].

The following plasmids have been published: pT7-7 (a derivative of pT7-1 which allows expression of genes under control of the T7-RNA polymerase-recognized φ10 promoter) [19], pGMI515 (RP4′, IncP, contains a 26 kb insert from Rm pSym*a* carrying *nodFEGHD3syrM*, Tc^r) [15], pGMI174 (pBR322′, ColE1, contains a 6.1 kb insert from Rm pSym*a* carrying *nodFEGH*) [20].

Synthesis and purification of [^{35}S]PAPS

An extract capable of synthesizing PAPS was prepared from 2.5 kg of Baker's yeast [21]. [^{35}S]PAPS was synthesized in a reaction volume of 150 μl containing 14.7 mM ATP, 16.7 mM $MgCl_2$, 0.1 M Tris/HCl buffer, pH 8.5, 1 mCi (4.5 μM) Na_2SO_4 and 60 μl of yeast extract (~ 10 mg of protein) for 60 min at 37 °C. The mixture was boiled for 2 min and centrifuged. To the supernatant was added 4 μl of 20% (w/v) glucose (29 mM) and 10 units of hexokinase. The ATP was then enzymatically hydrolysed by incubation for 30 min at 30 °C. The reaction mixture was boiled for 2 min and centrifuged.

The PAPS was purified by h.p.l.c. using a PVDI (100 mm × 4.6 mm, particle size 5 μm, pore size 300Å) column using a programme of 5 min of buffer (20 mM sodium phosphate, pH 7.1) followed by 40 min of a 0–1 M NaCl gradient in the same buffer. The flow rate was 0.5 ml/min and 1 min fractions were collected. The radiolabelled PAPS eluted at ~ 400 mM NaCl, ~ 5 min after the residual ATP. The quantity of PAPS obtained was estimated by recording A_{259}, and in these conditions a specific activity of the radiolabelled PAPS of ~ 100 Ci/mmol was obtained.

Cloning and expression of NodH

Plasmid pGMI174 containing the *nodFEGH* genes of *R. meliloti* was isolated from the *E. coli* strain HB101. An *Nde*I site was introduced at the first ATG of the *nodH* gene using a 17-mer primer containing three mismatches (ACA CGC CCA TAT GAC CC) and a 17-mer primer with two mismatches, (CGG

CGT GGA TCC ACA TG), that hybridizes 183 bp downstream of the NodH stop codon and introduces a *Bam*HI site at this position. The *nodH*-containing fragment of 901 bp was amplified by PCR [22] and, after digestion with the appropriate enzymes and gel purification, was cloned into the *Nde*1 and *Bam*HI sites of pT7-7 in *E. coli* TG1. The resulting plasmid (pT7H1) was verified by restriction analysis and then used to transform *E. coli* BL21. The resulting strain (GMI96019) was used as a source of NodH in all further experiments and was compared with a strain containing the vector pT7-7 in BL21.

To verify the expression and localization of NodH, exponential-phase cultures grown at 37 °C in LB medium [18] were centrifuged, washed twice with M9 minimal medium [18] supplemented with 0.5% (v/v) methionine assay medium, 0.2% (w/v) glucose, 50 μg/ml ampicillin, 0.5 μg/ml thiamine and 1 mM $MgSO_4$ and then resuspended in 20 ml of M9 complete medium to an A_{600} of 0.45. The cultures were divided into two parts and incubated at 37 or 18 °C for 65 min to ensure complete depletion of methionine. A non-induced sample (0.5 ml) was taken at this time from the 37 °C culture. The cultures were induced with IPTG (0.2 mM for the 37 °C culture and 0.4 mM for the 18 °C culture) and incubated for 75 min (37 °C) and 150 min (18 °C). Then 7.5 ml of the 37 °C culture was incubated with 0.2 mg/ml rifampicin for 40 min. The cultures were labelled with 10 μCi/ml [^{35}S]methionine for 2 min at 37 °C (for the 37 °C culture) or 5 min at 15 °C (for the 18 °C culture). Ten microlitres of 2% (w/v) methionine was added per ml of culture and the cells were harvested by centrifugation. Part of the cell cultures was fractionated by rupturing spheroplasts [23] by two cycles of freezing/thawing. The extracts were resuspended in SDS gel loading buffer and boiled for 5 min, and volumes corresponding to equivalent amounts of cells were loaded onto SDS/12% (w/v)PAGE gels according to the Laemmli method [24]. Fluorography was performed using En^{3}Hance.

Preparation of NodH extract

An exponential-phase culture of *E. coli* BL21(pT7H1) grown at 37 °C in LB medium was shifted to 15 °C, induced with 0.4 mM IPTG and incubated for 24 h. The cells were collected by centrifugation in a GS3 rotor (Sorvall) at 5000 rev./min (4200 *g*) for 10 min. The cell pellet was washed once with ice-cold 0.2 M sodium phosphate, pH 7.7. The cell pellet was quick-frozen in liquid nitrogen and stored at −70 °C. When needed the cells were resuspended on ice with 1 ml/g of cells of buffer H [10 mM potassium phosphate, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% (w/v) glycerol]. Phenylmethanesulphonyl fluoride (1 mM) was added and the mixture was sonicated on ice. Streptomycin sulphate (1%, w/v) was added, and the extract was left on ice for 1 h, with gentle shaking from time to time. The extract was centrifuged for 20 min at 20000 rev./min (35000 *g*) in a Ti50.2 rotor (Beckman). The supernatant was retrieved and dialysed overnight in a cold room against buffer H to get rid of bacterial PAPS. This crude extract was used as a source of NodH enzyme in the sulphation experiments.

Enzyme kinetic studies

Reactions were carried out in 30 μl containing 20 μl of NodH extract (20 μg of protein) and constant concentrations of PAPS (50 μM; containing 2.3×10^6 c.p.m. of purified [^{35}S]PAPS) and tetra-*N*-acetyl chitotetraose (60 μM). For determination of the apparent K_m for PAPS, the concentration was varied between 5 and 100 μM, for tetra-*N*-acetyl chitotetraose between 3.6 and 120 μM, and for Nod factors between 1.95 and 150 μM. The reaction mixtures were incubated for 10 min at 28 °C, and then boiled for 1 min and centrifuged for 2 min, and 2 μl of each reaction was loaded on to silica 60 t.l.c. plates. The eluting solvent for sulphated tetra-*N*-acetyl chitotetraose analysis was butan-2-ol/ethanol/water (4:3:3, by vol.), and for sulphated Nod factors analysis was chloroform/methanol/14% ammonium hydroxide (45:45:15, by vol.). After drying the plates, the PAPS and the sulphated substrates were located by autoradiography, and the radioactive spots were scraped off and counted by scintillation counting. The percentage incorporation of the PAPS into the sulphated substrates was calculated and used to determine the K_m. The best linear fits of the Lineweaver–Burk plots were determined using the Cricket-Graph software (Apple).

Isolation of Nod factors

Non-sulphated factors were isolated from the supernatant of 4 l of *nod*-genes induced culture of strain GMI6372 [11] essentially as described [3], except that the gel-permeation and ion-exchange chromatographic steps were omitted from the purification scheme. M.s. showed that this preparation contained primarily tetrameric NodRm factors which were non-sulphated and non-O-acetylated and contained the fatty acids $C_{16:2}$ and $C_{16:1}$.

Coupled two-step radiolabelling of NodRm factors

The reagents for the first step (synthesis of PAPS) were of the highest grade available in order to reduce the concentration of unlabelled sulphate. The yeast extract (2 ml) was dialysed for 3 h against 100 ml of 20 mM Tris/HCl buffer, pH 8. The reaction mixture (65 μl) contained 3.8 mM ATP, 38.5 mM $MgCl_2$, 5 mCi $Na_2{}^{35}SO_4$ (51 μM) and 60 μl of dialysed yeast extract (~ 10 mg of protein) and was incubated for 30 min at 37 °C. The reaction mixture was boiled for 2 min and centrifuged for 2 min. At this stage the extent of [^{35}S]PAPS synthesis was monitored by spotting 0.5 μl of the reaction mixture on to a PEI–cellulose F t.l.c. plastic plate and run with 1 M NaCl as eluting solvent. The radioactive spots, located by autoradiography, were cut out and counted by scintillation counting. The supernatant of the reaction was transferred to a fresh tube containing 10 nmol (87 μM final concentration) of non-sulphated Nod factors, and 50 μl of NodH extract (~ 0.75 mg of protein) was added. The reaction mixture was incubated for 45 min at 30 °C and then extracted twice with 150 μl of butan-1-ol. The organic phase was evaporated to dryness in a stream of nitrogen gas and resuspended in 100 μl of ethanol/water (1:1, v/v). The Nod factors were separated by h.p.l.c. on a 250 mm × 7.5 mm C18 Spherisorb ODS2, particle size 5 μm, column. The program was 25 min of 80% (v/v) buffer A (10 mM K_2SO_4, pH 4.1, acetonitrile; 80:20, v/v) and 20% (v/v) acetonitrile, followed by a gradient of 20 min from 80 to 50% buffer A, followed by 10 min of 50% buffer A. The flow rate was 1 ml/min and 2 ml fractions were collected. The column was calibrated with sulphated and non-sulphated NodRm-IV (C16:2) which elute at 19 and 36 min respectively. The radioactive peak between 18 and 20 min was collected and evaporated to dryness as before. The factors were dissolved in 1 ml of water and desalted by passage through a C18 Bond-elut, washing with 3 ml of water and eluting with 1.5 ml of ethanol/water (1:1, v/v).

Determination of protein

Protein concentrations were estimated by the method of Bradford [25] using a commercial reagent (Bio-Rad) and lysozyme as a standard.

Binding of labelled Nod factors to a particulate *Medicago truncatula* root fraction

Plants of *M. truncatula* cv. Jemalong were grown as described [13]. The methods used for the binding of labelled Nod factors to a high-density fraction (sedimenting at 3000 *g*) of a *M. truncatula* root extract were as described [13].

RESULTS AND DISCUSSION

Expression in *E. coli* of the *R. meliloti* NodH enzyme

In the plasmid pT7H1, the sulphotransferase NodH is under the control of the T7 RNA polymerase promoter $\phi 10$; the T7 RNA polymerase gene is itself transcribed from the *lac* promoter and harboured by a λ phage integrated as a prophage in the bacterial chromosome of *E. coli* strain BL21 [17].

E. coli BL21 cells transformed with pT7H1 and grown at 37 °C expressed NodH as a major cellular protein after induction with IPTG (Figure 1, lane 4). The position of the NodH protein can clearly be seen by comparison with labelling performed after treatment of the cells with rifampicin where *nodH* is the major plasmid gene expressed (lanes 7–9).

No sulphotransferase activity was detected in the supernatant of cells expressing NodH at 37 °C, after disruption; indeed, the expressed protein was found to be associated mainly with the pelletable fraction (inclusion bodies) in an insoluble form (Figure 1, compare lanes 8 and 9). In marked contrast, the enzyme was found mostly in the supernatant of disrupted cells which had been cultivated at 18 °C in the presence of IPTG (Figure 1, compare lanes 13 and 14) and was biologically active (see below). Low-temperature incubation of transformed cells preventing the formation of inclusion bodies and allowing recovery of the protein product in an active and soluble form has been described for several other proteins [26].

Enzymatic sulphation of tetra-*N*-acetyl chitotetraose and of a desulphated Nod factor

The sulphotransferase activity of the 18 °C soluble extract was first probed with tetra-*N*-acetyl chitotetraose as a model substrate, as crude extracts of wild-type *R. meliloti* could sulphate this compound (D. W. Ehrhardt and S. R. Long, personal communication). As expected, only extracts of bacteria containing the *nodH* gene could efficiently transfer the sulphate group from [^{35}S]PAPS to tetra-*N*-acetyl chitotetraose (Figure 2, lane 2). In this experiment 36% of the added PAPS was consumed.

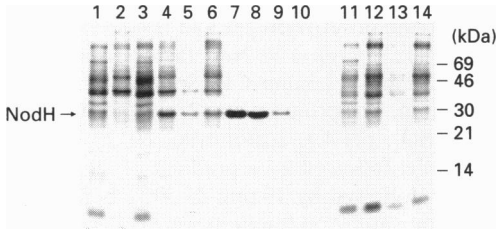

Figure 1 SDS/PAGE of [^{35}S]methionine-labelled proteins from *E. coli* expressing *R. meliloti* NodH

The control bacterial strain BL21/pT7-7 and the NodH strain BL21/pT7H1 were induced with IPTG in the presence or absence of rifampicin at either 37 or 18 °C, and the proteins were labelled with [^{35}S]methionine. The extracts from whole cells were then fractionated into the soluble and insoluble fractions. Control strain: 37 °C uninduced (lane 1); 37 °C induced (lane 2); 37 °C induced plus rifampicin (lane 10); 18 °C induced (lane 11). NodH strain: 37 °C uninduced (lane 3); 37 °C induced, whole cells (lane 4), insoluble fraction (lane 5), soluble fraction (lane 6); 37 °C induced plus rifampicin, whole cells (lane 7), insoluble fraction (lane 8), soluble fraction (lane 9); 18 °C induced, whole cells (lane 12), insoluble fraction (lane 13), and soluble fraction (lane 14). Cells were not treated with rifampicin at low temperature, because this antibiotic hardly penetrates the cells in these conditions.

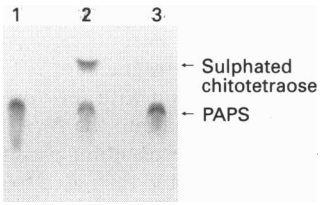

Figure 2 Sulphation of tetra-*N*-acetyl chitotetraose by the NodH enzyme

Separation of radiolabelled sulphated compounds by t.l.c. The reaction and chromatographic conditions are described in the Experimental section. The assays were carried out with the soluble extracts from the control strain BL21/pT7-7 (lane 1) or from strain BL21/pT7H1 (lanes 2 and 3), in the presence (lanes 1 and 2) or absence (lane 3) of tetra-*N*-acetyl chitotetraose. The same amount of protein was added for both extracts.

In order to assess the efficacy of the *E. coli* expression, we compared the activity of crude extracts obtained either from *E. coli* BL21 transformed with pT7H1, or from *R. meliloti* GMI357 (Δ*nodD1ABC*) complemented with pGMI515 (*nodFEGHD3-syrM*). We chose this strain because it is unable to synthesize Nod factors or a chitose core which might interfere with proper sulphation with the added tetra-*N*-acetyl chitotetraose substrate. It also expresses strongly the *nodH* gene after induction with luteolin, a known inducer of *nod* gene transcription [27]. For concentrations of PAPS and tetra-*N*-acetyl chitotetraose of 50 μM and 60 μM respectively we obtained a specific activity of 2320 pmol.min^{-1}.mg of protein^{-1} for the *E. coli* strain and 320 pmol.min^{-1}.mg of protein^{-1} for the induced *R. meliloti* strain. Thus the *R. meliloti* extract was ~ 7-fold less active than the *E. coli* extract.

Having established the sulphotransferase activity of the *E. coli* BL21/pT7H1 extract with tetra-*N*-acetyl chitotetraose, we looked for its ability to mediate sulphation of non-sulphated Nod factors; these factors [mainly NodRm-IV(C16:2)] were purified from the culture supernatant of a *R. meliloti nodH*$^-$ strain. The NodH extract could indeed transfer a sulphate group from PAPS to non-sulphated Nod factors, and at 50 μM concentrations of both PAPS and Nod factors the specific activity was 2700 pmol.min^{-1}.mg of protein^{-1}, i.e. similar to the value obtained with tetra-*N*-acetyl chitotetraose.

Initial velocity studies

The apparent K_ms of the NodH sulphotransferase for PAPS, tetra-*N*-acetyl chitotetraose and the non-sulphated Nod factors were determined from the corresponding $1/v$ versus 1/[substrate] plots (Figures 3a, 3b and 3d). The plots intersect on the horizontal axis, yielding an apparent K_m for PAPS of 56 μM, an apparent K_m for chitotetraose of 70 μM, and an apparent K_m for Nod factors of 28 μM. These values are in the range of those described for other sulphotransferases: for example, the K_m values of the choline sulphokinase of *Penicillium chrysogenum* for PAPS and choline are 12 and 17 μM respectively [28], and the ones of the oestrogen sulphotransferase of bovine adrenal glands for PAPS and 17-β-oestradiol are 70 and 14 μM respectively [29].

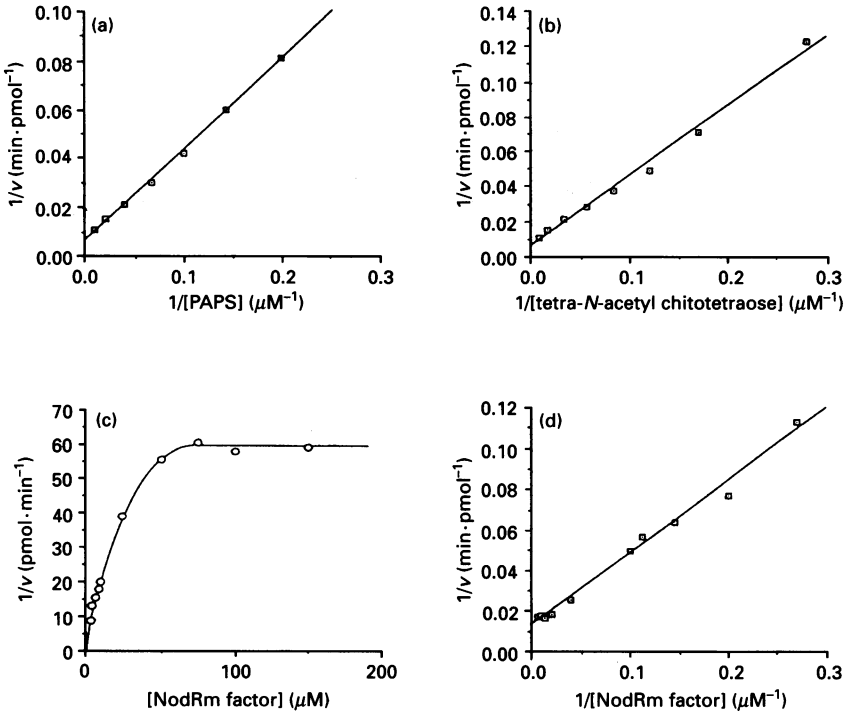

Figure 3 Affinity characteristics of NodH for its substrates and cofactor

Plots of reciprocals of forward velocity ($1/v$) versus 1/[PAPS] (**a**), 1/[tetra-*N*-acetyl chitotetraose] (**b**) and 1/[non-sulphated NodRm factors] (**d**), and plot of the change in forward velocity (v) versus the concentration of non-sulphated NodRm factors (**c**).

Labelling of NodRm-IV(S, C16:2) with ^{35}S to a high specific radioactivity

The radiolabelling of NodRm factors to a high specific activity comprises two sequential steps: firstly, the synthesis of the labelled cofactor of the NodH enzyme, [^{35}S]PAPS, using inorganic sulphate as labelled substrate and a yeast enzymatic extract; and secondly, the transfer of the labelled sulphate group from [^{35}S]PAPS to NodRm factors using the NodH enzyme expressed in *E. coli* (Scheme 1). To avoid as much as possible undesirable isotopic dilution with unlabelled sulphate ions, water and reagents of the highest grade available (those with the lowest sulphate content) were used; furthermore, enzymatic extracts were depleted of sulphate and PAPS by extensive dialysis. In the first step the [^{35}S]PAPS is synthesized from ATP, carrier-free [^{35}S]sulphate, and a yeast extract containing the necessary enzymes for PAPS synthesis: ATP-sulphurylase, inorganic pyrophosphatase and APS kinase. Under the conditions used, the fraction of the [^{35}S]sulphate incorporated into [^{35}S]PAPS is $\sim 15\%$ [Figures 4a (lane 2) and 4b (lane 5)]. Before adding NodRm factors and the NodH enzyme extract for the second step of the synthesis, the reaction is boiled and the denatured proteins are pelleted by centrifugation in order to eliminate yeast chitinases and N-acetylglucosaminidases. After completion of the second step, the NodRm factors are extracted with butan-1-ol, and then [^{35}S]NodRm-IV(S, C16:2) is purified by h.p.l.c. on a reverse-phase C_{18} column (Figure 4b, lanes 6–8). Of the synthesized [^{35}S]PAPS $\sim 70\%$ is consumed during the second step, so that the overall yield (fraction of $^{35}SO_4^{2-}$ incorporated into [^{35}S]NodRm factors) is $\sim 10\%$. A specific radioactivity of 800 Ci/mmol for the labelled Nod factors is obtained.

Tris and ethanol (solutions of Nod factors are alcoholic to avoid non-specific adsorption onto tube walls) were found to inhibit NodH action; NaCl and KCl also inhibit the sulphation but only at high concentrations ($\sim 50\%$ inhibition at 0.5 M).

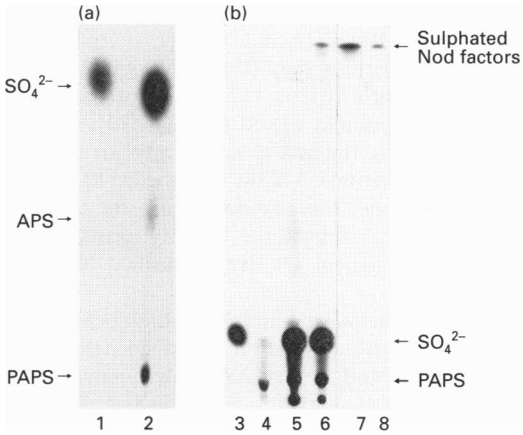

Figure 4 Coupled two-step radioabelling of NodRm factors

The figure shows t.l.c. of the products of the first step on PEI–cellulose (**a**) and after the second step on silica (**b**) as described in the Experimental section. Standards were loaded: [^{35}S]SO_4^{2-} (lanes 1 and 3), and purified [^{35}S]PAPS (lane 4). The spots above PAPS in lane 4 arose from degradation of PAPS by NH_4OH (2% final concentration in the eluting solvent). Samples were loaded from the reaction after the first step (lanes 2 and 5) and after the second step before (lane 6) and after (lane 7) the butan-1-ol extraction. An aliquot of the h.p.l.c.-purified fraction (lane 8) was loaded. In lane 2, the visualized APS is the residual product of the ATP-sulphurylase action on ATP, and is the substrate of APS kinase for PAPS generation.

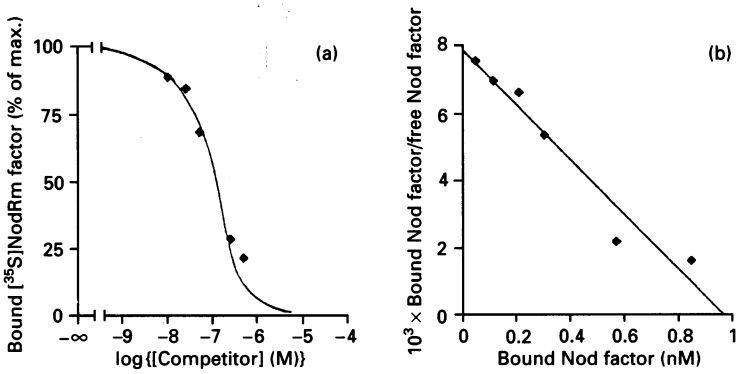

Figure 5 Binding of the [^{35}S]NodRm-IV(S,C16:2) ligand to a particulate fraction from *M. truncatula* roots and competition with unlabelled NodRm factors

(**a**) [^{35}S]NodRm-IV(S,C16:2) factor (6 nM) was incubated with 240 μg/ml of protein of the high-density root fraction from *M. truncatula*, and with increasing concentrations of unlabelled Nod factors (mainly NodRm-IV(Ac,S,C16:2)) ranging from 10 to 500 nM. Non-specific binding was determined at 1 μM of unlabelled ligand (zero binding). (**b**) Scatchard plot of the competition experiment.

Binding of [^{35}S]NodRm-IV(S, C16:2) to a Nod-factor-binding site in root extracts

We tested the sulphate-labelled factor for binding to the characterized Nod-factor-binding site 1 (NFBS1) displayed by a particulate fraction from roots of the legume *M. truncatula* [13]. In the presence of 6 nM [^{35}S]NodRm-IV(S,C16:2) the specific binding to the particulate fraction was 0.2 pmol.mg of protein^{-1}, a value identical to the one obtained with the chemically synthesized [^{3}H]NodRm-IV(Ac,S,C16:2) factor, whose specific activity is $\sim$ 56 Ci/mmol [13]. However, the d.p.m. signal with the ^{35}S-labelled factor was 14-fold greater. The specific binding of [^{35}S]NodRm-IV(S,C16:2) to the *M. truncatula* root fraction was inhibited by increasing the concentrations of unlabelled Nod factors [mainly NodRm-IV(Ac,S,C16:2)] (Figure 5a). Scatchard analysis of the competition data (Figure 5b) gave an equilibrium dissociation constant (K_D) of 117 nM, a value close to that obtained with the tritiated ligand (86 nM) [13]: this difference may be attributable to the lack of an O-acetyl group on the non-reducing sugar at position C6 of the ^{35}S-labelled ligand compared with the tritiated one, thereby decreasing the affinity of the cognate receptor (the lack of the O-acetyl group decreases to some extent the biological activity of purified Nod factors on *Medicago* [7,10]).

In contrast with various methods for radiolabelling of NodRm factors, the radioactive sulphation described herein is the only modification of the Nod factors that allows recovery of a highly radioactive but still biologically active ligand. Thus, this ^{35}S-labelled Nod factor is a promising tool in the search for a binding site with a higher affinity than NFBS1, and might allow the screening of a root cDNA expression library for putative receptor clones.

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