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Rhizobium meliloti NodP and NodQ Form a Multifunctional Sulfate-Activating Complex Requiring GTP for Activity

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The nodulation genes *nodP* and *nodQ* are required for production of *Rhizobium meliloti* nodulation (Nod) factors. These sulfated oligosaccharides act as morphogenic signals to alfalfa, the symbiotic host of *R. meliloti*. In previous work, we have shown that *nodP* and *nodQ* encode ATP sulfurylase, which catalyzes the formation of APS (adenosine 5′-phosphosulfate) and PP_i. In the subsequent metabolic reaction, APS is converted to PAPS (3′-phosphoadenosine 5′-phosphosulfate) by APS kinase. In *Escherichia coli*, *cysD* and *cysN* encode ATP sulfurylase; *cysC* encodes APS kinase. Here, we present genetic, enzymatic, and sequence similarity data demonstrating that *nodP* and *nodQ* encode both ATP sulfurylase and APS kinase activities and that these enzymes associate into a multifunctional protein complex which we designate the sulfate activation complex. We have previously described the presence of a putative GTP-binding site in the *nodQ* sequence. The present report also demonstrates that GTP enhances the rate of PAPS synthesis from ATP and sulfate (SO_4^{2-}) by NodP and NodQ expressed in *E. coli*. Thus, GTP is implicated as a metabolic requirement for synthesis of the *R. meliloti* Nod factors.

In *Escherichia coli*, *cysD* and *cysN* encode subunits of ATP sulfurylase (EC 2.7.7.4) and *cysC* encodes adenosine 5′-phosphosulfate (APS) kinase (EC 2.7.1.25) (13, 17). They catalyze the following reactions:

$$ATP + SO_4^{2-} \xrightarrow{\text{ATP sulfurylase}} APS + PP_i$$

$$APS + ATP \xrightarrow{\text{APS kinase}} PAPS + ADP$$

where PAPS is 3′-phosphoadenosine 5′-phosphosulfate. In *E. coli*, the PAPS-activated sulfuryl moiety is reduced to sulfite and then sulfide, for use in cysteine biosynthesis. Mutants that cannot make APS or PAPS are cysteine auxotrophs (13, 17).

The *nodP* and *nodQ* genes of *Rhizobium meliloti* are homologous to the *E. coli cysDNC* region as detected by Southern blot hybridization. *R. meliloti* strains mutated in *nodP* or *nodQ* are not auxotrophs. Originally identified because of their involvement in nodulation of host legume plants (2, 30, 36), *nodP* and *nodQ* are now proposed to be involved in the synthesis of nodulation (Nod) factors which are active on the roots of alfalfa (reviewed in references 4 and 9). These factors, NodRm-IV(S) and NodRm-V(S), are oligomers of *N*-acetylglucosamine (GlcNAc) substituted with an *N*-acyl group on the nonreducing end and a sulfate group on the C-6 position of the reducing-end GlcNAc residue. We and others have proposed (27, 32) that NodP and NodQ catalyze formation of an activated sulfate group, which is transferred to the factor by NodH, as *nodH* mutants make an unsulfated version of the factor (7, 8, 15, 32).

There are two copies of *nodPQ* in *R. meliloti*, each located on one of the Sym plasmids (30, 33). Double *nodQ* mutants, like *nodH* mutants, produce an unsulfated factor and have severely diminished ability to nodulate alfalfa (3a, 26, 33). Double *nodQ* mutants are not auxotrophs: there is evidence of a third sulfate-activating locus in *R. meliloti*, *saa*, which is involved in methionine and cysteine biosynthesis. It is likely to be less similar to *nodP* and *nodQ* than *nodP* and *nodQ* are to *E. coli cysDNC*, as it is not detected on Southern blots (33). There are also other organisms bearing homologs of *nodPQ*, in which these genes are not part of the primary cysteine biosynthetic pathway. The soil bacterium *Azospirillum brasilense* has *nodP* and *nodQ* genes, originally detected by Southern blot hybridization, in which mutations do not result in auxotrophic strains. Their role in this organism is unknown (38).

In this study, we show that NodQ has APS kinase activity in addition to its role in ATP sulfurylase, so that the NodP-NodQ assembly constitutes a sulfate-activating complex (SAC). Alignment of the amino acid sequences encoded by the *E. coli*, *R. meliloti*, and *A. brasilense* genes shows that they have striking similarity. Furthermore, yeast APS kinase (12) is very homologous to CysC and the C-terminal end of NodQ. Our previous finding of a consensus GTP-binding site in NodQ (30) correlates with our present report that GTP is required for formation of PAPS from sulfate by a semipurified NodP-NodQ complex, as found for the *E. coli* CysD-CysN complex (16, 19). Sulfate activation, and hence formation of correctly sulfated Nod factors, may thus have a specific requirement for GTP. Antibody to CysC reacts with NodQ, and use of this antibody to probe Western blots (immunoblots) of *R. meliloti* extracts shows that NodQ is not cleaved between its functional domains into two peptides.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* and *R. meliloti* strains and plasmids used in this study are listed in Table 1. Plasmids not previously described, pJSS53, pJSS54, and pJSS55, were constructed by PCR (29) as follows. As there are no convenient restriction sites for subcloning *nodP1* or *nodQ1*, we created *Hin*dIII sites immediately downstream of both *nodP1* and *nodQ1* and a *Kpn*I site just upstream of *nodQ1*. In our primer design, we took advantage of the naturally occurring *Kpn*I site

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or genotype	Source or reference
Strains		
E. coli		
TSL3	*cysD91 strA recA938::cat*	17
DM63	F⁻, *cysN96::kan proC leu thi ara gal lac hsd* Str^r	17
JM81A	F⁻, *cysC92 tfr8*	17
BL21(DE3)	IPTG-inducible T7 polymerase	34
R. meliloti		
Rm1021	Str^r derivative of wild-type RCR2011	21
DsAux7	*saa*::Tn*5*-233 (cysteine or methionine auxotroph)	33
Plasmids		
pRmS63[a]	pUC18 with 12-kb *Kpn*I fragment containing *nodDABC* through *nodPQ1*	30
pJSS53[a]	pUC118 with 1.4-kb PCR product containing *nodP1*	This study
pJSS54[a]	pUC118 with 2.1-kb PCR product containing *nodQ1*	This study
pJSS55[a]	pUC118 with 3.4-kb PCR product containing *nodP1* and *nodQ1*	This study
pJSS102[a]	pMB50 with 1.4-kb insert from pJSS53	This study
pJSS103[a]	pMB50 with 2.1-kb insert from pJSS54	This study
pJSS104[a]	pMB50 with 3.4-kb insert from pJSS55	This study
pJSS22[b]	pET3 with 3.4-kb *Kpn*I-*Mae*I fragment containing *nodP1* and *nodQ1*	32
pJSS23	Same as pJSS22, but *nodPQ1* insert is in the opposite orientation	32
pRmJT5	pLAFR3 with 20-kb insert containing host range *nod* genes	36

[a] *lac* promoter from vector is expressing *nodP1* and/or *nodQ1*.
[b] T7 promoter from vector is expressing *nodP1Q1*.

upstream of *nodP1*. We used the following four primers in the reactions (restriction sites are shown in boldface): no. 1895, 481 bp upstream of the start of *nodP1*, 5′GCGC**GGTAC**CGAAGTCGCGCTCCGCCGTTGCGTATCTC3′; no. 1896, 52 bp downstream of *nodQ1*, 5′GCGC**AAGCTT**CCGGGACCATATAGACAGAACATCCAGT3′; no. 1897, 125 bp upstream of *nodQ1*, 5′GCGC**GGTACC**CCGCTGACCGGGGCGGTCGAGTCCGACG3′; and no. 1898, immediately downstream of *nodP1*, 5′GCGC**AAGCTT**GCGGCGGTATAGATTGAACATACGACAT3′. We used *Hin*dIII-digested pRmS63 as a template for the PCR. The digestion was solely to linearize the plasmid, and none of the *Hin*dIII sites occur in the *nodP1Q1* region. We used 20 ng of this DNA with the PCR primers at 10 μM each. Three reactions were set up with primers as follows: no. 1895 and no. 1896, no. 1895 and no. 1898, and no. 1896 and no. 1897. These produced products of 3.4 kb (*nodP1* and *nodQ1*), 1.4 kb (*nodP1*), and 2.1 kb (*nodQ1*), respectively. As we wanted to increase the fidelity of the reactions, we used 12 μM deoxynucleoside triphosphates (dNTPs) (instead of 50 μM) and ran only 15 cycles (instead of 30 cycles) of 1.5 min at 97°C, 1 min at 55°C, and 5 min at 74°C. We used Replinase enzyme (NEN) with the buffer supplied by the company, and the temperature cycles were produced by a Hybaid thermal reactor. Control reactions with either no template or template plus only one primer produced no detectable product.

The PCR products were treated with Klenow fragment (Amersham) in the presence of excess dNTPs, sequentially digested with *Kpn*I and *Hin*dIII, and ligated into pUC118. The orientation in all cases is such that the genes are driven by the *lac* promoter of the vector. One cloned product from each PCR was sequenced. pJSS53, *nodP1* alone, has no PCR errors. pJSS54, *nodQ1* alone, has one C-to-T change at bp 3269, which does not change the amino acid sequence. pJSS55, which encodes *nodP1* and *nodQ1* together, has four sequence differences that must have arisen from misincorporation: T→C at bp 312, A→G at bp 327, C→G at bp 1612, and C→T at bp 1897. The first two changes are upstream of the coding regions. The second two encode conservative amino acid changes in NodQ, Ala→Gly and Ala→Val, respectively. The numbering system is per Schwedock and Long (30).

The *Kpn*I-*Hin*dIII fragments from pJSS53 through pJSS55 were subcloned into pMB50, a broad-host-range vector, to create pJSS102 through pJSS104, respectively. Again, the genes are driven by the *lac* promoter.

Sequencing. Single-stranded template DNA was made from pUC118-based plasmids by using the helper phage M13K07 as described by Vieira and Messing (39). We used the dideoxy chain termination method of Sanger et al. (27), as modified by Tabor and Richardson (37), with both single-stranded and double-stranded templates (40). For this procedure, we made use of the Sequenase kit, which supplies the modified T7 DNA polymerase (U.S. Biochemicals). In addition to using the universal and PCR primers for sequencing, we used four 15-mers, spaced approximately 500 bp apart, to sequence the cloned PCR products. Sequencing ladders were separated on 5% Long Ranger gels (A. T. Biochemicals).

Computer methods. We used the GCG sequence analysis software package version 6.2d. (5) on a VAX for sequence comparisons. Alignments were performed by using the TULLA program (35). The sequences for CysD and CysN have been established previously (18), and the sequence for CysC is derived from references 18 and 20a. The sequence for yeast ATP sulfurylase (MET3) was taken from the work of Cherest et al. (3), and that for yeast APS kinase (MET14) was from the work of Korch et al. (12). The sequence of *A. brasilense* NodP and NodQ was from the work of Vieille and Elmerich (38).

Western blots. Proteins were transferred from acrylamide gels to 0.2-μm-pore-size nitrocellulose membranes (Schleicher and Schuell) by using standard Western blotting techniques (6). The transfer was performed at 80 V for 4 h. Rabbit

anti-CysC antibodies were kindly provided by G. D. Markham and C. Satishchandran. We used the Vectastain ABC kit, according to directions, except that Nonidet P-40 replaced Tween, the primary antibodies were incubated with the blot overnight, and the secondary antibodies and ABC reagent were incubated with the blot for 1 h. Rainbow markers (Amersham) were used as molecular weight standards for gel electrophoresis.

Materials for biochemical assay. ATP, GTP, Na_2SO_4, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), Tris, isopropyl-β-D-thiogalactopyranoside (IPTG), carbenicillin, ampicillin, rifamycin, and streptomycin were obtained from Sigma Chemical Co. $Na_2{}^{35}SO_4$ (0.25 to 1 Ci/mmol) was purchased from NEN, DuPont Co. or from ICN Radiochemicals. The protein assay mix and protein native molecular weight standards were supplied by Bio-Rad Laboratories. Superose-12 and S-300 HR resin were purchased from Pharmacia LKB Co. Phenylmethanesulfonyl fluoride (PMSF), pepstatin, and inorganic pyrophosphatase were obtained from Boehringer Mannheim Biochemicals. PEI-F thin-layer chromatography (TLC) plates were obtained from E. M. Separations Co.

ATP sulfurylase and APS kinase assays. The assays used were similar to those described previously (17, 32). These assays monitor the incorporation of $^{35}SO_4$ into APS or PAPS.

(i) Assays for data in Fig. 1. Extracts for assays shown in Fig. 1 were prepared as follows. Culture volumes of 10 ml were washed in 50 mM Tris (pH 8) and resuspended in 200 μl buffer B (50 mM Tris, pH 8, 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% [vol/vol] glycerol, 13 mM 2-mercaptoethanol, and protease inhibitors). The cells were sonicated, and cell debris was removed by centrifugation. Extracts were kept on ice or at 4°C during their preparation and stored frozen at −70°C.

Reaction conditions for assays shown in Fig. 1 were as follows. The following components were added to the extracts in buffer B (final concentrations are given): 32 mM ATP, 39 mM $MgCl_2$, 1.3 mM Na_2SO_4 (25 Ci/mmol), 1 U of inorganic pyrophosphatase. Also, 0.6 U APS kinase (28) was added, depending on the assay. For assays measuring ATP sulfurylase activity, the purified APS kinase was added. For assays measuring the combination of endogenous ATP sulfurylase and APS kinase, purified APS kinase was not added. The reactions were stopped by boiling for 1 min and the mixtures were immediately placed on ice. The products of the reactions were separated on PEI-F cellulose plates (E. M. Separations) with 1 M LiCl and visualized by autoradiography. Assays were quantitated with an AMBIS two-dimensional radioactivity detector. To estimate the ATP sulfurylase activity of extracts, the amounts of PAPS formed from coupling the reactions with APS kinase were measured. This was done because APS is a less stable intermediate.

(ii) Assays used during purification of the SAC complex. Assays were similar to those previously described (17). Throughout each step of the purification, the assays produced predominantly PAPS; very little (less than or equal to 5%) APS was detected. The conditions were as follows: ATP, 0.50 mM; GTP, 0.50 mM; $Na_2{}^{35}SO_4$, 10 μM (5 mCi/mmol); $MgCl_2$, 2.0 mM; phosphoenolpyruvate, 2.0 mM; pyruvate kinase, 8.0 U/ml; adenylate kinase, 3.5 U/ml; inorganic pyrophosphatase, 5.0 mU/ml; HEPES-K^+, 70 mM (pH 8.0); temperature, 25°C ± 3°C. The inorganic pyrophosphatase was dialyzed against 50 mM HEPES-K^+ at pH 8, and its activity was determined prior to use. Reactions were stopped by placing the tubes containing reaction mixtures in boiling water for 1.0 min. The tubes were immediately chilled on ice and centrifuged to remove precipitate. The radiolabelled reactants were separated on PEI-F TLC plates by using a 1.0 M LiCl mobile phase (17) and quantitated with an AMBIS two-dimensional radioactivity detector (18).

Assays used for data in Fig. 4. The assay conditions used for the data shown in Fig. 4 were identical to those discussed for the purification except that GTP was excluded from the controls. The concentration of GTP used in these assays, 0.5 mM, appears to be at or near saturation since increasing the GTP to 5.0 mM did not result in a significant increase in the rate of the reaction; decreasing to 0.1 mM resulted in a slight decrease of the rate. Activity units were expressed as (micromoles of PAPS formed per minute) × 10^{-6}. The level of detection in these assays is approximately 5 nM PAPS or APS. The standard least-squares analysis of the curve for formation of activated sulfate in the presence of GTP (see Fig. 4) gives a slope of 4.7 μM/min, and 1 standard deviation unit = 0.00015 μM/min. The plot for formation in the absence of GTP is essentially at background everywhere and was not analyzed.

Partial purification of SAC. Cells for enzyme preparation were grown in 12 liters of media {SB(3)/LB(3) ratio, 1/1 [vol/vol], with 0.1% glycerol and 50 μg of carbenicillin per ml} (17), inoculated to an optical density at 550 nm of 0.1 with fresh cultures of BL21(DE3)(pJSS22). The doubling time at 37°C with shaking was 35 min. IPTG was added to a final concentration of 0.1 mM when cells reached an optical density at 550 nm of 2.2. After an interval of 15 min, fresh rifampin (stock of 30 mg/ml in methanol) was added to a final concentration of 45 μg/ml. Following a 45-min incubation with rifampin, cells were pelleted by centrifugation at 4°C; washed once with 120 ml of 50 mM Tris-Cl, pH 8.0; spun again to harvest; and frozen at −70°C. A 38-g amount (wet weight) of cells was obtained. Purification was always at 4°C unless stated otherwise. The 38 g of cells was suspended in 152 ml of cracking buffer (50 mM Tris-Cl, pH 8.0; 1 mM EDTA; 30 mM KCl; 0.3 mM PMSF; 1 μg of pepstatin per ml) and sonicated at or below 15°C. The cellular debris was pelleted by centrifugation (12,000 rpm, GSA rotor, relative centrifugal force = 15,000, 10 min), and the supernatant was collected. Solid streptomycin was added to 1% mass per volume. The solution was stirred for 30 min and centrifuged to remove the precipitate. Pulverized ammonium sulfate was added to the stirred supernatant to 35% saturation, and the mixture was stirred for 30 min. The solution was then centrifuged, and the pellet was suspended in 150 ml of cracking buffer. This suspension was loaded onto a 5.4-cm-diameter column containing 2.1 liters of S-300 HR resin (Pharmacia) equilibrated with cracking buffer. Column fractions were assayed by a modification of the assay used for extracts.

Apparent native molecular weight of the SAC was determined by using a Superose-6 column calibrated with protein standards obtained from Bio-Rad Laboratories. The calibration curve was linear from 1.3 to 670 kDa. The column was equilibrated at 4°C with 50 mM Hepes-K^+, pH 8, with or without 50 mM KCl. The KCl had no influence on the R_fs of the standards or SAC. The column fractions were assayed for APS- and/or PAPS-forming activity in the presence of GTP by using the $^{35}SO_4$ assays described above.

RESULTS

NodQ has APS kinase activity in vivo. Previously we had shown that a clone carrying *nodP1*, *nodQ1*, and several other *nod* genes (pRmS63) was able to complement *E. coli* cysteine auxotrophs deficient in both ATP sulfurylase and APS kinase activities. We now report that NodQ itself, in addition to being necessary for ATP sulfurylase activity, has APS kinase activity. Functions were tested by complementation for subclones car-

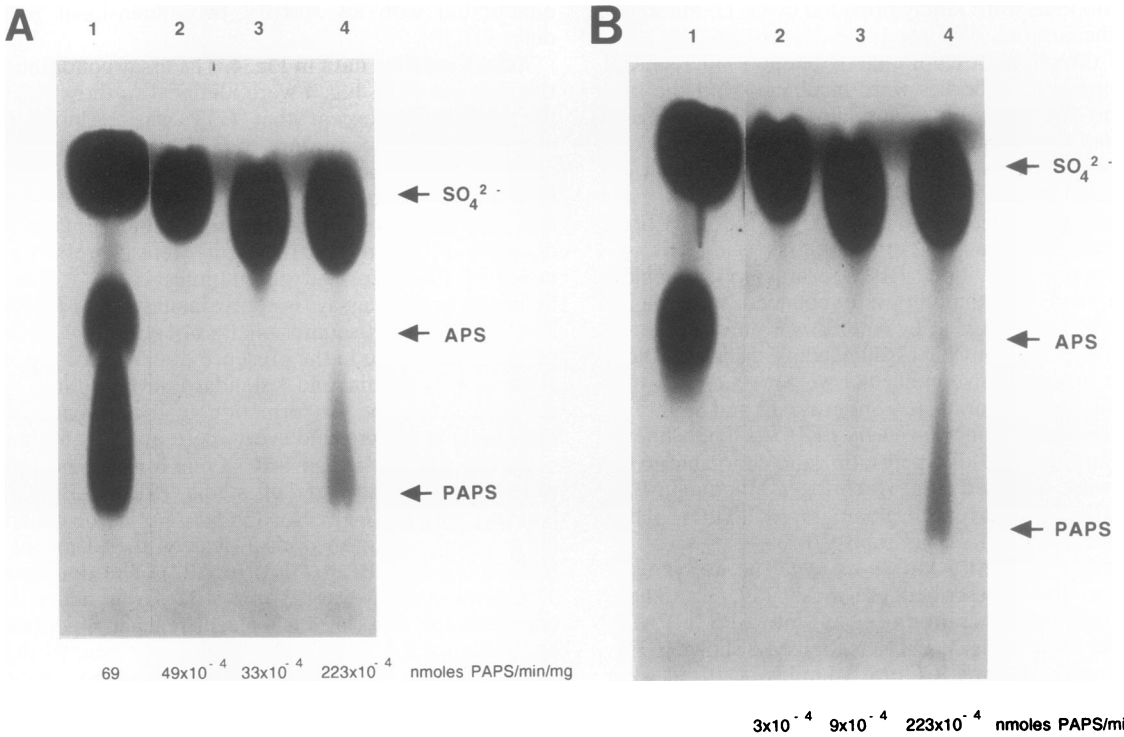

FIG. 1. ATP sulfurylase and APS kinase assays of an *R. meliloti* auxotroph containing plasmids expressing *nodP*, *nodQ*, or both *nodP* and *nodQ*. Extracts were tested for the ability to synthesize ^{35}S-labelled APS and/or PAPS from $^{35}SO_4$ and ATP; SO_4^{2-}, APS, and PAPS from the reactions were separated on TLC plates (positions indicated to the right of each panel) and visualized by autoradiography. All of the assay mixtures for panel A contained exogenously added APS kinase, isolated from *E. coli*; those for panel B had no exogenously added APS kinase. Inorganic pyrophosphatase was present in all assay mixtures. Lanes 1, purified yeast ATP sulfurylase (Sigma); lanes 2, crude extract of DsAux7/pJSS102 (*nodP* only); lanes 3, DsAux7/pJSS103 (*nodQ* only); lanes 4, DsAux7/pJSS104 (*nodP* and *nodQ*). PAPS was quantitated, and the results are indicated below each lane. For panel A, lane 1, the APS spot represents 0.3 nmol. For panel B, lane 1, the APS spot represents 0.5 nmol. The specific activities in lanes 4 are identical only by coincidence, as they represent different assays.

rying *nodP1* alone, *nodQ1* alone, and *nodP1* and *nodQ1* together (Table 2). *nodP1* alone (pJSS53) was unable to complement any of the *E. coli* auxotrophs, while *nodP1* and *nodQ1* together (pJSS55) complemented all three. *nodQ1* alone (pJSS54) complemented JM81A, the *cysC* mutant. Our previous observation that pJSS22 (*nodP1* and *nodQ1* in pTE3) complemented only TSL3 (*E. coli* auxotroph deficient only in ATP sulfurylase [32]) probably reflects expression differences, since the two clones use different promoters. The data reported here show that a sufficiently expressed *nodQ* does complement the *cysC* defects of JM81A and DM62.

APS and PAPS synthesis in extracts of isogenic strains containing *nodP*, *nodQ*, or *nodPQ* expression clones. Since the genetic data indicated that NodQ could substitute for CysC in vivo, we tested whether NodQ had APS kinase activity in vitro. Extracts of *R. meliloti* DsAux7 (cysteine mutant [33]) harboring either a *nodP*, *nodQ*, or *nodPQ* expression vector were assayed for the ability to synthesize radiolabelled APS and/or PAPS from $^{35}SO_4$ and ATP (Fig. 1). The assay mixtures shown in Fig. 1A contained exogenously added APS kinase and inorganic pyrophosphatase; those in panel B did not include exogenous APS kinase. Control experiments using purified ATP sulfurylase of *E. coli* (Fig. 1, lane 1 in both panels) show the expected result, that PAPS formation is dependent on APS kinase. Clones containing either the *nodP* or *nodQ* gene alone did not confer upon DsAux7 the capability to produce appreciable amounts of APS or PAPS (Fig. 1, lanes 2 and 3 of both panels). Figure 1B, lane 4, shows a small quantity of APS, demonstrating that in the presence of both subunits, both APS and PAPS are produced. The fact that no APS is visible in the corresponding lane in Fig. 1A is likely due to the fact that the assay mixtures for Fig. 1A, lane 4, contained exogenous APS kinase, resulting in an increased rate of PAPS formation with a concomitant decrease in the steady-state level of APS. The similar quantities of PAPS produced in lanes 4 of the gels shown in the two panels of Fig. 1 suggest that APS production may be rate limiting in these assays, consistent with the observed low levels of APS. Overall, the data showed that enhanced levels of ATP sulfurylase activity and APS kinase

TABLE 2. Complementation of *E. coli* cysteine auxotrophs by *nodP1* and *nodQ1*[a]

Plasmid	Plasmid-borne gene(s)	Result for: TSL3 CysD$^-$	DM62 CysN$^-$ CysC$^-$	JM81A CysC$^-$
pJSS53	*nodP1*	−	−	−
pJSS54	*nodQ1*	−	−	+
pJSS55	*nodP1 nodQ1*	+	+	+
pJSS22	*nodP1 nodQ1*	−	−	−
pJSS22 + pGP1-2[b]	*nodP1 nodQ1* T7 gene *1*	+	ND	−

[a] +, growth in the absence of cysteine; −, no growth; ND, not determined.
[b] Plasmid encodes a heat shock-inducible T7 RNA polymerase. Growth at 34 and 37°C gave the same results as did growth at 30°C.

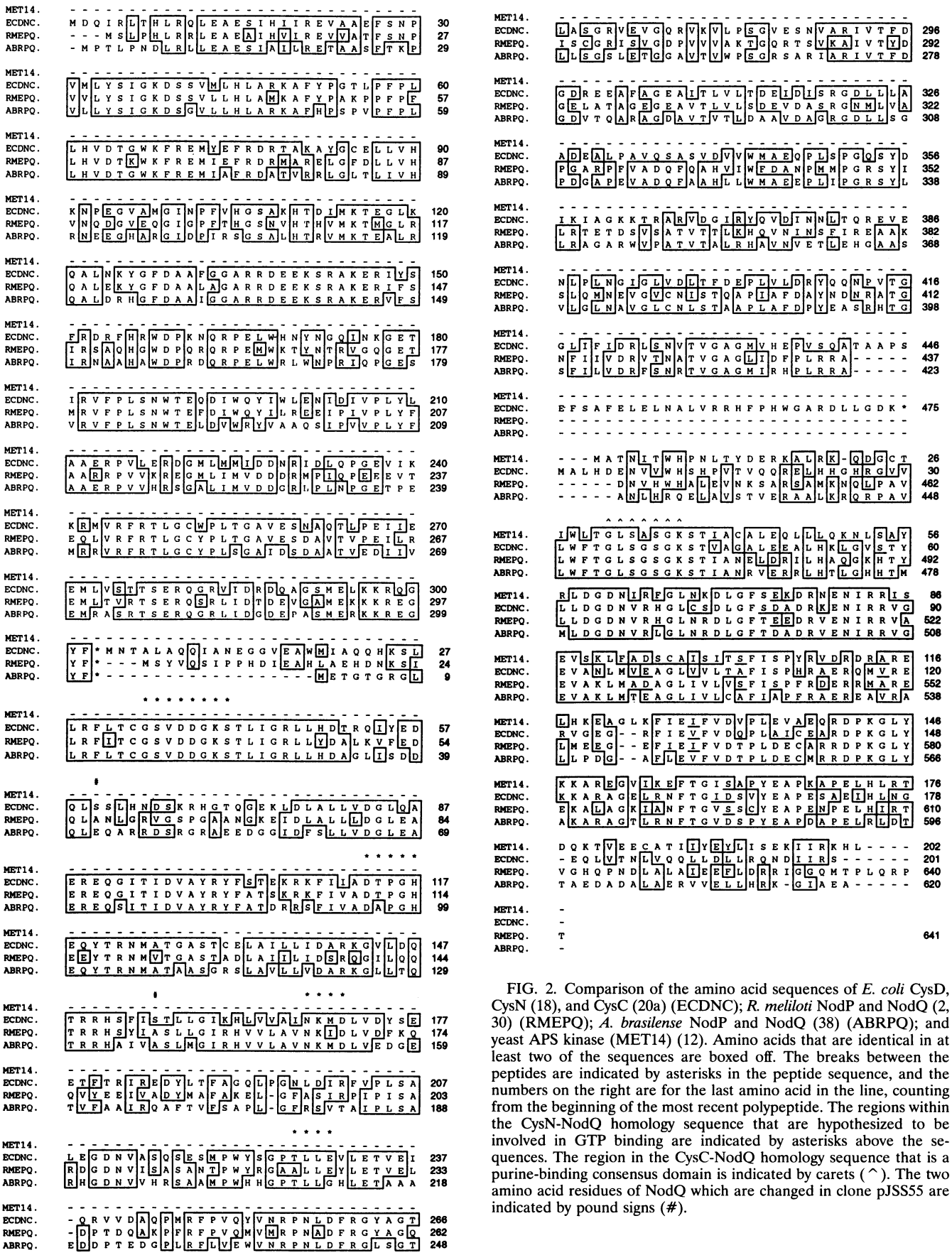

FIG. 2. Comparison of the amino acid sequences of *E. coli* CysD, CysN (18), and CysC (20a) (ECDNC); *R. meliloti* NodP and NodQ (2, 30) (RMEPQ); *A. brasilense* NodP and NodQ (38) (ABRPQ); and yeast APS kinase (MET14) (12). Amino acids that are identical in at least two of the sequences are boxed off. The breaks between the peptides are indicated by asterisks in the peptide sequence, and the numbers on the right are for the last amino acid in the line, counting from the beginning of the most recent polypeptide. The regions within the CysN-NodQ homology sequence that are hypothesized to be involved in GTP binding are indicated by asterisks above the sequences. The region in the CysC-NodQ homology sequence that is a purine-binding consensus domain is indicated by carets (^). The two amino acid residues of NodQ which are changed in clone pJSS55 are indicated by pound signs (#).

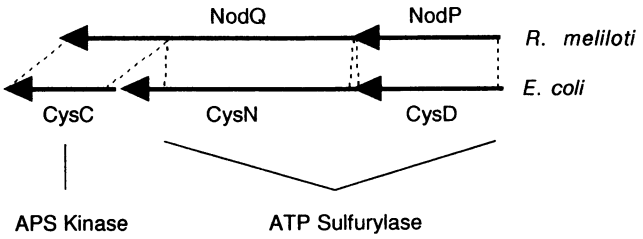

FIG. 3. Schematic of homology between *R. meliloti* NodP and NodQ and *E. coli* CysD, CysN, and CysC. The peptides are represented by arrows. NodP and the N-terminal two-thirds of NodQ are homologous to the *E. coli* proteins that together have ATP sulfurylase activity. The C-terminal one-third of NodQ is homologous to *E. coli* APS kinase.

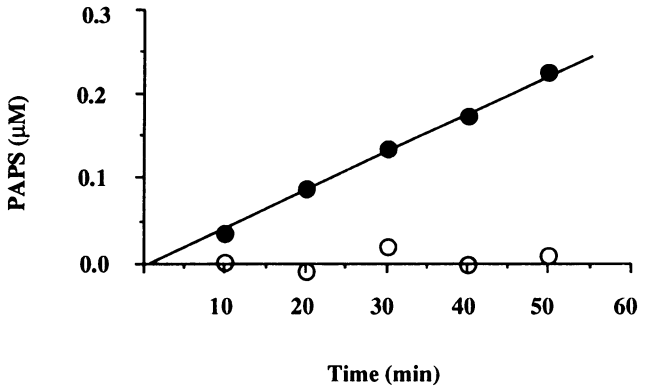

FIG. 4. GTP enhances the rate of activated sulfate formation. The lines represent the progress curves for the formation of activated sulfate in the presence (solid circles) and absence (unfilled circles) of GTP. APS and PAPS were detected by the ^{35}S-SO_4 assays described in Materials and Methods. The reaction mixtures were identical except for the presence of GTP. Only PAPS was detected as a product in the reaction mixtures containing GTP. In reaction mixtures lacking GTP, the levels of APS and PAPS formed were below the limits of detection. Each datum point represents the average of three determinations.

activity required plasmid-borne copies of both NodP and NodQ.

NodP and NodQ are highly homologous to CysD, CysN, and CysC. As previously shown, the amino acid sequences of NodP and NodQ are extremely similar to their *E. coli* homologues (18) (Fig. 2). The NodP and CysD sequences are 68% identical and 81% similar and are only 3 amino acids different in length. NodQ is homologous to both CysN and CysC, with 52% identity and 68% similarity overall. The regions of NodQ previously noted to share homology with the GTP-binding domains of elongation factor Tu (11) are conserved in CysN (18, 30). The C-terminal portion of NodQ shows 55% identity with CysC. A purine-binding consensus domain (GXXXGK) in this region (14) is consistent with the use of ATP by the kinase (Fig. 2). A schematic of the homology of these five polypeptides (Fig. 3) shows that NodQ is slightly smaller than the combined CysN and CysC and is missing what would correspond to the C-terminal region of CysN.

The NodQ activities and the *E. coli* and *R. meliloti* sequence comparison suggest that ATP sulfurylase and APS kinase may be covalently linked in *R. meliloti*. This assertion is further supported by observations, detailed in the following sections, that NodQ is not proteolytically cleaved in *R. meliloti* into CysN- and CysC-like proteins and that these proteins, which comigrate during size exclusion chromatography, catalyze synthesis of PAPS, not APS. These data imply that NodP and NodQ are likely associated in a multifunctional protein complex which we define here as the SAC.

Figure 2 also shows the alignment of NodP and NodQ of *R. meliloti* with NodP and NodQ of *A. brasilense* and yeast APS kinase. As previously reported (38), the NodPs showed 63% identity and the NodQs showed 57% identity. As with *E. coli* CysN, the putative GTP-binding domains were conserved. Yeast APS kinase (the *MET14* gene product) (12) was homologous to CysC and the C-terminal domain of NodQ; its sequence was 50% identical to the C-terminal domain of NodQ. All four sequences in the "CysC-NodQ" region displayed the purine-binding consensus sequence. In contrast with these highly conserved genes, yeast ATP sulfurylase (the *MET3* gene product) (3) was not significantly homologous to NodP, NodQ, CysD, or CysN (data not shown).

GTP is required for the activity of NodP-NodQ. NodQ and CysN amino acid sequences display a consensus GTP-binding site (2, 18, 30). *E. coli* ATP sulfurylase displays a GTPase activity that is tightly coupled to an enhancement in the rate of APS formation (16, 19). To determine whether SAC activity is influenced by GTP, SAC was partially purified and assayed in the presence and absence of GTP. Partial purification was achieved by two selective precipitation steps and ion-exchange chromatography (Table 3) of extracts from *E. coli* cells overexpressing the *R. meliloti nodP* and *nodQ* genes as previously described (32). The partially purified NodP-NodQ proteins were assayed for activity in the presence and absence of GTP (Fig. 4). We found that GTP enhanced the initial rate of PAPS formation by the NodP-NodQ fraction more than 50-fold. As with the initial assays, we found that the predominant product was PAPS, not APS; the fact that so little APS is seen in the in vitro reaction is consistent with the possibility that APS is not released as a free intermediate from the SAC.

It is important to rule out the possibility that the observed activity arises from contamination of the partially purified enzyme complex with the endogenous *E. coli* enzymes. The strongest evidence is that no APS or PAPS is detected unless the *R. meliloti nodP* and *nodQ* genes are present and expressed. Further support derives from size exclusion chromatography with the partially purified enzyme complex. ATP sulfurylase and APS kinase from *E. coli* are well separated during size exclusion chromatography, and their apparent molecular masses are quite different: ATP sulfurylase elutes at 390 kDa (15a), and APS kinase elutes at 40 or 80 kDa, depending on the oligomerization state (28). However, the *R. meliloti* NodP-NodQ ATP sulfurylase and APS kinase comigrate during size exclusion chromatography and show an apparent native molecular mass of 143 kDa (standard deviation = 8.8 kDa) (data not shown). The comigration of these activities at an apparent molecular weight distinct from that of the *E. coli* enzymes argues strongly against a contaminating artifact.

Detection of NodQ with antibodies to CysC. The *nodQ* gene encodes a protein with distinct functional domains for ATP sulfurylase and APS kinase. Since these activities are in separate polypeptides in the *E. coli* enzymes, we asked whether NodQ may be cleaved in *R. meliloti* to separate the functional domains. We reasoned that NodQ, which had domains for both ATP sulfurylase and APS kinase activities and had sequence similarity to CysN and CysC, might present epitopes in common with CysC, the APS kinase of *E. coli*. We therefore used antibodies against *E. coli* CysC (kindly provided by G. D. Markham) to detect NodQ. Western blot transfer and antibody reactions were performed on *E. coli* extracts carrying

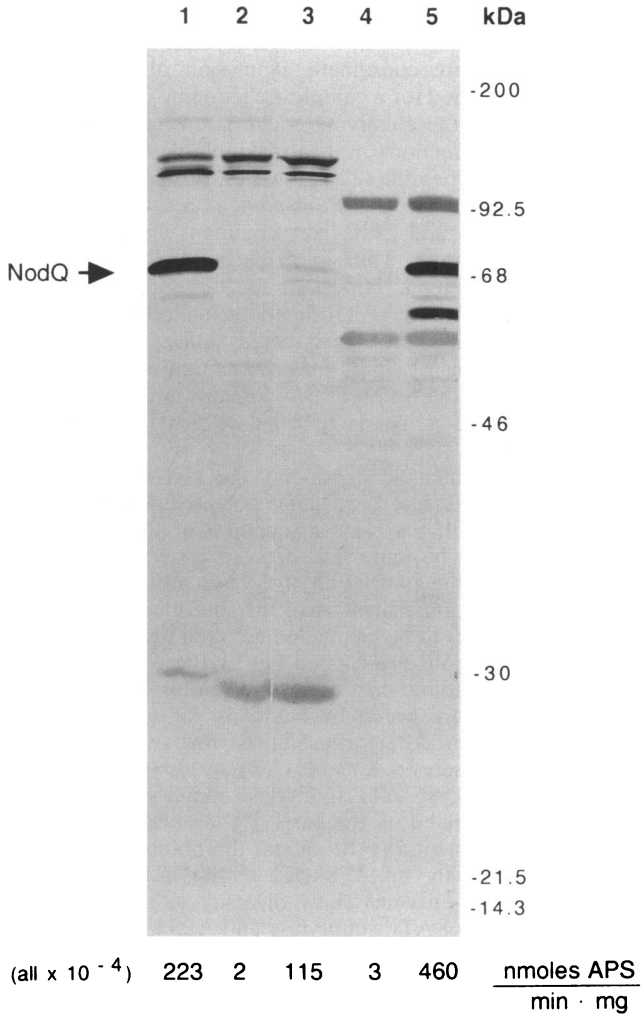

FIG. 5. Western blot of extracts from strains of *E. coli* and *R. meliloti* expressing NodQ, probed with anti-CysC antibodies. Extracts are as follows: lane 1, *R. meliloti* DsAux7/pJSS104; lane 2, DsAux7; lane 3, Rm1021 grown in minimal media; lane 4, *E. coli* BL21(DE3) (pJSS23); lane 5, BL21(DE3)(pJSS22). The numbers at the bottom indicate the ATP sulfurylase activities of the extracts used for the blot. The extract used in lane 1 is the same extract as that used for the assays shown in Fig. 1, lanes 4, and the activity reported here is from the same assay as that shown in Fig. 1A, lane 4. Positions of the molecular mass markers are indicated on the right.

cloned *nodPQ* genes, and on *R. meliloti* extracts. The two extracts were assayed in parallel for ATP sulfurylase activity. NodQ overexpressed in *E. coli* cross-reacted with anti-CysC antibodies (Fig. 5, lane 4 versus lane 5). NodQ appeared to be unstable in these *E. coli* extracts, as several bands smaller than 70 kDa also reacted specifically in the NodQ-overexpressing extract (Fig. 5, lane 5). This instability was also observed in in vitro transcription-translation experiments, in which NodQ was expressed in *E. coli* S-30 extracts (30). NodQ overexpressed in *R. meliloti* also cross-reacts with the antibodies (Fig. 5, lane 1), and only the full-sized NodQ band (68 kDa) was observed. NodQ was detectable in single copy neither without nor with luteolin induction of *nodPQ* expression (Fig. 5, lane 2, and data not shown). In sum, taken together, these data show that NodQ shares epitopes with CysC and that the NodQ protein in *R. meliloti* is not proteolytically processed into two polypeptides.

TABLE 3. Partial purification of *R. meliloti* NodP-NodQ SAC

Purification step	Total protein (g)	Total activity (units)[a]	Sp act (units/mg)[a]	Relative sp act	% Recovery
Crude extract	3.1	26,070	8.3	1.0	100
Streptomycin sulfate supernatant	2.5	11,628	4.7	0.6	45
20–35% $(NH_4)_2SO_4$ fraction	0.2	3,089	15.6	1.9	12
Pooled S-300 fractions	0.052	2,142	41.2	5.0	8

[a] Units are expressed as (micromoles of PAPS formed per minute) $\times 10^{-6}$. The assay protocols are described in Materials and Methods. Protein concentrations were determined by the method of Bradford (1) with reagents supplied by Bio-Rad.

R. meliloti has two almost-identical copies of *nodP* and *nodQ* and a third locus, *saa*, that activates sulfate for use in biosynthesis of sulfur-containing amino acids (33, 40a). A wild-type *R. meliloti* strain growing on SO_4^{2-} as sole sulfur source through action of the *saa* locus (33), and showing significant ATP sulfurylase activity, does not contain large amounts of any protein species that cross-reacts with the CysC antibody (a minor background band does not correlate genetically with the *saa* locus) (Fig. 5, lane 3). This suggests that the third set of sulfate-activating enzymes, encoded by *saa*, do not include a protein that is as closely related to CysC as is the NodQ polypeptide. A fragment including the *saa* functions has recently been cloned (40a), and this will permit physical and genetic analysis of this locus to be initiated.

DISCUSSION

NodRm-IV(S) is a novel sulfated lipo-oligosaccharide that is secreted into the supernatant of *R. meliloti* cultures (15). It depends on the action of *nod* genes for its synthesis and export (9). In this study, we show that NodP and NodQ together suffice to synthesize not only APS but also PAPS. The multifunctional NodP-NodQ assembly we term the SAC. We observed that the NodPQ complex produces primarily PAPS, with APS being almost undetectable; this is consistent with the possibility that APS is not released as a free intermediate. In *R. meliloti* Nod factor biosynthesis, it is likely that the sulfate from the PAPS is transferred to the Nod factor acylchitotetraose precursor by NodH (6a, 15, 32), forming NodRm-IV(S) (26). We found that GTP was required for activity of the SAC, which suggests that Nod factor synthesis in *R. meliloti* is dependent on GTP.

In assigning function to the *nodP* and *nodQ* genes, we found that *nodQ*, or *nodP* and *nodQ*, generated as PCR segments and cloned in pJSS54 and pJSS55, complement the *cysC* auxotrophy in JM81A (Table 2). Our initial observation, repeated here (Table 2), that a pET3-borne copy of *nodP* and *nodQ* cloned in pJSS22 did not complement the *cysC* mutant is likely due to differences in expression. We note that the vector for pJSS22 uses a polymerase different from that used by the vectors for pJSS54 and pJSS55 (T7 versus *E. coli*) to drive expression, and each provides distinct sequences 3′ to the cloned insert. Thus, mRNA stability, length, and abundance are among the factors that might account for the phenotypic differences. In any case, it is clear that *nodQ* can complement an *E. coli cysC* mutant if *nodQ* is expressed at a sufficiently high level.

It is interesting that *nodP* alone cannot complement a *cysD*

mutant, nor can *nodQ* alone complement a *cysN* mutant. However, both together can complement both mutants. This suggests that while NodP and NodQ can interact to form ATP sulfurylase, and CysD and CysN do the same, neither NodP and CysN nor CysD and NodQ can form functional enzymes. This is particularly interesting considering how highly conserved these enzymes are. Possibly, relevant protein-protein contacts could be revealed by a genetic search for compensatory mutations in NodP that permit NodP-CysN and other heterologous pairs to interact.

The complementation results shown here (Table 2), in conjunction with the combined ATP sulfurylase-APS kinase assays, demonstrate that NodP and NodQ together have both activities and should be considered a multifunctional complex carrying out the two-step sulfate activation process. Immunoblots confirmed that NodQ is not processed within the active *R. meliloti* cells into separate sulfurylase and kinase domains. As a biochemical test, we made a comparison with enzyme systems where the two activities are known to be separate. Yeast ATP sulfurylase can make only APS (Fig. 1B, lane 1). The addition of exogenous *E. coli* APS kinase permitted formation of both APS and PAPS. Our assays showed that extracts from *R. meliloti* auxotroph DsAux7 were deficient in ATP sulfurylase activity unless both NodP and NodQ were overexpressed. When both are present, it is evident that there is also APS kinase activity, as PAPS is produced whether or not exogenous APS kinase is added.

The sequence analysis of *nodQ* showed that it possessed a consensus GTP-binding site and displayed substantial similarity to EF-Tu (18, 30). The *E. coli* CysN protein also showed this site. This suggested that GTP might be important for the activity of the enzymes encoded by these genes. Leyh and Suo (16) have shown that in *E. coli*, ATP sulfurylase is activated by a GTPase activity. Here, we examined the properties of partially purified NodP-NodQ SAC. We found that the NodP-NodQ complex requires GTP for any significant level of activity, and thus that biosynthesis of sulfated Nod factors should require GTP in *R. meliloti.*

NodP and NodQ are highly similar to proteins of *A. brasilense*, a soil bacterium that fixes nitrogen and associates with plant roots. As with *R. meliloti*, mutations in the *A. brasilense nodP* and *nodQ* genes do not produce auxotrophic strains (38); thus, NodP and NodQ do not seem to be the major sulfate-activating enzymes for amino acid biosynthesis in these two organisms. There are also homologues of *nodP* and *nodQ* in *Rhizobium* species strain NGR234, as detected by Southern blot hybridizations (23, 30). It is unknown whether the predominant functions of these genes are for symbiosis or amino acid biosynthesis, but we note the recent report (24) that Nod factors synthesized by NGR234 carry sulfate modification.

The known eubacterial ATP sulfurylase genes are well conserved. In contrast, the amino acid sequence of yeast ATP sulfurylase is not homologous to CysD, CysN, NodP, or NodQ, even though yeast APS kinase is very similar to NodQ and CysC. The yeast ATP sulfurylase may function by a mechanism different from that of *E. coli*, as it is a single polypeptide and shows no putative GTP-binding domain.

The finding that NodQ includes domains for both ATP sulfurylase and APS kinase is interesting from both evolutionary and mechanistic standpoints. Fusions have been well documented for a number of biosynthetic pathways. One example is the two-polypeptide TrpB-TrpA complex in *E. coli* and other bacteria, which in *Neurospora* and yeast spp. is found to be a single polypeptide (41, 42). One explanation proposed for this difference is that single mRNAs are translated into many polypeptides in bacteria, while single mRNAs are translated into single polypeptides in eukaryotes. Thus, the prokaryote can assure coordinate expression of the different polypeptides needed for a complex reaction by linking them in one operon and the eukaryote could assure coordinate production of the functions by bearing multiple functional domains in one polypeptide (22). However, this argument would not apply to the eubacterial *R. meliloti*, as coordinate production of CysN-like and CysC-like proteins is assured by their linkage in an operon. Thus, perhaps the fusion of the two domains into NodQ is important for targeting NodQ to a macromolecular assembly that functions in Nod factor biosynthesis. One localization signal would suffice to bring both functional domains of NodQ into association with other Nod proteins. Finally, the fusion of two domains into one polypeptide may provide a means to regulate activity of the two functions.

From the mechanistic standpoint, the fact that two enzymatic domains exist fused in a single polypeptide domain in *R. meliloti* suggests that a close association of the two *E. coli* polypeptides may be important or even essential to activity. For TrpB-TrpA, the association of the two proteins correlates with the observation that in vivo, the intermediate indole-3-phosphate appears to be channeled between the two proteins, so that two reactions are carried out by the two-polypeptide complex. Our finding that both ATP sulfurylase and APS kinase domains are borne by NodQ is consistent with the suggestion that the APS formed in the first reaction may be channeled to the second domain. This may also be the case for the *E. coli* CysD-CysN ATP sulfurylase and CysC APS kinase, as has been suspected on the basis of the unfavorable K_{eq} of the sulfurylase reaction ($\approx 10^{-9}$) and the potent inhibition of APS kinase activity by APS ($K_i < 400$ nM). Eukaryotic sulfate-activating enzymes show diversity in this respect: a close association of ATP sulfurylase and APS kinase activities has been found in rat chondrocytes, and the APS displays channeling between the ATP sulfurylase and APS kinase activities (20). However, the enzymes of *Penicillium chrysogenum* (25) do not show channeling of the APS intermediate (20). While a fusion of an APS kinase-like domain to the ATP sulfurylase protein is seen in the *P. chrysogenum* sequence, this is interpreted as an allosteric domain for effects of PAPS on ATP sulfurylase activity (10). With respect to *R. meliloti* NodP-NodQ, it will be interesting to determine whether the APS intermediate is effectively channeled and thus to understand how the various forms of activated sulfate may be partitioned into the pathways for amino acid biosynthesis, sulfation of Nod factor NodRm-IV(S) and lipopolysaccharide (33, 40a). Further study of the kinetics of the NodPQ reactions may be valuable for examining this question.

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