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# Purification of *Rhizobium leguminosarum* HypB, a Nickel-Binding Protein Required for Hydrogenase Synthesis

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**The products of the *Rhizobium leguminosarum hyp* gene cluster are necessary for synthesis of a functional uptake [NiFe] hydrogenase system in symbiosis with pea plants, and at least for HypB and HypF, a role in hydrogenase-specific nickel metabolism has been postulated (L. Rey, J. Murillo, Y. Hernando, E. Hidalgo, E. Cabrera, J. Imperial, and T. Ruiz-Argüeso, Mol. Microbiol. 8:471–481, 1993). The *R. leguminosarum hypB* gene product has been overexpressed in *Escherichia coli* and purified by immobilized nickel chelate affinity chromatography in a single step. The purified recombinant HypB protein was able to bind 3.9 ± 0.1 $Ni^{2+}$ ions per HypB monomer in solution. $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ ions competed with $Ni^{2+}$ with increasing efficiency. Monospecific HypB antibodies were raised and used to show that HypB is synthesized in *R. leguminosarum* microaerobic vegetative cells and pea bacteroids but not in *R. leguminosarum* aerobic cells. HypB protein synthesized by *R. leguminosarum* microaerobic vegetative cells could also be isolated by immobilized nickel chelate affinity chromatography. A histidine-rich region at the amino terminus of the protein (23-HGHHHH DGHHDHDHDHDHHRGDHEHDDHHH-54) is proposed to play a role in nickel binding, both in solution and in chelated form.**

*Rhizobium leguminosarum* bv. viciae possesses an $H_2$ uptake system that is able to oxidize $H_2$ generated by the nitrogenase complex as a byproduct of the $N_2$ reduction reaction (8, 40). This system consists of an uptake [NiFe] hydrogenase and accessory proteins, and it is only expressed in the plant symbiotic state. The main features of the system have been studied by our laboratory in the *Pisum*-pea bacteroid symbiosis. The genetic determinants for the $H_2$ uptake system are clustered in a 15-kb DNA region (*hup* region) in the symbiotic plasmid (21, 22). This region has been sequenced, and 17 potential genes have been identified. The first six genes constitute the hydrogenase structural operon and include the genes *hupS* and *hupL*, encoding the hydrogenase polypeptides (13), and four additional genes, *hupCDEF* (14). A five-gene cluster containing *hupGHIJK* has been identified downstream the hydrogenase structural operon (38).

All of the above *hup* genes are only expressed in bacteroids (35). In contrast, a gene cluster located immediately downstream is also activated in microaerobic vegetative cells (35). This cluster contains six genes, *hypABFCDE* (39), which show homology to genes from the *Escherichia coli* hydrogenase pleiotropic operon (26) and are conserved in a number of uptake hydrogenase-containing bacteria (9, 47). These genes are absolutely required for hydrogenase activity in *R. leguminosarum* (39) and have been shown to be required for maturation of hydrogenase in *E. coli* (17). The *hypB* gene is particularly interesting. Early experiments showed that mutations in the *E. coli hydB* locus (identical to *hypB*) are phenotypically reverted by high nickel concentrations in the medium (48). Specific Δ*hypB* mutants were later shown to behave similarly (17). Analogous results have been obtained with *Azotobacter chroococcum* (7) and *R. leguminosarum* (34). The product of *E. coli hypB* has recently been purified and characterized (27). HypB from *E. coli* binds guanine nucleotides and shows GTPase activity. However, no nickel binding to the purified protein could be demonstrated. The sequence of the *R. leguminosarum hypB* product contains a histidine-rich region (39) which is absent from the *E. coli hypB* product. Taking advantage of this feature, we have developed a one-step purification procedure for the *R. leguminosarum* HypB protein expressed in *E. coli* and have been able to show that *R. leguminosarum* HypB binds $Ni^{2+}$ in solution.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *R. leguminosarum* bv. viciae UPM791 ($Str^r$ $Hup^+$) and its derivative AL18 (*hypB*::Tn*5*) were used as $Hup^+$ wild-type (21) and $HypB^-$ (39) strains, respectively. Plasmid pAL618 is a pLAFR1-derived cosmid containing the *hup* region from *R. leguminosarum* (21). Plasmid pAL18 is a pAL618 derivative carrying the AL18 Tn*5* insertion. *E. coli* BL21(DE3) was used as the host for T7 polymerase-dependent gene expression (43). Plasmids pBluescript SK+ (41), pRLH60 (pBluescript carrying *hypB* under control of the T7 promoter [39]), and pRLH47 (pBluescript carrying *hypBF* in the opposite orientation with respect to the T7 promoter [39]) have been described. Bacterial strains were grown in the media and conditions previously described (22).

**Overproduction of the HypB protein.** The protocol of Studier et al. (44) was used for T7 polymerase-dependent expression of *hypB* in *E. coli* BL21(DE3) containing plasmid pRLH60, with minor modifications. For specific radiolabeling experiments, 1-ml portions were induced in the presence of rifampin for 2 h and labeled with 3 to 5 μCi of Trans$^{35}$S-Label (ICN, Costa Mesa, Calif.). For protein purification experiments, terrific broth (45) was used, and induction in the absence of rifampin was allowed to continue for 5 h before harvesting. Induced cultures were stored at −80°C until necessary.

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**Purification of the HypB protein by Ni(II)-NTA-agarose chromatography.** The Ni(II)-nitrilotriacetic acid (NTA)-agarose matrix was obtained from Diagen (Düsseldorf, Germany), and the manufacturer's recommendations for its use (12) were followed, with minor modifications as follows.

**(i) Denaturing conditions.** Frozen cells from a 100-ml induced culture were lysed in the presence of 6 M guanidine-HCl (3.5 ml), and cell extracts were applied to an Ni(II)-NTA-agarose column (2 by 0.8 cm). Proteins were stepwise eluted by means of buffers of decreasing pH, 8.0, 6.3, 5.9, and 4.5, all of which contained 8 M urea, at a flow rate of 0.5 ml $min^{-1}$. Fractions (1 ml) were collected, and portions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (18).

**(ii) Nondenaturing conditions.** All the manipulations were carried out at 0 to 4°C. Frozen cell paste from a 500-ml induced culture was resuspended in 10 ml of native buffer (50 mM $Na_2HPO_4$, 300 mM NaCl, 1mM dithiothreitol) containing DNase I (50 ng $ml^{-1}$) and phenylmethylsulfonyl fluoride (1 mM). Cell extracts were obtained by passage through a French pressure cell (104 MPa) and subsequent centrifugation (12,000 × *g*, 30 min). The supernatant was applied to an Ni(II)-NTA-agarose column (4 by 0.8 cm) equilibrated in native buffer, and proteins were eluted by means of a 120-ml imidazole gradient at a flow rate of 1 ml $min^{-1}$. Trial runs with linear gradients showed a strong HypB tailing profile and were used to optimize the final gradient profile: 50 mM imidazole (24 ml); 100 mM imidazole (20 ml); 100 to 160 mM imidazole linear gradient (44 ml); 160 to 260 mM imidazole linear gradient (20 ml); and 300 mM imidazole (12 ml). Fractions (2 ml) were collected and analyzed as above.

**(iii) *R. leguminosarum* cells.** The *R. leguminosarum hyp* system was induced by microaerobic cultivation (35). After induction, 1.5-liter cultures (0.5 g of fresh cell weight) were collected by centrifugation, resuspended in ice-cold native buffer containing lysozyme (1 mg $ml^{-1}$) and DNase I (50 ng $ml^{-1}$), and incubated in ice for 15 min. Cells were lysed by four cycles of freezing and thawing followed by sonication for a total of 3 min in 30-s bursts. Cell debris was removed by centrifugation (12,000 × *g*, 15 min), and the supernatants were applied to an Ni(II)-NTA agarose column (1 by 0.8 cm). The column was developed at a flow rate of 2 ml $min^{-1}$ with a linear 0 to 0.3 M imidazole gradient (120 ml total volume), and 2-ml fractions were collected. Fractions were separated by SDS-PAGE in 12% gels. Gels were stained with Coomassie blue, and duplicates were immunoblotted as described below.

**Protein analysis.** Protein content was routinely determined by the bicinchoninic acid method (42), except for purified protein, which was estimated by means of its $A_{280}$. The molar HypB extinction coefficient was estimated from the known amino acid sequence ($E_{280}$ theoretical = 11,170). Amino acid analysis was performed with a Beckman Amino Acid Analyzer by standard procedures (1). Amino-terminal sequencing of HypB samples blotted onto an Immobilon-P (Millipore) membrane (28) was performed at the University of California–Riverside Biotechnology Instrumentation Facility with an Applied Biosystems gas-phase sequencer.

**Size exclusion chromatography.** Purified HypB preparations were applied to Sephacryl S-200 and S-300 (Pharmacia) columns (0.8 by 50 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.4 or 8.0) to which 0.1% Triton X-100, 1 mM dithiothreitol, and 0.3 M NaCl were added in different combinations. HypB was also applied to Superose 6 HR 30/10 and Superose 12 HR 30/10 (Pharmacia) columns equilibrated with the same buffers as above, except that 1 M NaCl and 10 mM EDTA additions were also tried. For size exclusion chromatography under denaturing conditions, 8 M urea was added.

**Nickel binding in solution.** Binding of $Ni^{2+}$ by HypB was determined by equilibrium dialysis in modified chambers (37). The chambers contained 260 μl of native buffer. The upper chamber contained different concentrations of HypB (2.5 to 5 μM), and the lower chamber contained 0.1 μCi of $^{63}NiCl_2$ (0.86 Ci $mmol^{-1}$; Amersham) per ml and increasing concentrations of $NiCl_2$. After overnight equilibration (16 h, 4°C), the contents of both chambers were collected for counting. Each experimental point was determined in duplicate chambers at least twice. Binding data were analyzed by means of the EBDA (29) and LIGAND (29, 32) computer programs. For competition experiments, increasing concentrations of $MnCl_2$, $CoCl_2$, $ZnCl_2$, or $CuSO_4$ were added to the lower chambers containing $^{63}Ni$.

**Nucleotide binding and nucleotidase activity.** Binding of [$\alpha$-$^{32}P$]ATP, [$^3H$]GDP, and [$\gamma$-$^{32}P$]GTP by HypB was assayed by two methods: equilibrium dialysis (27) in the presence or absence of 10 μM $NiCl_2$, and nonequilibrium gel filtration chromatography (30) with Sephadex G-25. ATPase and GTPase activities were assayed with [$\gamma$-$^{32}P$]ATP and [$\gamma$-$^{32}P$]GTP. Released $^{32}P_i$ was measured as charcoal-unadsorbed radioactivity (27).

**Immunoblotting.** For HypB-specific antiserum generation, preparations of HypB purified under denaturing conditions were subjected to SDS-PAGE in 12% gels, and the region of the unstained gel corresponding to ca. 39 kDa was excised, frozen in liquid $N_2$, and fragmented with mortar and pestle. New Zealand rabbits were immunized with this preparation following a standard schedule of intradermal injections (10). Antisera were used for immunoblots obtained by standard techniques (10). Cell extracts or column fractions were separated by SDS-PAGE in 12% gels, and resolved proteins were transferred to Immobilon-P membranes by wet electrotransfer. Blots were probed with a 1:1,500 dilution of HypB-specific antiserum and a secondary goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad). A chromogenic substrate (bromochloroindolyl phosphate-nitro blue tetrazolium) was used as the developing reagent.

**Two-dimensional gel electrophoresis.** Two-dimensional O'Farrell gel electrophoresis was carried out as originally described (33).

## RESULTS

**One-step purification of recombinant HypB protein by nickel chelate affinity chromatography.** The presence of a histidine-rich stretch of amino acid residues in the *R. leguminosarum* HypB predicted protein prompted us to investigate whether HypB could be isolated by nickel chelate affinity chromatography. This technique exploits the affinity of adjacent imidazole groups for Ni(II) complexed via NTA to an agarose matrix (16).

Expression of the *R. leguminosarum* HypB protein in sufficient amounts was achieved in *E. coli* by means of plasmid pRLH60 (39). This plasmid contains a 1.3-kb *Xho*I-*Cla*I DNA fragment of pAL618 that includes *hypB* (Fig. 1A) under the control of a T7 promoter. Controlled expression of insert DNA was accomplished by means of the T7 RNA polymerase in *E. coli* BL21(DE3) (44). Under conditions of specific expression and $^{35}S$ labeling, HypB was observed as a prominent labeled protein band after SDS-PAGE separation (Fig. 1B, lane 4). Total protein staining of the gels, however, did not reveal a HypB-specific band (Fig. 1B, lane 2). This is likely due to comigration of HypB with a major outer membrane protein of ca. 37 kDa (25).

HypB binding to the Ni(II)-NTA matrix was tested with a cell extract obtained from an induced culture of *E. coli* BL21(DE3) carrying plasmid pRLH60. In order to be able to follow the protein, the induced culture (200 ml) was spiked with a small portion (0.5 ml) of a selectively $^{35}$S-labeled, induced culture. In initial experiments, and to make sure that the histidine-rich sequence was exposed to the medium, cell extract isolation and column development were carried out under denaturing conditions in the presence of guanidine-HCl and urea, as described in Materials and Methods. A major radioactive peak eluted at pH 4.5 and contained a ca. 39-kDa radioactive protein, likely corresponding to *R. leguminosarum* HypB (data not shown).

Ni chelate affinity chromatography might be useful for purification of nondenatured HypB if the Ni-binding motifs in HypB were exposed to the medium. To test this possibility, cell extracts from induced *E. coli* BL21(DE3) cells containing plasmid pRLH60 were obtained under nondenaturing conditions and applied to an Ni(II)-NTA column. Bound proteins were eluted by means of an increasing imidazole gradient, and the radioactivity in the collected fractions was monitored (Fig. 1C). Most of the proteins in the extract did not bind to the matrix or interacted weakly with it (Fig. 1D). A major radioactive peak eluted from the column at ca. 200 mM imidazole (Fig. 1C), which corresponded to a single radioactive protein band of ca. 39 kDa (Fig. 1D, lanes 4 and 8) attributable to HypB. No ca. 39-kDa band was observed when extracts from induced cultures of *E. coli* BL21(DE3) containing plasmid pBluescript SK+ (no insert DNA) and *E. coli* BL21(DE3) containing plasmid pRLH47 (insert DNA in opposite orientation) were separated under the same conditions. Thus, one-step purification of recombinant HypB protein was achieved by means of Ni chelate affinity chromatography. A total of 2.5 mg of protein was recovered in pooled fractions 44 to 56, which accounts for 2.5% of the protein loaded into the column.

**Properties of purified recombinant HypB. (i) Amino acid analysis.** Limited N-terminal amino acid sequence analysis was performed on the ca. 39-kDa protein band isolated by denaturing Ni(II)-NTA chromatography and SDS-PAGE. The results obtained, (X)-T-V-(X)-G-(X)-G-T-S-A-I-G, correspond to the sequence expected for HypB (M-C-T-V-C-G-C-G-T-S-A-I-G) except for the processing of the N-terminal methionyl residue and for an undetermined blocking of the three cysteinyl residues. Total amino acid analysis of the same band resulted in an amino acid molar percent composition corresponding to that expected for HypB (data not shown).

**(ii) UV absorption spectrum.** Purified recombinant HypB showed a peak at 278 nm and another one at 260 nm (data not shown). The $A_{278}/A_{260}$ ratio was 1.10, which suggests that bound nucleotides copurify with the protein. However, no nucleotide release could be detected by measuring $A_{254}$ to $A_{260}$ in low-molecular-weight fractions after treatment of HypB protein with 8 M urea and gel filtration separation.

**(iii) Nucleotide binding and nucleotidase activity.** No GTPase or ATPase activities associated with purified recombinant HypB preparations in either the presence or absence of $Ni^{2+}$ were detected (data not shown). Similarly, no binding of ATP, GDP, or GTP by HypB could be demonstrated (data not shown).

**(iv) Molecular size and isoelectric point.** Both in extracts (39) and in purified form (Fig. 1B, lane 4, and D, lane 4), the recombinant HypB protein migrated as a ca. 39-kDa band in SDS-PAGE gels, whereas the molecular size predicted from the sequence is ca. 32 kDa. Native molecular size determination was attempted by different methods. First, native PAGE in 8% gels, under the conditions tested (basic and neutral buffer systems, low and high salt, and addition of dithiotreitol or 0.1% Triton X-100), resulted in lack of migration of the protein into the gel. Second, size exclusion chromatography with Sephacryl S-200 and S-300 as well as Superose 6 HR and 12 HR gave conflicting results. HypB protein was excluded from both Sephacryl S-200 and S-300 matrixes, suggesting that protein in the purified preparations is in the form of a complex of >1,000 kDa. However, HypB protein interacted very strongly with the Superose 6 HR and 12 HR matrixes and did not elute from the columns under native conditions. Addition of 8 M urea resulted in elution of the protein in the denatured state. Under these conditions, the HypB monomer eluted from the Superose 6 HR column with the same retention time as chymotrypsinogen A (25 kDa). This suggests that even in the presence of the denaturing agent, HypB still interacted strongly with the Superose matrix.

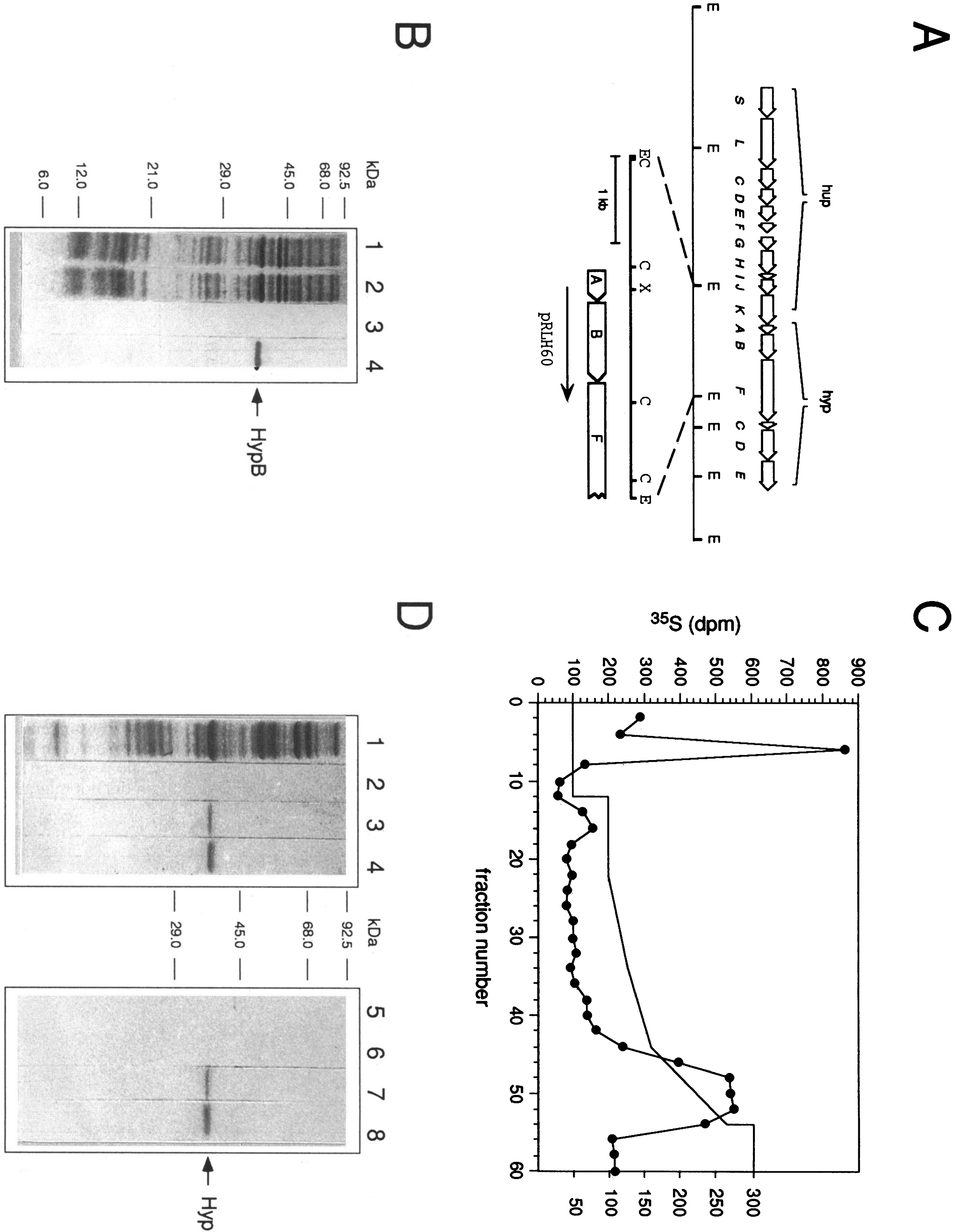
Two-dimensional O'Farrell gels of both purified protein and crude extracts (Fig. 2A and B) showed that recombinant HypB protein is produced as a heterogeneous mixture of forms of different isoelectric points, ranging from pI 5.7 to 6.2.

**Nickel binding by recombinant HypB.** Since HypB was able to bind NTA-chelated Ni(II), purified recombinant HypB protein was also assayed for its ability to bind $Ni^{2+}$ ions in solution. Equilibrium dialysis experiments (Fig. 3) showed that HypB binds 3.9 ± 0.1 $Ni^{2+}$ ions per monomer, with an apparent *Kd* of 2.5 ± 0.3 μM. An optimal computer fitting of the binding data (29, 32) was obtained when only one type of binding site was assumed, suggesting that all binding sites have a similar affinity for $Ni^{2+}$. Addition of ATP or GTP (1 mM) to HypB did not have any effect on its affinity for $Ni^{2+}$.

The specificity of $Ni^{2+}$ binding in solution was assayed by equilibrium dialysis competition with divalent metal cations (Fig. 4). $Mn^{2+}$ ions up to 0.4 mM had no effect on $Ni^{2+}$ binding. $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ ions competed with $Ni^{2+}$ ions with increasing efficiency. $Co^{2+}$ and $Cu^{2+}$ displaced ca. 25 and 75% of the $Ni^{2+}$ ions bound to HypB, respectively, under the conditions of the assay. $Zn^{2+}$ was able to displace most of the $Ni^{2+}$ bound to HypB, which showed an affinity for $Zn^{2+}$ similar to that for $Ni^{2+}$ ($Ki \approx 2$ μM).

**Identification of HypB in *R. leguminosarum* free-living and symbiotic cells.** The ca. 39-kDa protein band obtained by SDS-PAGE of fractions from denaturing Ni chelate affinity chromatography was used to raise specific antibodies in rabbits. Antisera were used in immunoblot assays of cell extracts

FIG. 1. Nickel chelate affinity chromatography of *R. leguminosarum* HypB protein expressed in *E. coli*. (A) Location of pRLH60 insert DNA, containing *hypB*, in the *hup* region cloned in cosmid pAL618. (B) T7 polymerase-dependent expression of *hypB* in *E. coli* BL21(DE3) containing plasmid pRLH60 (lanes 2 and 4) versus that in *E. coli* BL21(DE3) containing the control plasmid pBluescript SK+ (lanes 1 and 3); Coomassie blue staining (lanes 1 and 2) and autoradiogram (lanes 3 and 4) of SDS-PAGE gels loaded with extracts from induced, $^{35}$S-labeled cultures. (C) Ni(II)-NTA-agarose chromatography of $^{35}$S-labeled extracts from *E. coli* BL21(DE3) containing plasmid pRLH60 under nondenaturing conditions. The column was developed with an imidazole gradient as described in Materials and Methods. (D) Analysis of fractions corresponding to $^{35}$S peaks. Portions from fractions 6 (lanes 1 and 5), 16 (lanes 2 and 6), 42 (lanes 3 and 7), and 48 (lanes 4 and 8) were separated by SDS-PAGE, stained with Coomassie blue (lanes 1 to 4), and exposed to radiographic film (lanes 5 to 8). The positions of protein standards are indicated.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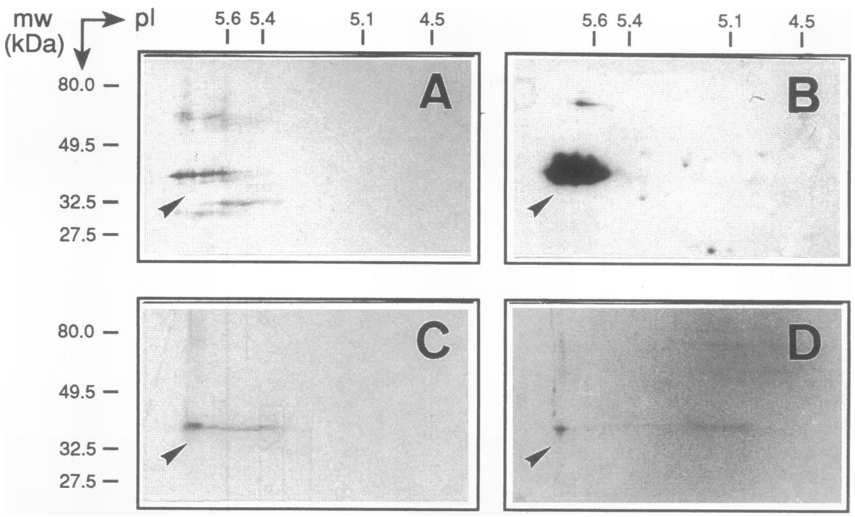

FIG. 2. Two-dimensional O'Farrell gels of HypB protein. (A) Purified recombinant HypB protein (immunoblot); (B) extract from induced, $^{35}S$-labeled *E. coli* BL21(DE3) cells containing plasmid pRLH60 (autoradiogram); (C) extract from *R. leguminosarum* UPM791 microaerobic vegetative cells (immunoblot); and (D) extract from *R. leguminosarum* UPM791 pea bacteroids (immunoblot). Arrowheads point to the ca. 39-kDa protein. The positions of size (mw) and isoelectric point (pI) protein standards are indicated.

from *R. leguminosarum* cultures (Fig. 5). A major immunoreactive band of ca. 39 kDa was present in extracts from microaerobically grown cells and pea bacteroids of wild-type *R. leguminosarum* (Fig. 5, lanes 2 and 3). This band was absent in extracts from aerobically grown cells of the same strain (Fig. 5, lane 1) and in extracts from *R. leguminosarum* AL18 (*hypB*:: Tn*5*) cells grown under any condition (Fig. 5, lanes 4 to 6). In addition to the ca. 39-kDa band, other immunoreactive bands of higher and lower molecular masses were observed. The bands with lower molecular masses are attributable to HypB, since they appeared only when the ca. 39-kDa band was present. Specific precautions were taken to minimize proteolytic degradation, but this did not eliminate the extra bands (data not shown). Immunoblots from two-dimensional O'Farrell gels of extracts from microaerobically grown cells and from pea bacteroids of the wild-type *R. leguminosarum* strain (Fig. 2C and D) further revealed additional heterogeneity, showing that the ca. 39-kDa band is present in *R. leguminosarum* as a heterogeneous mixture of forms with different charge, as was the case for the recombinant HypB protein (Fig. 2A and B).

This result prompted us to check whether the abnormal behavior of the recombinant HypB protein observed in native PAGE and in Sephacryl and Superose matrixes under nondenaturing conditions (see above) would also occur in *R. leguminosarum*. PAGE gels and column fractions were tested for HypB by immunoblot. Again, the protein did not migrate into

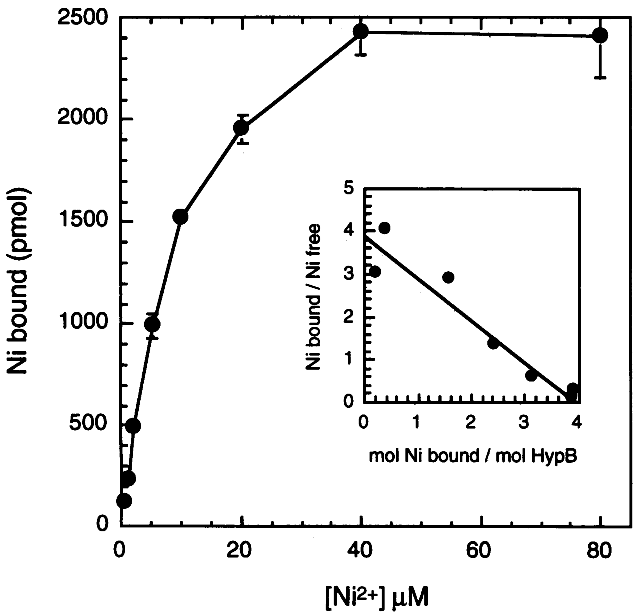

FIG. 3. $Ni^{2+}$ binding by purified recombinant HypB. Equilibrium dialysis was carried out with HypB (5 μM) and increasing concentrations of $NiCl_2$ solutions containing $^{63}NiCl_2$ (0.1 μCi ml$^{-1}$). Inset: Scatchard transformation of the data.

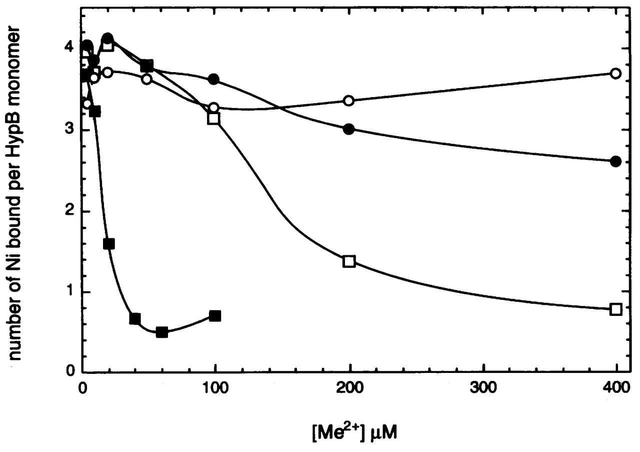

FIG. 4. Effect of divalent metal ions on nickel binding to recombinant HypB. Equilibrium dialysis was carried out with HypB (5 μM) at fixed $NiCl_2$ (40 μM) and $^{63}NiCl_2$ (0.1 μCi ml$^{-1}$) concentrations and increasing concentrations of $MnCl_2$ (○; 10 to 400 μM), $CoCl_2$ (●; 10 to 400 μM), $CuSO_4$ (□; 10 to 400 μM), or $ZnCl_2$ (■; 5 to 100 μM).

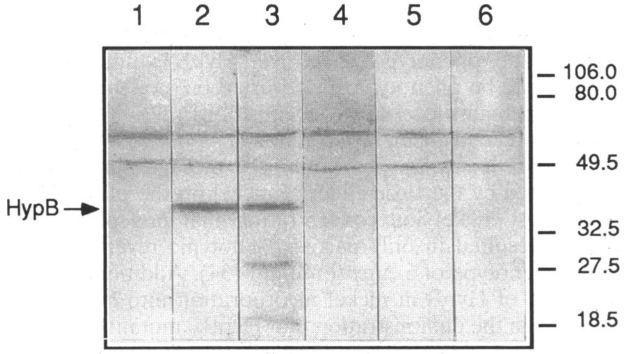

FIG. 5. Immunoblot identification of HypB in *R. leguminosarum*. Cell extracts (20 to 30 μg of protein) from wild-type strain UPM791 aerobic (lane 1) and microaerobic (lane 2) vegetative cells and pea bacteroids (lane 3) and from HypB⁻ mutant AL18 aerobic (lane 4) and microaerobic (lane 5) vegetative cells and pea bacteroids (lane 6) were loaded in each lane. The positions and sizes (in kilodaltons) of protein standards are indicated.

native 8% PAGE gels and was excluded from the Sephacryl S-300 matrix (data not shown), suggesting that this was not an artifact caused by overexpression of the protein in the *E. coli* host.

**HypB from *R. leguminosarum* binds to the Ni(II)-NTA matrix.** Nondenaturing cell extracts from microaerobically grown *R. leguminosarum* cultures were applied to an Ni(II)-NTA column and fractionated as described for the recombinant HypB protein (Fig. 1). Portions from the fractions corresponding to 200 mM imidazole were separated by SDS-PAGE and either stained (Fig. 6A) or immunoblotted (Fig. 6B). Three protein bands were observed in the fraction corresponding to *R. leguminosarum* UPM791(pAL618). One of them, of ca. 39 kDa, was absent in the fraction from *R. leguminosarum* AL18(pAL18) and immunoreacted with the HypB antiserum.

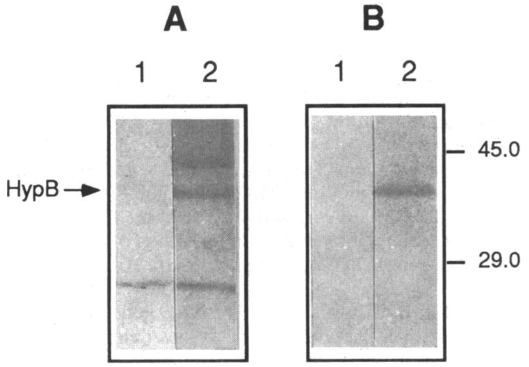

FIG. 6. Interaction of HypB from *R. leguminosarum* cell extracts with the nickel chelate affinity matrix. Cell extracts from microaerobic vegetative cultures were applied to an Ni(II)-NTA-agarose column and developed under nondenaturing conditions as described in Materials and Methods. Fractions containing ≈0.15 M imidazole and higher were pooled, acetone precipitated, and resolved by SDS-PAGE. (A) Coomassie blue staining. (B) Immunoblot with HypB-specific antibodies. Lane 1, HypB⁻ mutant AL18; lane 2, wild-type strain UPM791. The positions and sizes (in kilodaltons) of protein standards are indicated.

## DISCUSSION

One-step purification of recombinant *R. leguminosarum* HypB protein has been achieved by means of Ni(II)-NTA-agarose chromatography. The amino acid sequence of *R. leguminosarum* HypB shows a long histidine-rich stretch near its amino terminus (23-HGHHHHDGHHDHDHDHDHHR GDHEHDDHHH-54 [39]). This sequence is likely responsible for the strong retention of HypB by the metal matrix. The behavior of proteins and peptides in immobilized metal ion affinity chromatography (36), of which Ni(II) chelate affinity chromatography is just an example, has been shown to closely reflect the availability of exposed histidine residues for interaction with the metal (11). A few naturally occurring histidine-rich proteins have been purified by immobilized metal ion affinity chromatography (4). In addition, a method for purification of recombinant proteins by addition of a six-histidine tail has been developed (15). The quadridentate chelating matrix NTA-agarose is the basis of this method. When charged with $Ni^{2+}$ (coordination number of six), this matrix showed strong specificity for peptides and proteins with contiguous histidines (16). A number of engineered proteins have been purified in this way (see, for example, references 20 and 24).

Denaturing conditions were not required for strong interaction of HypB with the matrix, suggesting that enough histidine residues are normally exposed and available for interaction with the immobilized metal. This is congruent with the high hydrophilicity of the histidine-rich region (39). Native *R. leguminosarum* HypB protein produced in its cognate host under microaerobic conditions was also strongly retained by the Ni(II)-NTA-agarose matrix. Only two contaminating proteins copurified with HypB, which should make the eventual purification of the protein from *R. leguminosarum* cells easy.

Purified recombinant HypB protein was able to bind $Ni^{2+}$ ions in solution as well as in chelated form. It is likely that the maximum of ca. 4 $Ni^{2+}$ ions bound per monomer interact with the histidine-rich stretch, although it is possible that additional histidine or cysteine residues might be required for ligation. The *Klebsiella aerogenes ureE* gene product, which has been shown to have a C-terminal histidine-rich sequence (10 histidyl residues out of 15, HGHHHAHHDHHAHSH [31]), has been purified and shown to bind ca. 3 $Ni^{2+}$ ions per monomer (19). Extended X-ray absorption fine-structure and variable-temperature magnetic circular dichroism analyses of Ni-saturated UreE protein are compatible with a coordination sphere of three to five imidazole rings for each UreE-bound nickel ion (19). Assuming that all the histidyl side chains are available for ligation and that $Ni^{2+}$ ion binding by HypB is similar to that by UreE, the 20 histidyl residues in and around the *R. leguminosarum* HypB histidine-rich stretch could account for the ca. 4 $Ni^{2+}$ ions bound per HypB monomer. Two lines of evidence support the previous assumptions. First, computer fitting of the binding data suggests that all four Ni sites in HypB have similar binding constants, which is compatible with a similar ligand environment for all of them. Second, the pattern of competition of HypB binding of $Ni^{2+}$ in solution by other divalent cations (Fig. 4) was similar to that found with UreE (19), suggesting that Ni-binding sites in both proteins are similar. However, unlike HypB, *K. aerogenes* UreE does not bind to an Ni(II)-iminodiacetic acid-agarose matrix and could not be isolated by immobilized metal ion affinity chromatography. This, in turn, suggests that there are differences in exposure of the histidine-rich stretch of both proteins to the medium.

The *E. coli* HypB protein has recently been purified and characterized (27). The recombinant *R. leguminosarum* HypB protein, as isolated, differs from *E. coli* HypB in several respects.

(i) *E. coli* HypB, unlike *R. leguminosarum* HypB, does not bind $Ni^{2+}$ in solution. This is probably related to the histidine-rich amino-terminal region. Optimal alignment of HypB sequences from different microorganisms (39) shows very high conservation throughout the sequences with the exception of the histidine-rich region. Histidine-rich regions are present in *Rhodobacter capsulatus* HypB (5, 50) and equivalent gene products from *Azotobacter vinelandii* (3), *A. chroococcum* (46), and *Alcaligenes eutrophus* (6). However, *E. coli* HypB lacks a similar region (only two histidines among the first 50 residues). Since different HypB proteins likely carry out similar functions, either the histidine-rich region must not be necessary for this common function or its role must be bypassed in the *E. coli* system. In this respect, it is interesting that a recent database search for *R. leguminosarum* HypB homologs revealed a recently sequenced *E. coli* gene which codes for a protein with a highly homologous histidine-rich region and $Ni^{2+}$-binding capacity (49).

(ii) No GDP-binding or GTPase activity could be demonstrated with our purified *R. leguminosarum* HypB preparations, whereas both activities have been demonstrated with *E. coli* HypB, despite the fact that the proposed G domains in *E. coli* HypB are absolutely conserved in *R. leguminosarum* HypB (27, 39). It is possible that our inability to demonstrate these activities with purified recombinant *R. leguminosarum* HypB is due to the anomalous behavior of the protein, which most likely aggregates into a multimeric form. This behavior did not appear to be a result of overexpression and purification in *E. coli*, since HypB protein expressed in *R. leguminosarum* under its cognate promoter gave similar results. It is tempting to speculate that the aggregation properties of HypB have a biological meaning as has recently been shown for Era, an *E. coli* GTPase (23), which associates with the membrane and forms large intermolecular aggregates upon nucleotide binding. Alternatively, it is possible that the protein as isolated is tightly bound to GDP (GTP), which might preclude detection of any of the activities. The existance of a UV absorption maximum at 260 nm in purified preparations of the protein supports this possibility. Tight binding of nucleotides and the requirement for GTPase-activating proteins and guanine nucleotide release proteins for the GTPase cycle are well documented (2).

The availability of HypB-monospecific antibodies allowed us to follow HypB expression in *R. leguminosarum*. The protein was detected in microaerobic vegetative cells and in bacteroids but not in aerobic vegetative cells. This is consistent with previous results on *hyp* gene expression based on mRNA detection and *lacZ* fusions (35). Synthesis of HypB (and presumably other Hyp proteins) in microaerobic vegetative cells is puzzling, since no hydrogenase expression takes place under these conditions (35). The *R. leguminosarum* HypB protein, expressed in either *E. coli* or *R. leguminosarum*, was always observed as a ca. 39-kDa band in SDS-PAGE gels (versus a theoretical value of ca. 32 kDa). In addition, multiple forms of different isoelectric points were observed, both in extracts and in purified form. This situation might be artifactual and due to the histidine (and acidic residue)-rich region or might result from modifications of the protein. Possible modifications include (i) metal ions bound to the histidine-rich region, (ii) nucleotides bound to the G domains (23), and (iii) unknown groups modifying the three cysteinyl residues at the amino terminus (see above). These possibilities are currently under investigation.

What is the role of the HypB protein in the assembly of active hydrogenase? An implication of HypB in nickel metabolism for hydrogenase synthesis had first been proposed on the basis of the phenotype of *E. coli* $HypB^-$ mutants; these mutants can be phenotypically reverted by growth in medium containing high nickel ion concentrations (27, 48). A similar result has recently been reported for *A. chroococcum* (7). With the *R. leguminosarum*-pea symbiosis, these experiments are complicated by the toxic effect of nickel on the plant. In fact, the highest nickel ion concentration that pea plants could tolerate resulted in only partial phenotypic reversion of the $Hup^-$ phenotype of a Δ*hypB* mutant (34). Additional support for a role of HypB in nickel incorporation into hydrogenase comes from the demonstration that $HypB^-$ mutants synthesize a nickel-free, unprocessed form of the hydrogenase large subunit. This has been shown for *E. coli* hydrogenase 3 (27) and *A. eutrophus* soluble hydrogenase (6). We have also been able to show that *R. leguminosarum* $HypB^-$ mutants accumulate an unprocessed form of the hydrogenase large subunit (34). Our results with *R. leguminosarum* constitute the first demonstration of a direct interaction between HypB and nickel and are compatible with a possible role of HypB in either the scavenging of $Ni^{2+}$ for hydrogenase synthesis ($Ni^{2+}$ binding in solution) or the chelation of Ni(II) previously bound to another protein (interaction with immobilized nickel).

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