Characterization of *Helicobacter pylori* Nickel Metabolism Accessory Proteins Needed for Maturation of both Urease and Hydrogenase

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Received 3 October 2002/Accepted 4 November 2002

Previous studies demonstrated that two accessory proteins, HypA and HypB, play a role in nickel-dependent maturation of both hydrogenase and urease in *Helicobacter pylori*. Here, the two proteins were purified and characterized. HypA bound two Ni\(^{2+}\) ions per dimer with positive cooperativity (Hill coefficient, approximately 2.0). The dissociation constants \(K_1\) and \(K_2\) for Ni\(^{2+}\) were 58 and 1.3 \(\mu\)M, respectively. Studies on purified site-directed mutant proteins in each of the five histidine residues within HypA, revealed that only one histidine residue (His2) is vital for nickel binding. Nuclear magnetic resonance analysis showed that this purified mutant version (H2A) was similar in structure to that of the wild-type HypA protein. A chromosomal site-directed mutant of hypA (in the codon for His2) lacked hydrogenase activity and possessed only 2% of the wild-type urease activity. Purified HypB had a GTPase activity of 5 nmol of GTP hydrolyzed per nmol of HypB per min. Site-directed mutagenesis within the lysine residue in the conserved GTP-binding motif of HypB (Lys59) nearly abolished the GTPase activity of the mutant protein (K59A). In native solution, both HypA and HypB exist as homodimers with molecular masses of 25.8 and 52.4 kDa, respectively. However, a 1:1 molar mixture of HypA plus HypB gave rise to a 43.6-kDa species composed of both proteins. A 43-kDa heterodimeric HypA-HypB complex was also detected by cross-linking. The cross-linked adduct was still observed in the presence of 0.5 mM GTP or 1 \(\mu\)M nickel or when the mutant version of HypA (altered in His2) and HypB (altered in Lys59) were tested. Individually, HypA and HypB formed homodimeric cross-linked adducts. An interaction between HypA and the Hp0868 protein (encoded by the gene downstream of hypA) could not be detected via cross-linking, although such an interaction was predicted by yeast two-hybrid studies. In addition, the phenotype of an insertion mutation within the Hp0868 gene indicated that its presence is not critical for either the urease or the hydrogenase activity.

*Helicobacter pylori* is a spiral, gram negative, microaerophilic bacterium that has been shown to be the etiological agent of gastritis and peptic ulcer disease (3, 4). It expresses two distinct nickel-containing enzymes, both of which are important for its virulence. These are a membrane bound [Ni-Fe] hydrogen-uptake hydrogenase, which permits respiratory-based energy production for the bacteria in the mucosa (19, 30), and an uptake hydrogenase, which permits respiratory-based energy production for the bacteria in the mucosa (19, 30), and an enzyme critical for early steps in colonization, urease (23, 25).

Synthesis of metal-containing enzymes often requires the participation of accessory proteins, and the maturation of hydrogenase and urease are no exceptions. Indeed, the complete genome sequence of *H. pylori* reveals the presence of a full complement of urease (ureEFGH) and hydrogenase (hypAB CDEF) accessory genes (36). A number of studies exist that address these accessory genes in other bacteria and their role in the Ni-dependent maturation of urease and hydrogenase apoenzymes (7, 9, 13, 14, 15, 20, 22, 27, 28, 34, 39). Although the specific role of each of these proteins has not been clarified to date, the existing data indicate that an efficient Ni enzyme maturation process involves a concerted effort of the accessory proteins, likely involving sequential Ni-metabolizing steps. By studying gene-directed mutants in *H. pylori*, it was found that two of the hydrogenase accessory genes, hypA and hypB, are required for both hydrogenase and urease activities (29). That hypA plays a role in *H. pylori* urease maturation was confirmed by another research group (F. Wöhl et al., Int. J. Med. Microbiol., vol. 291, suppl. 31, abstr. K-22, 2001), also by use of a gene-directed mutation approach. The lack of urease activity could not be attributed to the lack of hydrogenase activities in these two (hypA and hypB) mutants, since a hydrogenase structural gene mutant, hypD, and other hyp accessory mutants, hypD, hypE, and hypF, showed wild-type levels of urease activity although they were all deficient for hydrogenase activity. Also, the expression levels of the urease apoenzyme in the hypA and hypB mutants were comparable to those of the wild type; however, the nickel content of the urease from the hypA mutant was fourfold less, and that from the hypB mutant was fivefold less, than that of the wild type (29). Therefore, it was proposed that in addition to being involved in the maturation of the hydrogenase apoenzyme, HypA and HypB are also involved in the maturation of the urease apoenzyme into its Ni-containing active form.

Both hydrogenase and urease maturation independently require an accessory protein with a functional nucleotide-binding domain and also one that is capable of binding and/or donating nickel to the active site (10, 11, 14, 16, 20, 26, 28). Mutation in the GTP-binding domains of ureG (*Klebsiella aerogenes*) and hypB (*Bradyrhizobium japonicum*) resulted in the production of an inactive urease and hydrogenase, respectively (26, 28). Studies with the UreDFG-apourease complex from *K. aerogenes* showed that it could be activated in vitro only if GTP (200 \(\mu\)M)
and nickel (20 μM) were present (34). Initial studies with UreE from K. aerogenes showed that it could bind approximately 6 Ni²⁺ ions per dimer, and this was attributed to the presence of a histidine-rich C terminus. However, later studies with a truncated UreE, lacking 15 C-terminal residues, showed that, in the absence of the histidine-rich region, the protein could still bind 2 Ni²⁺ ions per dimer (6). Similar to K. aerogenes, the nickel-metabolizing accessory complexes of H. pylori were expected to have both nickel-sequestering and GTP-hydrolyzing proteins for the mobilization of nickel into the active site of the nickel enzymes. H. pylori HypB has the characteristic GTP-binding domain but lacks the histidine-rich region. There are no such domains in HypA, but there are scattered histidine residues within the protein. Since a mutation in hypA and hypB resulted in a deficiency in urease and hydrogenase activities in H. pylori (29), it is possible that these mutants are deficient in nickel sequestering, GTP hydrolysis, or both functions.

In the present study, we have purified and characterized HypA and HypB proteins from H. pylori and determined their GTP-hydrolyzing and nickel-binding abilities and their ability to interact in vitro. This is the first report showing that a HypA protein is capable of binding nickel and that HypA and HypB intimately interact in vitro. Site-directed mutagenesis of hypA was done in five histidine residues, and the nickel-binding ability of each of the five purified mutant proteins was investigated. A site-directed mutant protein in the conserved lysine residue within the GTP-binding motif of HypB was also purified, and its GTPase activity was determined. A chromosomal site-directed mutation was also introduced into the hypA gene (the codon for His2 was replaced for that for alanine). Urease and hydrogenase activities of the mutant strain were measured.

The hydrogenase and urease activities of the Hp0868 (gene downstream of hypA) mutant were measured, and cross-linking studies between HypA and Hp0868 protein were also carried out.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Escherichia coli DH5α (BRL) was used for all genetic manipulations. E. coli BL21(DE3) RIL (Stratagene) was used as the host for expression of the recombinant proteins. E. coli XL1-Blue supercompetent cells (Stratagene) were used as the hosts for site-directed mutagenesis. E. coli DH10B (BRL) was used as host for the phyA::KSF construct. H. pylori strain ATCC 43504 was used as the wild type and parent strain for obtaining the Hp0868 and hypA::KSF mutant strains. The H. pylori mutant strain hypA::KSF was used as the host for obtaining the hypA::H2A chromosomal site-directed mutant strain (Table 1). E. coli strains were grown in Luria-Bertani medium or plates, supplemented with 100 μg of ampicillin/ml or 30 μg of kanamycin/ml as required. H. pylori was routinely grown on Brucella agar plates supplemented with 10% defibrinated sheep blood with and without 25 μg of kanamycin/ml. For chromosomal site-directed mutants, Brucella agar plates with either 25 μg of kanamycin/ml or 5% sucrose were used.

**Construction of plasmids for overexpression of wild-type HypA, HypB, and Hp0868 proteins.** The hypA, hypB, and Hp0868 genes were PCR amplified in the presence of the appropriate primers, HypA F1 and HypA R1, HypB F1 and HypB R1, and 868 F1 and 868 R1 (IDT) (Table 2), respectively, with genomic DNA from wild-type H. pylori as the template. These primers engineer an NdeI restriction site at the 5’ end prior to the start codon for each gene. The PCR products were then cloned into the Smal site of pBluescript KS+, yielding pKS-hypA, pKS-hypB, and pKS-868. pKS-hypA, pKS-hypB, and pKS-868 were

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each digested with NdeI and BamHI, and the fragment containing the coding region for each gene was purified and ligated into NdeI- and BamHI-digested pET-21A, yielding pET-hypA, pET-hypB, and pET-868, respectively, (Table 1). These recombinant pET plasmids were then transformed into electrocompetent BL21(DE3) RIL cells by using a pulse of 2.5 kV in a TransPorator Plus (BTX) apparatus.

Construction of plasmids for overexpression of HypA and HypB mutant proteins with single amino acid substitutions. Site-directed mutagenesis of hypA (histidine at positions 2, 17, 24, 79, and 95) and hypB (lysine at position 59 located within the GTP-binding motif) was done by using the QuikChange HR 10/30 column (Pharmacia). The column was pre-equilibrated with 50 mM Na2HPO4 (pH 7.5) plus 25 mM Tris-Cl (pH 7.5) was used as the elution buffer for anion-exchange chromatography. Na2HPO4 (50 mM, pH 7.5) was used as the start buffer, and 400 mM NaCl plus 10 mM Tris-Cl (pH 7.5) followed by incubation for 15 min at 25°C. The cell pellets were washed once with buffer 4°C. The cell pellet was washed once with buffer 

Cross-linking assay. A 1:1 molar mixture of the wild-type HypA and HypB proteins (after a 10-min preincubation at 4°C) was also loaded onto the column to look for any interaction under native conditions.

N-terminal amino acid sequencing and determination of molecular mass. N-terminal sequencing of purified HypA, HypB, and Hp0868 proteins was carried out at the MGIF (University of Georgia). The molecular mass was determined by liquid chromatography (LC)-mass spectrometry at the Chemical and Biological Sciences Mass Spectrometry facility (University of Georgia).

Size exclusion chromatography. The native sizes of the HypA and HypB wild-type proteins were determined by using a fast-performance LC Superose 6 HR 10/30 column (Pharmacia). The column was pre-equilibrated with 50 mM Tris-Cl (pH 7.5) plus 100 mM KCl. Calibration was done by using the molecular weight of GF 200 kit (Sigma) markers β amylase, alcohol dehydrogenase, bovine serum albumin (BSA), carbonic anhydrase, and cytochrome c (Mr, 200,000, 150,000, 60,000, 29,000, and 12,400, respectively). The buffer used for protein elution was the same as that used for equilibration. A 1:1 molar mixture of the wild-type HypA and HypB proteins (after a 10-min preincubation at 4°C) was also loaded onto the column to look for any interaction under native conditions.

Purification of HypA, HypB, and Hp0868 proteins. Purification of HypA and HypB (wild-type and mutant versions) was achieved in two steps: Q-Sepharose anion exchange followed by Sephacryl S-100 gel filtration chromatography. TN buffer was used as the start buffer, and 400 mM NaCl plus 10 mM Tris-Cl (pH 7.5) was used as the elution buffer for anion-exchange chromatography. For gel filtration chromatography, TN buffer was used.

The Hp0868 protein was purified by SP-Sepharose cation exchange followed by a high-resolution chromatography. NaCl-HPO4 (50 mM, pH 7.5) was used as the start buffer, and 50 mM NaCl-HPO4 (pH 7.5) plus 1 M NaCl was used as the elution buffer for cation-exchange chromatography. Gel filtration chromatography was performed in the presence of 20 mM NaCl-HPO4 (pH 7.5) plus 25 mM NaCl buffer.

Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the extent of purity. The protein concentration was estimated by using the Coomassie Plus protein assay reagent, and the protocol followed was that suggested by the manufacturer (Pierce). Pure fractions were then pooled and dialyzed for 48 h against different buffers depending on the type of assay.

Puriﬁcation of HypA, HypB, and Hp0868 proteins. Purification of HypA and HypB (wild-type and mutant versions) was achieved in two steps: Q-Sepharose anion exchange followed by Sephacryl S-100 gel filtration chromatography. TN buffer was used as the start buffer, and 400 mM NaCl plus 10 mM Tris-Cl (pH 7.5) was used as the elution buffer for anion-exchange chromatography. For gel filtration chromatography, TN buffer was used.

The Hp0868 protein was purified by SP-Sepharose cation exchange followed by a high-resolution chromatography. NaCl-HPO4 (50 mM, pH 7.5) was used as the start buffer, and 50 mM NaCl-HPO4 (pH 7.5) plus 1 M NaCl was used as the elution buffer for cation-exchange chromatography. Gel filtration chromatography was performed in the presence of 20 mM NaCl-HPO4 (pH 7.5) plus 25 mM NaCl buffer.
washed five times with the same buffer. Bound antibody was detected by the addition of the chromogenic reagents nitroblue tetrazolium (0.25 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.125 mg/ml) (Sigma). A 1:1 molar mixture of the wild-type HypA and Hp0868 proteins was also tested similarly.

Nickel-binding assay. The ability of the purified HypA and HypB proteins to bind nickel was determined by equilibrium dialysis followed by electrophoretic mobility shift assay (Shimadzu). Briefly, 6 to 7 μM protein was dialyzed versus increasing concentrations of NiCl₂ (for concentrations, see Fig. 3) taken in 1 liter of 50 mM NaCl (pH 8.25) for 48 h. Following dialysis, the nickel concentrations of the protein solution (bound plus free Ni²⁺) and the dialysis buffer (free Ni²⁺) were determined. The bound nickel was estimated by subtracting the two values. The nickel-binding abilities of the HypA mutant proteins (H2A, H17A, H24A, H79A, and H95A) were determined similarly. A 1:1 molar mixture of the wild-type HypA and HypB proteins was also assayed for nickel binding.

NMR spectroscopy for HypA (wild-type and H2A mutant proteins). Nuclear magnetic resonance (NMR) analysis was performed at the Chemical Sciences Magnetic Resonance Facility (University of Georgia). The NMR data were collected on a Varian Inova 500 spectrometer (499.8 MHz, 1H). Water signal suppression was achieved by using flip-back pulses (12) and pulsed-field gradients (31). The data were processed by using vnmr (Varian, Inc.) without solvent subtraction.

GTase assay. The ability of the purified HypA and HypB proteins to hydrolyze ATP was measured by quantitatively using a previously described protocol (11). A 1:1 molar ratio of HypA to HypB was also tested similarly. The ability of HypB to hydrolyze ATP or dGTP was determined by a method similar to that described for GTP. The GTase activity was also measured for the HypB mutant protein (K59A).

Construction of H. pylori chromosomal site-directed mutant of hypA. The H. pylori chromosomal site-directed mutant of hypA was constructed by using a previously described protocol (8). Briefly, a kan-sacB-flaC fragment (KSF) was excised from pKSF-II (kindly provided by M. Copass) and inserted into an SpH site within the hypA gene previously cloned into pBluescript KS+ (pphyt4) (Table 1). The KSF fragment has the upkaA kanamycin resistance cassette from Campylobacter coli (38), a promoterless sacB gene from Bacillus subtilis (35), and the flaC primer from H. pylori (17). The resulting construct (pphyt5-KSF) was transformed into the H. pylori ATCC 43504 wild-type strain by natural transformation. The hypA gene was interrupted by the insertion of the KSF fragment due to allelic exchange, giving rise to the hypA::KSF mutant strain. This strain was kanamycin resistant and sucrose sensitive. The desired mutation was confirmed by PCR amplification of chromosomal DNA from the hypA::KSF strain with the primers HypAF and HypAR (Table 2) followed by agarose gel electrophoresis (data not shown). This mutant was used as the parent strain for the transformation of the pphyt4-H2A construct.

The pphyt4-H2A construct (with a site-directed mutation in the codon for His2) was prepared by using the QuiKChange site-directed mutagenesis protocol (Stratagene) and pphyt4 as the template. Primers (HypA:H2AF1 and HypA: H2AR1) were specifically designed to introduce the desired mutation (Table 2). The transformation of the H. pylori mutant strain hypA::KSF with the construct pphyt4-H2A was done by natural transformation. A site-directed mutant of hypA (hypA::H2A) was obtained as a result of allelic exchange. This mutant was sucrose resistant and kanamycin sensitive. The mutation was confirmed by PCR amplification of the chromosomal DNA with primers HypAF and HypAR (Table 2), followed by sequencing of the product at the MGIF (University of Georgia).

Hydrogenase and urease activities were assayed for the wild-type and mutant strains.

Insertional mutagenesis of the Hp0868 gene. Mutagenesis of the Hp0868 gene was done by using a previously described protocol (29). Primers 886 F and 886 R were used for PCR amplification. The construct p886kan (Table 1) was electroporated into the H. pylori ATCC 43504 wild-type strain. Allelic exchange resulted in the insertion of the kanamycin resistance cassette (upkaA) into the Hp0868 gene. The mutation was confirmed by PCR amplification of chromosomal DNA with primers 886 F and 886 R followed by agarose gel electrophoresis to confirm the increased size of the interrupted gene (data not shown). Hydrogenase and urease activities were assayed for the wild-type and mutant strains.

Hydrogenase assay. Hydrogen uptake activity was determined amperometrically for whole cells with O₂ as the final electron acceptor. The protocol followed was described previously (19).

Urease assay. Cells were harvested after 72 h of growth, washed twice with 50 mM HEPES buffer (pH 7.5), and broken by two passages through a French pressure cell at 12,000 lb/in². The lysate was centrifuged at 28,000 × g to remove the cell debris, and the supernatant was assayed for urease activity by using the phenol-hypochlorite method (24, 40).

RESULTS AND DISCUSSION

Purification of HypA, HypB, and Hp0868 proteins. Earlier observations that the HypA and HypB proteins were involved in urease maturation prompted us to purify and characterize these two proteins. The HypA and HypB proteins (wild-type and mutant versions) were overexpressed as soluble proteins and purified to near homogeneity. The yield for all of these proteins was between 25 to 30 mg/liter. On SDS-PAGE gels, wild-type HypA and HypB migrated at approximately 13 and 30 kDa, respectively (Fig. 1A). Mutant HypA and HypB proteins also migrated at 13 and 30 kDa, respectively (Fig. 1A). The molecular mass of HypA seen here almost matched its predicted mass of 13.2 kDa. However, HypB ran as a slightly larger polypeptide since its predicted mass is 27.5 kDa.

A yeast two-hybrid study had predicted a strong interaction of HypA with another protein, Hp0868 (encoded by a gene downstream of hypA) (32). To address any interaction, the Hp0868 protein was overexpressed and purified to near homogeneity. The yield was approximately 20 mg/liter. The Hp0868 protein migrated at approximately 21 kDa on SDS-PAGE gels (the predicted mass is 18.5 kDa) (Fig. 1B).

N-terminal sequence and molecular mass determination. The N-terminal sequences of wild-type HypA, HypB, and Hp0868 proteins matched exactly with that of the deduced amino acid sequence as seen in The Institute for Genomic Research database for H. pylori. The amino-terminal methionine was present in all three proteins. The molecular masses (± standard deviations) for HypA, HypB, and Hp0868 proteins as determined by LC-mass spectrometry were 13.2 ± 0.003, 27.3 ± 0.03, and 18.7 ± 0.03 kDa, respectively.

Size exclusion chromatography. The native molecular masses of HypA and HypB as determined by gel filtration chromatography were around 25.8 and 52.4 kDa, which is consistent with them existing as dimers in solution. Interestingly, a 1:1 molar mixture of HypA and HypB gave rise to a species with a molecular mass of 43.6 kDa. Immunoblot analysis of this species with anti-HypA and anti-HypB antibodies from H. pylori showed the presence of both the proteins (data not shown). This indicates the existence of an intermolecular interaction between the two proteins in native solution. This conclusion is further supported by cross-linking studies.

Cross-linking assays. Since a mutation in either hypA or hypB resulted in a phenotype in which both hydrogenase and urease activities were markedly reduced, we proposed that these two proteins might be acting in a coordinated fashion for the activation of both of these enzymes (29). To investigate this possibility, cross-linking studies were carried out in the presence of a 5 mM concentration of the homobifunctional cross-linker DMS. Mixing equimolar concentrations of wild-type HypA and HypB resulted in the formation of a unique 43-kDa heterodimeric complex, which was not seen when either of these two proteins was tested individually (Fig. 2A). This size corresponds to a 1:1 molar ratio of HypA to HypB. This observation is in agreement with the size exclusion results. It therefore appears that HypA and HypB may be forming a biological complex aiding in the nickel enzyme maturation
process. The wild-type HypA-plus-HypB mixture also showed the formation of both a monomer and a dimer in the case of HypA and a monomer, a dimer, and perhaps an additional larger oligomer in the case of HypB. These latter results matched with that which was observed when the two proteins were tested individually; HypA showed the formation of a monomer (13 kDa) and a dimer (30 kDa), and HypB showed the formation of a monomer (30 kDa), a dimer (55 kDa), and a larger species (83 kDa) (Fig. 2A). Although HypA and HypB are individually capable of forming dimers, together they did not give rise to a heterotetrameric complex, which might indicate that the strength of the interaction between the HypA and HypB monomers supercedes that between the dimers. The addition of equimolar concentrations of BSA in the reaction mixture did not perturb the interactions (data not shown).

Cross-linking reactions involving wild-type HypA, HypB, and DMS in the presence of 0.5 mM GTP or 1 mM nickel also gave rise to this 43-kDa heterodimeric complex, with no additional complexes detected (Fig. 2B). A mixture of the HypA mutant (H2A) and HypB mutant (K59A) proteins formed a similar complex (Fig. 2C). So did a mixture of the HypA mutant protein (H2A), the wild-type HypB protein, and 1 mM nickel or a mixture of HypB mutant protein (K59A), wild-type HypA protein, and 0.5 mM GTP (Fig. 2C). No new complexes were seen in any of these reactions. These results indicate that the contact point for forming the HypA-HypB heterodimer does not require the nickel-binding residue in HypA (His2) nor the conserved lysine residue (Lys59) within the GTP-binding motif of HypB.

Since a strong interaction was predicted between HypA and its downstream gene (Hp0868) in yeast two-hybrid studies (32), a cross-linking reaction was attempted between Hp0868 and HypA proteins in the presence of 5 mM DMS. HypA did not show any cross-linked products with the Hp0868 protein (data not shown).

Nickel-binding assay. Earlier studies had shown that nickel supplementation in the growth media restored the urease activity of the hypA and hypB mutants, and partially purified urease from hypA and hypB mutants had four- and fivefold-lower nickel contents, respectively, than that from the wild type (29). Here we determined the number of Ni2+ ions bound to wild-type HypA and HypB proteins over a range of nickel concentrations. Equilibrium dialysis of HypB showed that it is incapable of binding nickel (Fig. 3A). This is in contrast to the documented nickel-binding ability of HypB from B. japonicum and Rhizobium leguminosarum, which bound 9 and 3.9 Ni2+ ions per monomer, respectively (11, 33). Equilibrium dialysis of HypA showed its ability to bind up to 2 Ni2+ ions per dimer (Fig. 3A). Another plot of the data (Fig. 3B) showed positive cooperativity in nickel binding, and a Hill coefficient of approximately 2.0 was calculated (2). The dissociation constants $K_1$ and $K_2$ (± standard deviations) for nickel were 58 ± 6 and 1.3 ± 0.2 μM, respectively, with half saturation at around a 15 μM Ni2+ ion concentration (2). In the presence of equimolar concentrations of HypB, the nickel-binding ability of HypA remained unaltered (data not shown). The ability of HypA to bind Ni2+ ions is a novel observation, although it may not be the only protein performing this function in H. pylori. Yet due to the presence of two Ni-dependent enzymes, urease and hydrogenase, this observation seems significant, especially in light of the inability of a HypA site-specific mutant protein to bind nickel (see below). HypA from E. coli has been overexpressed and purified, but its nickel-binding ability has not been addressed (18).

The Institute for Genomic Research database showed the presence of five histidine residues distributed within the HypA protein. Since the wild-type HypA protein showed the ability to bind Ni2+ ions, we carried out site-directed mutagenesis of each of the five histidine residues within hypA (His2, -17, -24, -79, and -95). The sequence of the recombinant pET plasmids showed the presence of an intact initiation codon and also a site-directed mutation at the targeted location (codon for His...
to Ala) within the open reading frame of hypA. The nickel-binding ability of each of the overexpressed and purified mutant proteins was investigated at different NiCl₂ concentrations. Four of the mutant proteins (H17A, H24A, H79A, and H95A) could bind around 2 Ni²⁺ ions per dimer, similar to the wild-type protein, with half saturation at a 15 μM Ni²⁺ ion concentration (data not shown). However, the H2A mutant protein lacked the ability to bind nickel. Sequence comparison studies showed that this histidine residue (His2) is well conserved among other HypA proteins as well. The Ni²⁺ ions bound by each of the HypA mutant proteins after equilibrium dialysis against 60 μM Ni²⁺ (which falls in the saturation range) are shown in Fig. 4. From these results we can conclude that among the five histidine residues, only the His2 is critical for nickel binding by HypA.

NMR spectroscopy for HypA (wild-type and H2A mutant proteins). Since we were concluding that His2 is critical for nickel binding based on characteristics of the H2A mutant protein, both the wild-type HypA and H2A mutant proteins were compared for their NMR spectra. The one-dimensional NMR spectra for the two proteins in the fingerprint NH region (6 to 10 ppm) were similar to each other, indicating that there is no significant difference between the two structures (Fig. 5). The spectra also indicated that the two proteins were in the folded state (Q. Teng, Chemical Sciences Magnetic Resonance Facility, University of Georgia, personal communication). The inability to bind nickel by the H2A mutant protein can therefore be attributed to the replacement of the His2 with alanine and not due to any structural changes.

FIG. 2. (A) Western blot analysis of cross-linked products arising from a mixture of wild-type HypA and HypB proteins. Reaction mixtures included HypB plus DMS (lane 1), HypA plus HypB plus DMS (lane 2), HypA plus HypB plus DMS (lane 4), and HypA plus DMS (lane 5). The concentration of each protein in the reaction mixture was 26 μM. A 43-kDa heterodimeric complex was seen only when the reaction mixture contained both proteins (lane 2 and 4). HypA and HypB tested individually and as a mixture gave rise to monomers and dimers. (B) Western blot analysis of cross-linked products arising from a mixture of wild-type HypA and HypB proteins in the presence and absence of 1 μM NiCl₂ or 0.5 mM GTP. Reaction mixtures included HypA plus HypB plus 0.5 mM GTP plus DMS (lane 1), HypA plus HypB plus DMS (lane 2), HypA plus HypB plus 1 μM NiCl₂ plus DMS (lane 3), HypA plus HypB plus 1 μM NiCl₂ plus DMS (lane 4), HypA plus HypB plus DMS (lane 6), and HypA plus HypB plus 0.5 mM GTP plus DMS (lane 7). The concentration of each protein in the reaction mixture was 31 μM. The 43-kDa heterodimeric complex was seen in all of the lanes. (C) Western blot analysis of cross-linked products arising from a mixture of wild-type (WT) and mutant HypA (H2A) and HypB (K59A) proteins. Reaction mixtures included K59A plus HypA (WT) plus DMS (lane 1), K59A plus HypA (WT) plus 0.5 mM GTP plus DMS (lane 2), K59A plus HypA (WT) plus 0.5 mM GTP plus DMS (lane 3), K59A plus HypA (WT) plus 0.5 mM GTP plus DMS (lane 4), K59A plus H2A plus DMS (lane 6), H2A plus DMS (lane 7), H2A plus HypB (WT) plus 1 μM NiCl₂ plus DMS (lane 8), H2A plus HypB (WT) plus DMS (lane 9). The concentration of each protein in the reaction mixture was 29 μM. The 43-kDa heterodimeric complex was seen in all lanes except those in which the mutant proteins were tested individually (lanes 3 and 7). Panel I was probed with anti-HypB antibody, and panel II was probed with anti-HypA antibody. A 1:1 molar mixture of proteins was used for these studies. Prestained protein markers (lane 3 in panel A, lane 4 in panel B, and lane 5 in panel C) were composed of phosphorylase B (101 kDa), BSA (70 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.7 kDa), soybean trypsin inhibitor (28.4 kDa), and lysozyme (20.8 kDa).
GTPase assay. A large number of GTP-binding proteins show the presence of a well-conserved GTP-binding motif which has been speculated to bind and hydrolyze GTP (5). It has therefore been hypothesized that these proteins may be involved in a switch mechanism, whereby some intracellular signal or reaction may be turned on in the GTP-bound form and off following GTP hydrolysis. The deduced amino acid sequence of HypB from *H. pylori* also shows the presence of a GTP-binding motif. Therefore, purified HypB was assayed for its GTPase activity. *H. pylori* HypB showed a GTPase activity of 5 nmol of GTP hydrolyzed per nmol of HypB per min, and the rate of hydrolysis was linear over 140 min (Fig. 6). Since *H. pylori* HypB did not possess any nickel-binding ability and the hypB mutant was still deficient in urease activity (29), it is reasonable to speculate that its GTP-hydrolyzing activity may be initiating some steps leading to the insertion of nickel into the urease apoenzyme. Since a lysine residue (Lys59) in the GTP-binding motif of HypB is also well conserved in other HypB proteins, a site-directed mutation was introduced that replaced this residue with alanine. Unlike the wild-type HypB protein, however, the HypB K59A mutant protein showed negligible GTPase activity (0.1 nmol of GTP hydrolyzed per nmol of HypB per min) (Fig. 6). This result is not unexpected because it has been shown that substitution of the conserved lysine residue in the GTP-binding motif of HypB in both *B. japonicum* and *E. coli* also greatly lowered its GTPase activity (21, 28). HypB showed slight dGTPase and negligible ATPase activities (data not shown). Purified HypA showed negligible GTPase activity, and the GTPase activity of HypB remained unaltered in the presence of equimolar concentrations of HypA (data not shown).

Hydrogenase and urease activities of the chromosomal site-directed mutant of *hypA*. Since the H2A mutant protein of HypA showed an inability to bind nickel, a site-directed mutation was introduced within the *hypA* gene so as to substitute the codon for His2 with that for alanine. The *H. pylori* wild-type and mutant strains were assayed for hydrogenase and urease activities. The mutant completely lacked hydrogenase activity (0.01 nmol of H2 oxidized per min per 10^8 cells) compared to the wild type (0.4 ± 0.2 nmol of H2 oxidized per min per 10^8 cells). The urease activity of the *hypA*-H2A mutant strain was around 2% (0.05 ± 0.05 mol of NH3 evolved per min per mg of protein) of that of the wild type (39 ± 8.1 mol of NH3 evolved per min per mg of protein). These results show the significant role played by His2 of the HypA protein in urease and hydrogenase activities and indicate that specific Ni

FIG. 3. (A) Ni^{2+}-binding ability of HypA and HypB (wild-type proteins). Nickel-binding ability was determined by equilibrium dialysis with 6 to 7 μM HypA (•) or HypB (○) against different concentrations (Conc.) of NiCl2, as indicated on the x axis, followed by atomic absorption spectrophotometry. HypA bound 2 Ni^{2+} ions per dimer, and HypB lacked Ni^{2+}-binding ability. (B) Sigmoidal curve for Ni^{2+} binding by HypA (wild type). A sigmoidal ligand binding curve was obtained with the same set of data as shown in panel A for HypA when a graph of percent fractional saturation (Y) was plotted against the log of Ni^{2+} concentration (Log [L]). This plot indicated the presence of positive cooperativity for Ni^{2+}-binding by HypA. A SigmaPlot, version 8.0, graphical program was used to fit this curve, with the equation Y = minimum + (maximum - minimum)/1 + 10^{logEO_{50} -6Hillslope}, where EO_{50} is the effective concentration of Ni^{2+} at 50% saturation and Hillslope is the slope factor which describes the steepness of the curve.

FIG. 4. Ni^{2+}-binding ability of HypA (wild-type and mutant proteins). The nickel-binding ability was determined for wild-type and mutant HypA proteins after equilibrium dialysis against a 60 μM NiCl2 concentration. Proteins indicated on the x axis are the HypA wild type (WT) and H2A, H17A, H24A, H79A, and H95A mutant proteins. Except for the H2A mutant protein, all other proteins are capable of binding approximately 2 Ni^{2+} ions per dimer.
The activities were similar for the wild-type and mutant strains, it indicates that the presence of the Hp0868 gene is not critical for the hydrogenase and urease activities. This is in contrast to the results seen in the case of the hypA mutant, which was deficient for both activities (29). These results are in agreement with the cross-linking results that also do not support an interaction between HypA and the Hp0868 protein.

Our results constitute the first demonstration of the nickel-binding ability of HypA and the significant role played by His2 of HypA for nickel binding and also for the activation of hydrogenase and urease. Another significant observation was that of the ability of the HypA and HypB proteins to interact and form a heterodimeric complex in solution as well as in the presence of DMS. The size of this complex corresponds to a 1:1 molar ratio of HypA to HypB. Although individually both proteins formed dimers, a heterotetrameric complex was not seen, indicating that the strength of the interaction between the monomers supercedes that between the dimers. HypB lacked the nickel-binding ability but was capable of hydrolyzing GTP. The lysine residue (Lys59) in the well-conserved GTP-binding motif of HypB was shown to be vital for the GTP-hydrolyzing activity of HypB. These results are compatible with the role of nickel sequestration by HypA, and the initiation of steps leading to nickel incorporation via the GTP-hydrolyzing ability of HypB. The histidine located in the extreme N terminus of HypA (His2) and the lysine in the GTP-binding motif of HypB (Lys59) play vital roles in nickel binding and GTP hydrolysis, respectively.

REFERENCES


