HybF, a Zinc-Containing Protein Involved in NiFe Hydrogenase Maturation

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Escherichia coli possesses four distinct hydrogenases, designated hydrogenases 1, 2, 3, and 4 (1, 5, 6, 47, 48; for a general classification of hydrogenases see reference 53). Isoenzymes 1 and 2 cleave elemental hydrogen into protons and electrons, which are used for energy conservation by feeding into an anaerobic respiratory chain. Hydrogenase 3, on the other hand, is a component of the classical formate-hydrogen lyase complex and reduces protons to elemental hydrogen with the electrons derived from formate (11, 42, 46). Hydrogenase 4 is part of a separate formate-hydrogen lyase system and appears to be a proton-translocating enzyme, thereby contributing to the electrochemical gradient across the cytoplasmic membrane (1); however, its exact physiological role is still unknown (3, 4, 49). The genes coding for structural components and redox partners are located in four operons, hya (hydrogenase 1), hyb (hydrogenase 2), hyc (hydrogenase 3), and hyf (hydrogenase 4) (1, 12, 35, 36). Except for the exceptions discussed below, lesions within these operons lead to loss of activity of the specific isoenzymes, leaving the activities of the other hydrogenases unaffected.

In addition to the products of the genes in the structural gene operons, hydrogenase activity requires the products of genes with functions in enzyme maturation. All four hydrogenases belong to the class of hydrogenases possessing an NiFe metal center coordinated by the thiolates of four cysteine residues from the protein backbone of the large hydrogenase subunit (54). Two of the cysteines function as ligands bridging the Fe and Ni atoms (54, 55; for a review see reference 20). In the classical type of NiFe hydrogenases the Fe carries two cyanide groups and one carbon monoxide group (2, 23). In E. coli, the products of seven genes were determined to have functions in the synthesis of this metal center (25, 28). Four of these products have been demonstrated (HyfF and HyfE) or supposed (HyfC and HyfD) to be involved in CN synthesis, in the attachment of the ligands to the iron (9, 41, 43; unpublished data), and in the putative transfer to the apoprotein of the large hydrogenase subunit (9).

The incorporation of nickel into the large subunit is independent of the incorporation of iron and seems to succeed the incorporation of iron. Evidence shows that a precursor of the large subunit synthesized under nickel deprivation conditions can be converted in vitro into the active enzyme by incubation with the metal (30, 34). Mutational studies revealed that insertion of nickel requires the activity of two additional maturation proteins, HybB and HybA. Inactivation of the genes encoding these proteins blocks the formation of active hydrogenases; however, this deficiency can be partially rescued by inclusion of high nickel concentrations in the growth medium (24, 40, 58). It appears, therefore, that nickel incorporation can occur spontaneously, but for functioning at low metal concentrations it is dependent on the activity of the two proteins (25). Protein HybB has been purified from E. coli (31), Rhizobium leguminosarum (45), Bradyrhizobium japonicum (21), and Helicobacter pylori (33). HybB is a guanine nucleotide binding protein, and for functioning in nickel insertion it must possess GTPase activity (32). Intriguingly, there are homologous pro-
metal-free apoprotein requires recognition of the correct target. It is plausible to assume that specific donation of nickel to the metal-free apoprotein requires recognition of the correct target by a protein-protein interaction. The fact that the sequences of the large subunits of isoenzymes 1 and 2 are very similar to each other but very different from that of hydrogenase 3 (53) may have required the evolution of two separate nickel-delivering proteins. HypB from E. coli, on the other hand, is involved in the maturation of all hydrogenase isoenzymes, which reflects a more general role in the maturation process (25). To gain more insight into the function of HybF and thereby also the function of its homolog HypA, we purified and characterized HybF from E. coli.

MATERIALS AND METHODS

Bacterial strains and plasmids. The E. coli strains and plasmids used in this study are listed in Table 1. Strains DPAPB and NIM were obtained by using the gene disruption method of Hamilton et al. (22) with the aid of the pMAK700 derivative pDB705 and pMDHBF. E. coli DH5α was used as the host for cloning procedures.

Media and growth conditions. E. coli cells were grown anaerobically at 37°C in buffered TGYEP medium (7) supplemented either with 15 mM formate plus 1 mM sodium molybdate, 1 mM sodium selenite, and 5 µM nickel chloride or with 15 mM fumarate plus 0.8% glucose or with 15 mM fumarate plus 0.8% glyceral, as described previously (10); 1 µM sodium molybdate, 1 µM sodium selenite, and 5 µM nickel chloride were used as supplements unless indicated otherwise. The cells were harvested at an optical density at 600 nm of 1.0, and crude extracts were prepared as described previously (17). The antibiotics chloramphenicol and ampicillin were added to the medium at concentrations of 30 and 50 µg per ml, respectively, when they were needed for maintenance of plasmids.

Site-directed mutagenesis and cloning procedures. To replace amino acid residues of HybF-Strep, site-directed mutagenesis was performed with plasmid

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>DH5α</td>
<td>F′ (ΔlacZΔM15) recA1 endA1 gyrA96 thi-1 hsdR17 (rK mK) supE44 relA1 deoR Δ(lac-proAB)U169</td>
<td>59</td>
</tr>
<tr>
<td>MC4100</td>
<td>F′ araD139 Δ(lacF1599)U169 pEC25 deoC1 relA1 (hiB5301 rpsL150)</td>
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</tr>
<tr>
<td>BL21(DE3)</td>
<td>F′ ompT hsdS (rK mK) gal dcm (DE3)</td>
<td>50</td>
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<tr>
<td>SLM101</td>
<td>MC4100, ATG codon of hypA mutated into TAA</td>
<td>25</td>
</tr>
<tr>
<td>DHP-B</td>
<td>MC4100 ΔhypB</td>
<td>25</td>
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<tr>
<td>DPABF</td>
<td>SMP101 ΔhypF</td>
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<tr>
<td>DPBF</td>
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<tr>
<td>NIM</td>
<td>DPAFB ΔhypF</td>
<td>This study</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap′ Te′</td>
<td>13</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm′ Te′</td>
<td>15</td>
</tr>
<tr>
<td>pDB705</td>
<td>pMAK700, ΔhypB (0.8-kb RsaI-AvaII fragment deleted), Cm′</td>
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<tr>
<td>pMDHBFP</td>
<td>pMAK700 with 252-bp deletion in hybF, Cm′</td>
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</tr>
<tr>
<td>pBHBF-Strep</td>
<td>pBR322 hybF-Strep, Ap′</td>
<td>24</td>
</tr>
<tr>
<td>pBHBF-Strep[H2A]</td>
<td>pBR322 hybF [H2A]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[H2O]</td>
<td>pBR322 hybF [H2O]-Strep, Ap′</td>
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</tr>
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<td>pBR322 hybF [E3O]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[E3L]</td>
<td>pBR322 hybF [E3L]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[C73A]</td>
<td>pBR322 hybF [C73A]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[C76A]</td>
<td>pBR322 hybF [C76A]-Strep, Ap′</td>
<td>This study</td>
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<td>pBHBF-Strep[C73/76A]</td>
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<td>This study</td>
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<td>pBHBF-Strep[C89A]</td>
<td>pBR322 hybF [C89A]-Strep, Ap′</td>
<td>This study</td>
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<tr>
<td>pBHBF-Strep[C89S]</td>
<td>pBR322 hybF [C89S]-Strep, Ap′</td>
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<td>pBHBF-Strep[P90A]</td>
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<td>pBR322 hybF [C92A]-Strep, Ap′</td>
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<td>pBR322 hybF [C92E]-Strep, Ap′</td>
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<tr>
<td>pBHBF-Strep[C92Q]</td>
<td>pBR322 hybF [C92Q]-Strep, Ap′</td>
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<td>pBHBF-Strep[C92H]</td>
<td>pBR322 hybF [C92H]-Strep, Ap′</td>
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<td>pBHBF-Strep[C92N]</td>
<td>pBR322 hybF [C92N]-Strep, Ap′</td>
<td>This study</td>
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<tr>
<td>pBHBF-Strep[C92E]</td>
<td>pBR322 hybF [C92E]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[C92Q]</td>
<td>pBR322 hybF [C92Q]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[C92H]</td>
<td>pBR322 hybF [C92H]-Strep, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pBHBF-Strep[C92N]</td>
<td>pBR322 hybF [C92N]-Strep, Ap′</td>
<td>This study</td>
</tr>
</tbody>
</table>

The HypA protein has been purified from H. pylori (33). This protein binds nickel stoichiometrically and also appears to interact with the HypB protein, as indicated by detection of heterodimers when stoichiometric mixtures of the two proteins were cross-linked chemically (33). From the results obtained thus far, two models can be proposed for the function of HypA and HypB. Either HypB is the nickel-donating protein and HypA guides HypB to the apoprotein, or HypA is the nickel-binding partner and HypB has a solely regulatory function in facilitating the switch required to release HypA after the nickel is donated. Circumstantial evidence that favors the latter model comes from the fact that purified HypB protein from H. pylori does not bind nickel (33) and the fact that HypA from E. coli functions only in the maturation pathway of hydrogenase 3 and there is a homolog of this protein, HybF, that has a role in the maturation of hydrogenases 1 and 2 instead (24).
pBBH-Strep by using inverse PCR as described previously (29, 38). To do this, the appropriate primers listed in Table 2 were used. The authenticity of the hybFStrep variant genes was verified by DNA sequencing. Plasmid pThybFStrep and its derivative pThybFStrep [H2O] were constructed by ligation of the EcoRI- and NdeI-digested vector pT7-7 with the EcoRI- and NdeI-digested PCR fragment carrying the hybFStrep or hybFStrep[1H2O] gene. The hybFStrep and hybFStrep[2H2O] genes were obtained with primers pNdeIhybFup and tet-inv.+=EcoRI and with primers pNdeIhybF2H2Qup and tet-inv.+=EcoRI, respectively; pTHbF-Strep or its [H2O] derivative was used as the template.

**TABLE 2. Oligonucleotide primers used in this work**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>His2Ala-hybF</td>
<td>5′-CCCGAGCTCTCTTTTGGCCAGAGGCCT-3′</td>
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<tr>
<td>His4CA-hybF</td>
<td>5′-CAAGAGGCCGCTCCCTTGAGGCCG-3′</td>
</tr>
<tr>
<td>hybF3EQ-MunI</td>
<td>5′-GGAGTGGGAAATGCTCATTGTCCTTGGCCAGAC-3′</td>
</tr>
<tr>
<td>hybF3EQL-A/II</td>
<td>5′-GATTGTCGAAATTGCTCATTGTAAGGCTTGGCCAGAC-3′</td>
</tr>
<tr>
<td>hybFH2E3-inv.</td>
<td>5′-CATTTTCCGGCACTCTTGCCGACCAAAACAGC-3′</td>
</tr>
<tr>
<td>Cys89Ala-hybF</td>
<td>5′-CGGAGCTGCTGGCATCTGCTGTGATGAAATCTCC-3′</td>
</tr>
<tr>
<td>Cys89Ser-hybF</td>
<td>5′-CGGGCTGCTGGCATCTGCTGTGATGAAATCTCC-3′</td>
</tr>
<tr>
<td>Pro98CA-hybF</td>
<td>5′-CAGACACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>hybFC89P90-inv.</td>
<td>5′-CTTCGGCAAGGGTTCGTTGATGAGCGACATGGCTTTCC-3′</td>
</tr>
<tr>
<td>HybFC73A</td>
<td>5′-CCCAGGGCCGTTTGGGATTTGACGACATGGCTTTCC-3′</td>
</tr>
<tr>
<td>HybFC76A</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>HybFC73/76A</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>HybFC73/76 inv.</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>HybFC92A</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>HybFC90/92 inv.</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>tet-inv.+=EcoRI</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>NdeIhybFup</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
<tr>
<td>NdeIhybF2H2Qup</td>
<td>5′-CAGGCACTGCGATCGTGAATCTCC-3′</td>
</tr>
</tbody>
</table>

* Bases that are different from the bases in the wild-type sequence are underlined; newly generated restriction sites are indicated by boldface type.

**RESULTS**

Hydrogenase activity staining in gels. To localize active hydrogenases 1 and 2 in polyacrylamide gels, cells were grown in TGYEP medium in the presence of 0.8% glycerol and 15 mM fumarate. The proteins in the crude extracts were separated by SDS-PAGE under native conditions, which meant that the loading buffer lacked DTT and 2-mercaptoethanol and that heat treatment was omitted. After separation, the gels were soaked in 100 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM benzyl viologen and 1 mM triphenyltetrazolium chloride and incubated either under an H2 atmosphere at 37°C or in an anaerobic chamber (5% H2, 95% N2) at room temperature. The activity-stained bands were determined to be hydrogenases 1 and 2 by using extracts of E. coli strains HDK103 and HDK203 (25) (data not shown).

**In vitro maturation assay.** Crude extracts of the strains were mixed with the appropriate amounts of NiCl2 and incubated for 2 h at 30°C. The newly generated hydrogenase 1 and 2 activities were evaluated by activity staining in polyacrylamide gels and Western blot analysis with antibodies raised against the large subunit of hydrogenase 2 (HybC).

**Nickel binding assay.** Filter binding assays were used to investigate the binding of nickel by HybF-Strep or its [H2O] variant. To do this, 65NiCl2 (specific activity, 697 mCi/mmol; NEN Life Science Products, Inc., Boston, Mass.) was diluted with unlabeled NiCl2 to obtain a 0.5 mM stock solution with a specific activity of 121.6 μCi/mmol. The proteins were incubated with different nickel concentrations for 30 min at room temperature, and this was followed by filtration through nitrocellulose filters (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) and extensive washing. The radioactivity was determined by scintillation counting with a Tri-Carb 2100TR liquid scintillation analyzer (Canberra Packard GmbH, Dreieich, Germany).

**Cross-linking experiments.** To investigate a possible interaction between HybF and HypB, cross-linking studies were performed. This was done as described by Merta et al. (33) by using dimethyl suberimidate, a homobifunctional imidoester cross-linker specific for primary amines (57). Alternatively, the sulfhydryl-specific cross-linking reagent p-phenylenediamine was used (56).

**Purification and properties of HybF-Strep.** The initial aim of this work was to purify the HypA protein from E. coli. However, despite extensive variation of the expression conditions, our efforts failed since the gene product was present exclusively in an insoluble form. As an alternative, we attempted to express hybF, which codes for the homolog of HypA involved in maturation of hydrogenases 1 and 2 (24). For this we used a variant which contains StreptagII as a C-terminal extension and has been shown previously to be fully active in hydrogenase maturation in vivo (24). As shown in Fig.
1A, most of the HybF-Strep formed was probably insoluble, even when the protein was expressed at a low temperature. The fraction present in the 100,000 × g supernatant, however, was still sufficient to recover a suitable amount of highly purified protein by affinity chromatography on a Streptactin column, followed by gel filtration in a Superdex 75 column and concentration by ultrafiltration. From 28 g (wet weight) of cells 1 mg of HybF-Strep was obtained. The purified protein was amino terminally sequenced and was found to possess an N-terminal methionine residue. In contrast to the HypA protein from *H. pylori* (33), HybF-Strep from *E. coli* migrated during gel filtration as an apparent monomer (Fig. 1B). Determination of the metal content of the purified protein (Table 3) showed that it contains an approximately stoichiometric amount of zinc, a very small amount of nickel, and an undetectable amount of iron. Taking into account that the medium in which expression occurred was not supplemented with zinc but was supplemented with 5 μM nickel chloride and that iron, which usually is present as a contaminant together with zinc, was not found, we concluded that HybF is a zinc-containing protein.

**Properties of HybF variants with replacements of amino acids putatively involved in metal coordination.** In the search for amino acid residues which are involved in the coordination of nickel, all of the histidine residues of the HypA protein from *H. pylori* have been replaced by alanine (33). Of the five variant proteins generated and purified, only the H2A form was unable to bind nickel, and the corresponding mutant strain lacked hydrogenase activity (33). Since a single histidine residue is not sufficient to coordinate nickel and in light of the unexpected finding that zinc is a cofactor of the HybF protein, a more extensive search for residues of the protein involved in coordination of metals was conducted. Figure 2 shows the residues of HybF which are conserved in the HypA-HybF protein family.

![FIG. 1. Purification of HybF-Strep. (A) Proteins from the different purification steps were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, lysate from cells before induction with IPTG; lane 2, lysate from cells after induction with IPTG; lane 3, extract after ultracentrifugation; lane 4, eluate from the Streptactin column; lane 5, concentrated HybF-Strep protein after gel filtration on a Superdex 75 column. (B) Migration behavior of HybF-Strep in the gel filtration experiment. The marker proteins (with sizes in kilodaltons) were ovalbumin (triangle 1), carbonic anhydrase (triangle 2), chymotrypsinogen (triangle 3), lysozyme (triangle 4), and cytochrome c (triangle 5). The position of the HybF-Strep protein is indicated by the arrow.](http://jb.asm.org/)

![TABLE 3. Metal contents of purified HybF-Strep and its [H2Q] variant](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Metal contenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
</tr>
<tr>
<td>HybF-Strep</td>
<td>0.0</td>
</tr>
<tr>
<td>HybF-Strep [H2Q]</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

a The values are numbers of metal ions per monomer and are means ± standard deviations.

![FIG. 2. Schematic representation of the HybF protein: primary structure of the HybF protein showing the conserved N terminus and the two cysteine motifs. The amino acids that were replaced by site-specific mutagenesis are indicated.](http://jb.asm.org/)
and might fulfill such a function. By using inverse PCR the codons for these residues were exchanged in order to insert the amino acids indicated in Fig. 2. The corresponding plasmids were used to transform strain DPABF, which does not express hypA and has a deletion in hybF. The transforms were grown to an optical density at 600 nm of 1, extracts were prepared and separated electrophoretically, and the gels were grown to an optical density at 600 nm of 1, extracts were prepared and separated electrophoretically, and the gels were developed with antisera directed against StreptagII (Fig. 3).

HybC was fully processed in the extract from the transformant carrying the wild-type hybF (lane 1), was not processed in the host strain lacking HypA and HypF (lane 2), and was processed in the transformants carrying the wild-type hybF gene and hybF with the replacements E3Q, C89A, C89S, and P90A (lanes 3, 6, 8, 9, and 10). No processing was observed with the variants of HybF having the replacements H2A, H2Q, and E3L (lanes 4, 5, and 7). The results suggest that C89 is not essential since its replacement is not detrimental to activity, although the gene product is present at a very low level. On the other hand, the consequence of replacement of E3 depends on the chemical nature of the newly inserted residue. Replacement by the non-polar amino acid leucine results in an inactive protein, whereas replacement by glutamine results in retention of activity. Analysis of the generation of active hydrogenases 1 and 2 by activity staining of polyacrylamide gels in which extracts from the transformants had been separated fully corroborated these results (Fig. 4B).}

In order to investigate whether the H2 exchanges had an effect on the binding of zinc, HybF-Strep [H2Q] was purified. The protocol that was used for purification of the wild-type HybF-Strep protein was used, and similar yields of apparently homogeneous proteins were obtained (results not shown). The results of metal analysis are shown in Table 3. Again, approximately one zinc per HybF-Strep [H2Q] monomer was detected. In this preparation, iron was present, although the ratio was substoichiometric; again, nickel was undetectable.

**C-terminal cysteine motif is not essential for HybF function.**

The replacement of Cys-89 by either alanine or serine showed that this residue has a structural role rather than a functional role, which could involve the coordination of zinc. To obtain further evidence for this hypothesis, the other three cysteine residues of the two motifs were replaced either singly or in pairwise combinations. Analysis of the levels of gene products in the different transformants showed that they all contained reduced amounts of HybF compared with the amount in a transformant carrying the wild-type hybF gene on the plasmid (Fig. 5A). In addition, all variants exhibited HybC processing, albeit to different extents, and formed active hydrogenases 1 and 2 (Fig. 5B and C). The attempt to purify a HybF-Strep variant with altered conserved cysteine motifs in order to confirm the probable role of these motifs in zinc binding was not successful because of the complete insolubility of the products.

**Maturation defect of a triple mutant devoid of proteins HypA, HypB, and HybF can be phenotypically reversed by a high nickel concentration in the medium.** Waugh and Boxer (58) were the first workers to show that lesions in hypA can be phenotypically suppressed by high nickel concentrations in the medium. This was also demonstrated later for a mutant with defects in both hypA and hybF (24), suggesting that the products of these genes are also involved in nickel insertion. These findings, however, did not rule out the possibility that the products of hypB and of hypA and hybF could mutually compensate for each other in this process. To test this possibility, a mutant was constructed in which the expression of intact products of all three genes was blocked, and this mutant was tested for phenotypic suppression of the defect in hydrogenase maturation by nickel in vivo. It was found that the defect in the triple mutant was rescued by nickel in vivo. An analysis of the approximate concentration of the metal that was sufficient for compensation showed that this concentration was not different.
From the concentration to which the strain with the hypB deletion responded (data not shown).

Is there an interaction between the HybF and HypB proteins? The postulated cooperative action of HybF and HypB in nickel insertion into the hydrogenases from *E. coli* might involve a physical interaction. Formation of such a complex has been demonstrated for the homologous proteins from *H. pylori* by chemical cross-linking (33). To test whether this is also true for the *E. coli* proteins, we employed two different cross-linking reagents. This experiment was done by using either the sulfhydryl-specific cross-linking reagent *p*-phenylenediamine or dimethyl suberimidate, a homobifunctional imidoester cross-linker specific for primary amines. The products of the reaction were separated by SDS-PAGE, and the gels were developed by immunoblotting. However, no evidence for specific cross-linking was obtained (results not shown).

In a second approach to detect an interaction between the two proteins, we analyzed whether purified HybF can modulate the intrinsic GTP hydrolysis catalyzed by the HypB protein. Again, no effect was observed, either in the presence or in the absence of nickel (data not shown), which is in accordance with the results of Mehta et al. (33).

In vitro maturation of hydrogenases. In vitro processing and maturation systems for hydrogenases have been reported for *Azotobacter vinelandii* (34) and *E. coli* (30). These systems require the presence of nickel ions at unphysiologically high concentrations, in the range of several hundred micromolar. Since this could be the consequence of the lack of functional HybF and/or HypA proteins in the extracts, we tested whether addition of the purified proteins made the system functional at low nickel concentrations. However, addition of the proteins, even at high concentrations, in the presence of GTP did not change the maturation pattern (results not shown).

However, a dramatic effect was observed when the amount of thiol compounds in the assay mixture was reduced (Fig. 6). The hydrogenase activity that developed indicated that the in vitro incorporation of nickel is diminished or even eliminated in the presence of equimolar (Fig. 6, lane 3) or excessive (lanes 4 to 6) amounts of DTT.

Nickel binding of the HybF-Strep protein. To test the nickel binding capability of HybF, a nickel binding assay was performed by using $^{63}$Ni. In this filter binding assay, specific interaction of nickel with the wild-type HybF-Strep protein was observed (Fig. 7A). In contrast, the HybF-Strep [H2Q] variant displayed reduced binding activities which varied greatly in independent experiments. The corresponding Scatchard plot of the binding characteristics of the wild-type protein is shown in Fig. 7B. A $K_d$ of 1.87 $\mu$M for nickel and a maximal binding of 0.87 nickel atom per HybF-Strep protein were determined. The Scatchard plot of the binding data for HybF [H2Q] (data not shown) indicates that binding to this protein variant is not saturable, suggesting that there is a nonspecific interaction.

To differentiate between specific binding and nonspecific binding of nickel, competition experiments with zinc as a competitor were performed. Addition of 25 $\mu$M ZnCl$_2$ to the incubation mixture had no effect on the nickel binding ability of the HybF-Strep protein (Fig. 7A), whereas it drastically reduced the amount of nickel bound by the HybF-Strep [H2Q] variant (Fig. 7A).

**DISCUSSION**

The results of genetic and biochemical studies have indicated that nickel insertion into the metal center of NiFe hydrogenases depends on the activity of two proteins, HypA and HypB. HypB from *E. coli* and other organisms has been thoroughly studied; it is a guanine nucleotide binding protein, and GTP hydrolysis activity is essential for nickel incorporation in vivo (31, 32). Whereas the amino acid residues involved in GTP binding and hydrolysis are strictly conserved in all members of the HypB family, the proteins from several selected organisms also possess a polyhistidine motif close to the N terminus, which appears to have a function in nickel storage. This domain is absent from the HypB protein of *E. coli*. Whether HypB (apart from nickel storage in some species)
also has a function in delivering the metal to the hydrogenase apoprotein has not been determined unequivocally yet. Removal of the polyhistidine stretch from the protein of *B. japonicum* eliminated the storage function but not the activity in the maturation process (21, 39). These variants still possessed a capacity for stoichiometric binding of nickel. On the other hand, recent studies on the nickel insertion systems involved in urease and hydrogenase formation of *H. pylori* redirected this function to another member of the maturation system, namely, HypA (33, 40).

**HybF binds nickel.** The results for HybF of *E. coli*, a member of the HypA protein family, presented in this paper support the contention that HypA is a nickel chaperone involved in hydrogenase maturation. As determined by equilibrium dialysis experiments (data not shown) and by the filter binding assays described above, binding of nickel to wild-type HybF is stoichiometric, which correlates with the finding that HypA of *H. pylori* binds two nickel ions per homodimer. Moreover, the binding is not competed out by zinc, which support the hypothesis that there are separate binding sites. Finally, the H2Q variant of HybF contains the same amount of zinc as the wild-type protein contains, but it has a strongly reduced capacity to bind nickel. The residual activity could be chased out in the presence of zinc, indicating that there is nonspecific binding. Similarly, HypA [H2A] from *H. pylori* does not bind nickel in equilibrium dialysis experiments (33).

The conclusion based on the results reported previously for HypA from *H. pylori* (33) and the results presented above for HybF from *E. coli* is that the H2 residue of these proteins seems to be involved in the coordination of nickel. Replacement of other conserved residues of HybF did not yield any clear evidence that other amino acid side chains serve as ligands for nickel. An exception may be Glu-3 since replacement by Gln, which can provide a coordination site, did not affect activity, whereas replacement by Leu eliminated activity completely. The possibility that two monomers of HybF can interact to provide the binding site(s) cannot be eliminated, although our results show that HybF, in contrast to HypA from *H. pylori* (33), is a monomeric protein and that the presence of nickel does not stimulate dimerization (results not shown).

One finding that initially contradicted such a nickel binding function was the finding that the purified HybF protein contained zinc stoichiometrically but only insignificant levels of nickel when the cells were cultivated anaerobically, conditions which lead to expression of the high-affinity nickel transport system (19, 60) in a medium supplemented with nickel but not with zinc. The same results were obtained when HybF was overproduced under aerobic conditions (results not shown). An argument could be made that zinc occupies the nickel site in the purified protein, a situation reported for the UreE protein from *Bacillus pasteurii* (44). UreE from this organism contains one zinc atom per dimer, and this zinc could be replaced by nickel in crystals soaked with nickel chloride. The results presented in this paper, however, although not disproving this possibility, strongly argue in favor of the existence of separate binding sites for nickel and zinc.

**HybF contains a zinc finger domain.** Genes coding for HypA-like proteins are present in all microorganisms that synthesize NiFe hydrogenases. Mutational analysis showed that HupA, the homolog in *Azotobacter chroococcum*, is also essential for maturation of hydrogenases. A Tn5 insertion in *hupA* could be partially compensated for by high nickel concentrations in the medium, leading to the first suggestion that the gene product is involved in nickel sequestering (18). Polar effects on the expression of downstream genes by the insertion could not be ruled out by Du and Tibelius, however.

The members of the HypA family are rich in cysteine, containing 3.5 to 8.6% cysteine, and the HypA of *E. coli* is on top of the list. The minimum number of residues is four, and these residues are strictly conserved at their positions in all HypA
sequences. They are present in the motif CXXC-X12/13-CXXC, which is characteristic for the formation of a zinc finger domain (8, 16). The mutational screening analysis performed in this study supports the contention that HybF is a zinc finger protein. Single replacements of each of the Cys residues grossly affected the stability and solubility of the variants. The products were, however, still active in the maturation process. It is therefore plausible to assume that these sites are the coordination sites for zinc. This agrees with the conclusion of Vallee and Auld that zinc atoms fully coordinated by four ligands are structurally important, whereas catalytic site zinc is coordinated by three protein ligands (52). A structural, previously purely speculative role of the zinc finger domain could involve the interaction with other components of the maturation system or with the large hydrogenase subunit. In view of the higher propensity of zinc (compared to the propensity of nickel) to interact with thiolate groups, the results obtained in the competition experiments also suggest that the nickel is bound by nonthiolate ligands.

Possible function of HybF. Nickel can be inserted into hydrogenases chemically both in vivo by supplying hypA, hyfB, or hypB mutants with an excess of nickel in the medium (24, 40, 58) and in vitro maturation assay mixtures challenged with nickel (30, 34). Attempts to demonstrate any activity of each of these gene products under in vitro conditions have failed so far. This may have been due to inactivation during purification, to the lack of a required metabolite, or to the lack of other components of the insertion machinery with which these proteins must interact. It is therefore premature to propose a detailed model for the function of HybF, HybA, and HybB in the maturation process; rather, a working model is presented here. It is plausible to assume that the nickel sequestered by HybF (or HypA) is donated to the apoprotein of the large hydrogenase subunit either directly or via some still unknown macromolecular component. In the former instance it is further plausible to assume that specific recognition of the cognate large subunit must take place, which necessitates a direct interaction between the nickel donor and the target protein. This interaction (docking or release) may be controlled by HybB, which acts as a molecular switch in accordance with the general function of GTPases. Arguments supporting this notion are that the hydrolysis of GTP is required for nickel incorporation into the large subunit of formate hydrogen lyase from E. coli (27). This reaction is coupled with potassium uptake (28). Participation of HypA and HypB in the insertion of nickel into hydrogenase from E. coli has been demonstrated by chemical cross-linking (33). Our own attempts to cross-link HybF and HybB from E. coli failed, which may have been due to the fact that E. coli HybF has only 4 Lys residues, compared to the 10 Lys residues in the H. pylori protein. Other reasons could involve the possibility that the interaction requires binding to the precursor of the large subunit.

Since nickel can be inserted chemically into the target proteins, why has such a complex system developed? The answer, of course, requires in vitro demonstration of the activities of these proteins, but it may lie in shielding of the metal from the complexing activity of thiols in the cytoplasmic space. High thiol concentrations can completely bind the trace element and make it unavailable for the insertion process (Fig. 6). Other explanations are that the system guarantees that the correct metal is inserted and that the kinetics of insertion are improved compared to those of the pure chemical process.

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