Structural Insights into HypB, a GTP-binding Protein That Regulates Metal Binding*

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HypB is a prokaryotic metal-binding guanine nucleotide-binding protein that is essential for nickel incorporation into hydrogenases. Here we solved the x-ray structure of HypB from *Methanocaldococcus jannaschii*. It shows that the G-domain has a different topology than the Ras-like proteins and belongs to the SIMIBI (after Signal Recognition Particle, MinD and BioD) class of NTP-binding proteins. We show that HypB undergoes nucleotide-dependent dimerization, which is apparently a common feature of SIMIBI class G-proteins. The nucleotides are located in the dimer interface and are contacted by both subunits. The active site features residues from both subunits arguing that hydrolysis also requires dimerization. Two metal-binding sites are found, one of which is dependent on the state of bound nucleotide. A totally conserved ENV/IGNLV/ICP motif in switch II relays the nucleotide binding with the metal ion-binding site. The homology with NiFH, the Fe protein subunit of nitrogenase, suggests a mechanistic model for the switch-dependent incorporation of a metal ion into hydrogenases.

Maturation of nickel-containing enzymes, like carbon monoxide dehydrogenase, urease, or hydrogenase, requires participation of numerous accessory proteins. Sequencing of several bacterial genomes combined with genetic and biochemical analysis revealed a set of such proteins for each metalloenzyme (1, 2). Although the basic functions of nickel storage, GTP hydrolysis-dependent nickel mobilization, and nickel insertion have been demonstrated, the specific steps performed by these proteins in the assembly of the active site are not completely understood (3). In the case of hydrogenases, the generation of active enzyme that also involves nickel incorporation is achieved by the activities of six proteins designated HypA–F (4). In most organisms the corresponding genes are encoded in a single operon. However, genes for additional maturation proteins, also including a maturation endopeptidase, are normally organized in an operon that also houses the hydrogenase structural genes (5). Two of these auxiliary proteins, namely HypA and HypB, are required for the nickel insertion reaction as judged by the fact that the deficiency of HypA and hypB mutants in hydrogenase maturation could be complemented chemically by the addition of nickel to the medium in high concentrations or by the addition of the metal to maturation assays employing crude extracts (6–8). The same mutations also reduce efficiency of urease maturation in *Helicobacter pylori* showing the more general importance of the two proteins (9).

Genetically, HypA and HypB are the key factors for enzymatic activity because mutations in one of these results in hydrogenase deficiency (7, 8). The same mutations also reduce maturation efficiency of urease showing the more general importance of the two proteins (9). HypA is capable of binding two nickel ions per dimer with micromolar affinity (10) and two zinc ions with much higher affinity, which are supposed to be necessary for structural integrity of the molecule (11). Because of its nickel binding capacity and its ability to form a dimeric complex with HypB, HypA is expected to be a nickel chelator rather than the deliverer to the hydrogenase active site. Instead, this central function is attributed to HypB, a G-nucleotide-binding protein, whose GTPase activity is reportedly essential for nickel insertion into hydrogenases (12). As for most other small guanine nucleotide-binding proteins, the intrinsic GTP hydrolysis reaction is very slow (*K₆₅ = 0.17 min⁻¹* and *K₅₇ = 4 μM* for HypB from *Escherichia coli*) (13), and no accelerating factor has been described so far.

Based on metal-binding properties, HypB proteins can be divided into three different subgroups. The first group exhibits a mostly N-terminal polyhistidine stretch in addition to the G-domain. In *Bradyrhizobium japonicum* this stretch is capable of binding 16 nickel ions per dimer with a *K₆₅* of 2.3 μM (14). Deletion of the histidine-rich region results in a truncated protein that nevertheless is able to bind one nickel ion per monomer and still functions in mobilizing nickel in vivo (15, 16). The second group, including HypB from *E. coli*, also shows an extended N-terminal region but contains no polyhistidine sequence. A study of metal-binding properties of HypB from *E. coli* showed that this protein binds two nickel ions per monomer. The first ion is bound by the conserved N-terminal CXX-CGC sequence with sub-picomolar affinity and high specificity, whereas the second metal-binding site, located in the G-domain (including residues Cys-166, His-167, and Cys-198), has lower specificity for nickel over zinc and a lower metal affinity (17). The affinity of this site for zinc was determined to 1 μM and 10 times higher for nickel. In vivo mutational studies of this metal-binding site show that replacement of any of these residues to alanine completely blocks hydrogenase maturation in vivo (18). The third HypB subgroup, mainly composed of HypB proteins from thermophilic organisms, lacks the N-terminal...
nickel-binding part and shows only a short stretch of 20 amino acids prior to the G-domain.

In order to gain insight into the mode of action of HypB and to understand the relation between nickel incorporation and GTP binding/hydrolysis, we solved the crystal structure of HypB from \textit{Methanocaldococcus jannaschii} (HypB subgroup 3), which is a short version of the protein family and thus should represent the essential features of HypB function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The encoding DNA fragment of HypB from \textit{M. jannaschii} was amplified by genomic PCR and cloned into the pGex4T3 plasmid using BamHI and XhoI restriction sites. The construct for expression of HypBΔN (residues 72–290) from \textit{E. coli} was amplified by PCR using the pGex4T3-plasmid containing HypB (full length) as template and cloned into a modified pGexTx1 plasmid (EcoRI and XhoI) containing a tobacco etch virus protease cleavage site. HypB was expressed as glutathione S-transferase fusion protein in \textit{E. coli} strain Rosetta DE3. HypBΔN and HypBΔN(L242A,L246A) were expressed as glutathione S-transferase fusion proteins in \textit{E. coli} strain BL21DE3.

The following protocol serves for all purified proteins. At A_{600} of 0.6 in TB-medium expression was induced by addition of 100 \(\mu\)M isopropyl \(\beta\)-D-1-thiogalactopyranoside, and cells were harvested after 4 h of incubation at 37 °C by centrifugation. The cells were lysed in standard buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl\(_2\), 5 mM \(\beta\)-mercaptoethanol (\(\beta\)-ME))\(^2\) containing 0.15 mM phenylmethylsulfonyl fluoride using a microfluidizer. Cell lysate supernatant was applied to a GSH column (Amersham Biosciences) pre-equilibrated with a protease buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM KCl, 5 mM Ca\(_2\)\(_{2}\), 5 mM Mg\(_{2}\)\(_{2}\), 5 mM \(\beta\)-ME) containing 0.15 mM phenylmethylsulfonyl fluoride using a microfluidizer. Cell lysate supernatant was applied to a GSH column (Amersham Biosciences) pre-equilibrated with standard buffer at 4 °C. Unspecifically bound protein was removed by washing with high salt buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 300 mM KCl, 5 mM Mg\(_{2}\)\(_{2}\), 0.1 mM ATP, 5 mM \(\beta\)-ME). Fusion protein was cleaved with 400 units of thrombin (bovine; Serva) or 0.5 mg of tobacco etch virus protease in protease buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM KCl, 5 mM Ca\(_{2}\)\(_{2}\), 5 mM Mg\(_{2}\)\(_{2}\), 5 mM \(\beta\)-ME) on the column. After elution, HypB from \textit{M. jannaschii} was incubated with 0.5 units/ (mg of protein) alkaline phosphatase (bovine; Roche Diagnostics) in standard buffer containing 1 mM Zn\(_{2}\) and 200 mM \((NH\_4\)_2\)SO\(_4\) to remove bound nucleotide. HypBΔN from \textit{E. coli} was incubated with 0.5 units/(mg of protein) alkaline phosphatase in standard buffer only, because precipitation was observed in the presence of Zn\(_{2}\) or \((NH\_4\)_2\)SO\(_4\). After 20 h the proteins were further purified by gel filtration on Superdex S-75 (Amersham Biosciences) with standard buffer to remove unbound nucleotide. Purified protein was concentrated to a final concentration between 40 and 70 mg/ml, flash-frozen in liquid nitrogen, and stored at −80 °C.

Overexpression of HypB from \textit{M. jannaschii} for selenomethionine incorporation was performed in minimal medium containing 50 mg/liter selenomethionine. Purification was analogous to the purification of native HypB except for using 10 mM \(\beta\)-ME.

**Crystallography**—Crystals of \textit{M. jannaschii} HypB were obtained using the hanging drop/vapor diffusion method. 2 \(\mu\)l of 20 mg/ml protein solution containing 3 mM GTP\(_\gamma\)S were mixed with 2 \(\mu\)l of reservoir solution (native: 150 mM maleic acid disodium salt, 42.5 mM HCl, 20% PEG3350; Se-Met: 175 mM maleic acid disodium salt, 52.5 mM HCl, 14% PEG3350). Crystals grew after 1–2 days to a dimension of 0.15 × 0.15 × 0.05 mm. For data collection, crystals were cryoprotected in a buffer containing 217.5 mM maleic acid disodium salt, 61.6 mM HCl, 31.9% PEG3350, 14.5% xylitol, 14.5% sucrose, 25 mM Tris, pH 7.5, 25 mM NaCl, 2.5 mM Mg\(_{2}\)\(_{2}\), and 2.5 mM \(\beta\)-ME.

Native and Se-Met data sets were collected in Grenoble at ID14-1 and at SLS (X105A), respectively. Data were processed with XDS (19), and initial heavy atom sites for SAD phasing were identified with SHELXD (20). Refinement of the initial sites and search for additional sites were performed using SOLVE (21). Phase determination, density modification, and automated model building with SOLVE/RESOLVE (22, 23) led to an interpretable density map and an initial model. The atomic model was further built using COOT (24), and refinement was carried out with Refmac5 (25). The native model was obtained, using MOLREP (26) and Refmac5. Because Se-Met crystals showed higher resolution, they were used for determination of anomalous signals of zinc and nickel. Data sets were collected at wavelengths of 1.488 and 1.281 Å for nickel and zinc, respectively. Anomalous maps were generated using MOLREP to obtain initial phases and CAD and FFT (CCP4-package) to calculate electron density. The occupancies of metal ions have been defined by adjusting the occupancies such that the \(B\)-factors approximate the mean \(B\)-factor of the surrounding atoms. Additionally, the occupancies were determined by alternate refinement of occupancies and \(B\)-factors with MLPHARE (CCP4 package).

Overlays of structural models were performed using COOT or BRAGI (27). All figures were created using Pymol (DeLano Scientific).

**Size Exclusion Chromatography**—All analytical gel filtration experiments were performed in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM Mg\(_{2}\)\(_{2}\), and 5 mM \(\beta\)-ME. 300 \(\mu\)M protein was incubated with 300 \(\mu\)M GDP or GTP\(_\gamma\)S prior to application onto the Superdex 75 10/30 column. The column was pre-equilibrated with 500 \(\mu\)M GDP or 500 \(\mu\)M GTP in standard buffer.

**Isothermal Titration Calorimetry**—Binding affinity of GDP or GTP\(_\gamma\)S to HypB was determined by isothermal titration calorimetry. The binding energy upon titration of nucleotide (400 \(\mu\)M) to HypB (40 \(\mu\)M) was measured at 20 °C in standard buffer. All experiments were at least performed twice. The data obtained were fitted using the MicroCal-ITC implementation of ORIGIN 7.

**ICPMS and TXRF**—For determination of bound metal ions, Se-Met-containing protein was used. Samples were diazylated

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\(^2\) The abbreviations used are: \(\beta\)-ME, \(\beta\)-mercaptoethanol; GTP\(_\gamma\)S, guanosine 5’-(\(\beta\)-\(\gamma\)-thiotriphosphate; ICPSM, inductively coupled plasma mass spectrometry; Se-Met, selenomethionine; SIMIBI, after signal recognition particle, MinD and BioD; TRAFAC, after translation factors; SRP, the signal recognition particle; SR, serum receptor; TXRF, total x-ray reflection fluorescence; PDB, Protein Data Bank; r.m.s.d, root mean square deviation; mol, molecule.
against buffer containing 25 mM Tris, pH 7.5, 1 mM β-ME to reduce the salt concentration. ICPMS and TXRF were performed at the Institute for Analytical Sciences, Dortmund, Germany.

RESULTS AND DISCUSSION

Overall Structure—The crystal structure of HypB from M. jannaschii in the GTPγS-bound state was solved to 1.9 Å resolution (Table 1). The final refined structural model contains two HypB molecules in the crystallographic asymmetric unit, consisting of 211 (mol A) and 209 (mol B) residues. At the N terminus, electron density for the first 10 amino acids of the 24.3-kDa polypeptide is not defined, because electrospray ionization-mass spectra show the presence of full-length HypB.

The overall structure of each monomer is globular, with dimensions of (40 Å)³, whereas the dimer has a more elongated shape (Fig. 1A). Dimer formation involves a buried surface of 2527 Å². Each polypeptide consists of a seven-stranded parallel β-sheet (Fig. 1B). By sequence comparison HypB was categorized as belonging to the SIMIBI class of GTP-binding proteins (28), which exhibit a different topology as compared with the TRAFAC class. Although members of the TRAFAC class feature a six-stranded β-sheet containing one antiparallel strand, with an occasional nucleotide state-dependent extra (parallel) strand (29), SIMIBI G-proteins exhibit a seven-stranded parallel β-sheet. The structural analysis of HypB fully supports this classification, which puts HypB into a close structural and functional (see below) neighborhood to the signal recognition particle (SRP) and its receptor (SR).

Calculations of the electrostatic surface of HypB using the adaptive Poisson-Boltzmann equation show that the protein surface is mainly negatively charged with a remarkable exception of helix α1 (Fig. 1B). This amphipathic helix has all positively charged amino acids located on the accessible surface. This pattern of positively charged residues prior to the G-domain is highly conserved in HypB proteins, indicating that it might be involved in interacting with as yet to be identified partner proteins or other negatively charged molecules.

One GTPγS nucleotide is bound to each monomer (Fig. 1A). The affinity of HypB from M. jannaschii toward GDP and GTPγS was determined by isothermal titration calorimetry (Fig. 1C) and for HypBΔN from E. coli (data not shown). The dissociation constants for guanosine di- and triphosphate are in the micromolar range, with 3.4 μM for GDP and 0.89 μM for GTPγS. These affinities are much lower than those found for most Ras proteins, consistent with observations for other SIMIBI class G-proteins, indicating that this type of proteins can in principle freely exchange nucleotide without the help of guanine nucleotide exchange factors.

Active Site- and Nucleotide-dependent Dimerization—The overall structure shows that two GTPγS molecules are tightly sandwiched in the dimer interface, in a parallel orientation. Because the nucleotide-binding sites are formed by both monomers, they appear to mediate dimer formation (Fig. 3, A and B).

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**HypB and Metal Binding**

| **TABLE 1** Crystallographic data collection and refinement statistics |
|--------------------------|--------------------------|--------------------------|--------------------------|
| **Data collection** | **Native data set** | **Se-Met** | **Zinc** | **Nickel** |
| Space group | P2₁,2₁,2₁ | P2₁,2₁,2₁ | P2₁,2₁,2₁ | P2₁,2₁,2₁ |
| Cell dimensions | | | | |
| a, b, and c (Å) | 43.46, 68.11, 155.84 | 43.12, 68.02, 156.08 | 43.12, 68.02, 156.08 | 43.19, 68.10, 156.50 |
| Resolution (Å) | 20.0-1.90 (2.0-1.9) | 20.0-2.10 (2.2-2.1) | 20.0-2.6 (2.8-2.6) | 20.0-2.4 (2.6-2.4) |
| Rsym (%) | 6.2 (23.3) | 4.8 (8.4) | 4.8 (8.0) | 8.8 (29.8) |
| Completeness (%) | 21.9 (7.9) | 20.5 (9.1) | 18.6 (13.2) | 10.1 (4.2) |
| No. of reflections unique | 242,599 (33,238) | 194,893 (15,945) | 80,948 (15,945) | 109,523 (22,753) |
| Phasing | | | | |
| Se sites | 12 | 27,106 (5419) | 27,106 (5419) | 34,310 (7221) |
| FOM after SOLVE | 0.38 | 0.38 | 0.38 | 0.38 |
| No. of reflections observed | 242,599 (33,238) | 194,893 (15,945) | 80,948 (15,945) | 109,523 (22,753) |
| No. of reflections unique | 242,599 (33,238) | 194,893 (15,945) | 80,948 (15,945) | 109,523 (22,753) |
| Rfree (%) | 22.0/24.1 | 22.0/24.1 | 22.0/24.1 | 22.0/24.1 |
| Refinement | | | | |
| Rwork/Rfree (%) | 22.0/24.1 | 22.0/24.1 | 22.0/24.1 | 22.0/24.1 |
| No. of atoms | | | | |
| Protein | 3245 | 3245 | 3245 | 3245 |
| GTPγS | 64 | 64 | 64 | 64 |
| Magnesium | 2 | 2 | 2 | 2 |
| Zinc | 0.5 + 0.5 | 0.5 + 0.5 | 0.5 + 0.5 | 0.5 + 0.5 |
| Water | 174 | 128 | 128 | 128 |
| r.m.s.d. | | | | |
| Bond lengths (Å) (weight) | 0.007 (0.022) | 0.007 (0.022) | 0.007 (0.022) | 0.007 (0.022) |
| Bond angles (°) (weight) | 1.105 (2.011) | 1.105 (2.011) | 1.105 (2.011) | 1.105 (2.011) |

* Friedel pairs are treated as different reflections.
Binding from monomer A involves two of the consensus motifs of the superfamily: the P-loop (GXXXXGK(S/T)), which wraps around the β,γ-phosphates, and the NKXD motif that is involved in the guanine-specific recognition of the base. The P-loop Lys-46 contacts the β,γ-phosphate oxygens, and the Thr-47 side chain is a ligand of Mg2+.

Almost all GTP-binding proteins of the SIMIBI and TRAFAC classes contain the canonical DXXG sequence followed by the catalytically important glutamine in most TRAFAC class G-proteins (28). HypB has an EXXG motif, also found in BioD-related proteins, which is part of the totally invariant ENXGNLXCP motif connecting switch II with zinc binding (see below). Glu-120 is a direct ligand of Mg2+, rather than a water-mediated one formed by Asp from DXXG. There is another direct ionic interaction with Asp-75 from helix α3. The magnesium coordination sphere of HypB is complemented by Thr-47 from the P-loop, the β,γ-phosphate oxygens, and a water-mediated contact to the β-phosphate, very similar to what has been described for BioD (30). This is different from the coordination sphere of Ras and Ras-like proteins, which possess only two direct ionic interactions coming from two phosphate oxygens, two hydroxyl side chains, and two water molecules as ligands (Fig. 2C). It is also different from the SIMIBI protein SRP, where Mg2+ is liganded by two phosphate oxygens, one threonine side chain (from P-loop), and three water molecules.

The conserved glycine is close to the γ-phosphate and could potentially form a similar main chain hydrogen bond as the glycine from the DXXG motif. The α-phosphate is contacted by the guanidino group of a highly conserved Arg-78 not found in other SIMIBI and TRAFAC NTPases. A similar positioned arginine in SMC ATPases (ABC cassette) is essential for ATP hydrolysis (31).

Monomer B contributes to the binding of the base, the ribose, and the γ-phosphate with participating residues that are highly conserved (Fig. 3B). The guanine base is bound by the carbonyl group of Ala-174 of monomer B (helix α9). The lysine from the NKXD motif is stabilized by the main chain of Thr-145 (mol B), and the ribose is bound by the main chain of Val-175 and a water-mediated contact of Asp-148 from the other monomer.

The Active Site—Both monomers contribute to the active site around the γ-phosphate and shield it from solvent. Apart from the canonical interaction with the P-loop and the magnesium ion (mol A), the γ-phosphate is additionally fixed by Asp-75 (mol A), and a water-mediated contact to Thr-150 (mol B). Monomer B also supplies an invariant lysine (Lys-153), which directly contacts the γ-phosphate and is itself bound by two water molecules, one of which is a ligand to Mg2+. Judging from
the number of interactions, monomer B via Lys-153 is crucial for stabilizing the active site elements around the γ-phosphate. As is commonly found in GTP-binding proteins, there is a water molecule in a position opposite to the scissile Pβ–O–Pγ bond (3.6 Å) to act as nucleophile, and it is contacted by His-154 (mol B) and the invariant Asp-69 (mol A). Asp-69 in the second β-strand is highly conserved in all SIMIBI family proteins and is proposed to act as general base in nucleophile activation in the SRP-SR GTPase (32), the Fe protein of nitrogenase (33), and the DNA-binding protein Soj ATPase (homologue of ParA) (34) reactions.

To investigate the oligomeric state of HypB in solution, we performed size exclusion chromatography with HypB from *M. jannaschii* and HypB from *E. coli* in the presence of GDP, GTP, or in nucleotide-free buffer (Fig. 3). The results indicate that HypBΔN from *E. coli* is a monomer in the absence of any nucleotide. In the presence of GTP this protein eluted with lower retention time, suggesting a higher molecular mass, most
likely because of dimerization. In GDP-containing buffer HypBΔN elutes at a volume between monomer and dimer indicative of a fast equilibrium between the two states. Mutation of residues Leu-242 and Leu-246 to Ala, which contribute to dimerization in a hydrophobic patch (Fig. 3A, blue area), completely abolishes nucleotide-dependent dimerization (Fig. 3C, dashed lines). HypB from *M. jannaschii* eluted independent of the nucleotide state as one species at retention times similar to those of GDP-bound HypBΔN from *E. coli*. We thus assume that under the conditions used, HypB from *M. jannaschii* is in a fast monomer/dimer equilibrium.

**Metal Binding Properties**—HypB is essential for nickel incorporation into hydrogenases. A recent determination of metal-binding properties of HypB showed that the *E. coli* protein harbors two metal-binding sites, one located at the N-terminal CXXCGC motif and a second site in the G-domain, including residues Cys-166, His-167, and Cys-198 (17, 18). HypB from *M. jannaschii* lacks the N-terminal sequences and thus the N-terminal nickel-binding site. Sequence alignments show that the second nickel- or zinc (as an analogue of nickel)-binding site, located in the G-domain is completely conserved among members of the HypB family (Fig. 2A, black arrows).

Examination of the electron density map revealed two putative metal-binding sites per monomer. The first site consisting of His-100 and His-104 is exposed to solvent and points away from the dimer interface (Fig. 4A, blue). The second site includes residues Cys-95, His-96, and Cys-127, which coincide with the predicted metal-binding site of HypB from *E. coli*. These residues are located directly in the dimerization interface (Fig. 4A, brown). For a more direct determination of the binding site, anomalous data sets for nickel at 1.488 Å and zinc at 1.281 Å were collected. The Se-Met crystals already contained zinc,
which remained bound to the protein during purification, and were not further soaked with zinc. Crystals for the nickel anomalous data set were soaked in 25 mM NiCl₂.

A high signal (35σ/H9268) for zinc was found at the first site consisting of His-100 and His-104 (Fig. 4B, ZnC). Here the tetragonal coordination of zinc is completed by the same residues of a second HypB molecule of the adjacent asymmetric unit in the crystal. Free in solution, HypB is dimeric at most, and thus the zinc ion binding would, if it also exists in solution, be completed by two water molecules. His-100 and -104 are not conserved over all HypB proteins (Fig. 2A, white arrows), indicating that nickel binding at this site is eventually not involved in the nickel delivery function of HypB. If this site is not a crystal packing artifact, its most probable function is nickel storage because of the fact that bacterial cells attempt to maintain a low concentration of free heavy metals. A similar structural motif, consisting of two histidines, is found for copper ions in UreE from Klebsiella aerogenes, the analogue of HypA in the urease pathway. Although the structure was solved in the presence of copper, the sites identified are presumed to be representative of nickel-binding sites (35). There, the histidines (His-110 and His-112) involved were also assumed to act as a nickel reservoir to facilitate nickel delivery to urease via other metal-binding sites.

The second zinc-binding area (Fig. 4C) uses residues invariant for all HypB proteins and shows two binding sites for zinc positioned asymmetrically in the dimer interface. Determination of the occupancies gives values between 80 and 100% of ZnA (B-value, 25–28) and 64–84% of ZnB (B-value, 28–36). The first zinc ion (ZnA) is coordinated by Cys-95 and Cys-127 of monomer A and Cys-95 of monomer B. The second ion (ZnB) is exclusively bound by monomer B using Cys-95, His-96, and...
Cys-127. Low density was observed for less defined water molecules, which might contribute to zinc coordination. This might suggest the existence of a binuclear [45:1N:2Zn:(2O)] cluster (Fig. 4D) asymmetrically coordinated by both monomers. The unequal binding is most obviously displayed by His-96 and Cys-95. In monomer A, His-96 does not participate in binding of ZnA but rather forms a tight aromatic stacking interaction with Phe-131 to stabilize both loops involved in binding of the cysteines. In monomer B, His-96 additionally coordinates ZnB. Cys-95 of molecule B resembles the function of a bridging sulfur atom which is not fulfilled by Cys-95 of molecule A. Nevertheless, the positions of these cysteines are confirmed by the presence of Se-Met-phased, unbiased density maps. Coordinates ZnB. Cys-95 of molecule B resembles the function of a bridging sulfur atom which is not fulfilled by Cys-95 of molecule A. Nevertheless, the positions of these cysteines are confirmed by the presence of Se-Met-phased, unbiased density maps.

Although the natively bound ion is most probably nickel, the arrangement identified might suggest a mechanism for nickel delivery. Because the metal ions are bound highly unsymmetrically in this cluster, one could speculate that after release of one metal ion the other is bound tightly and symmetrically by the HypB dimer. The energy difference between these two states would be the driving force for the nickel-delivery reaction.

Data sets for nickel-soaked crystals were measured, and nickel anomalous density was found at the His-100/His-104-binding site (ZnA) with considerably lower intensity (6σ) than for zinc. This indicates a displacement of zinc by nickel or a lower occupancy at this site by zinc. Because of the presence of zinc at the Cys-95/His-96/Cys-127-binding sites combined with the 10 times higher affinity of HypB for zinc than for nickel (17), a displacement of these zinc atoms by nickel is unlikely. Nevertheless, a small peak of anomalous density was observed at the position of atom ZnA (5.25σ). It is unclear why the conserved metal-binding site has higher affinity for zinc than for nickel. A full explanation and further mechanistic analyses most likely require the participation of additional components of the nickel delivery system such as SlyD (see below) and HypA.

Electron density obtained from native crystals does not show additional density at the cysteine residues in the dimer interface indicating that no metal ions are bound at this site in native crystals. Instead, extended density is found between Cys-95 and Cys-127 of one monomer indicating intramolecular disulfide bonds (data not shown). Nevertheless, additional globular density at the His-100/His-104 metal-binding site, suggesting the existence of a metal ion, could also be observed in the native model.

The Switch Regions—The function of Ras proteins is to act as molecular switches, which use the GTP-dependent conformational changes in the switch regions, switch I and II, to interact with downstream effector molecules (29, 36). SIMIBI type proteins such as SRP and SR use the GTP-dependent conformational change for hetero- or homodimerization. Dimerization in turn is required to mediate the biological function, which in case of the SRP-SR interaction is to couple ribosomal translation with membrane translocation of signal peptide-containing proteins.

In TRAFAC G-proteins, the magnesium ion along with the γ-phosphate determine the position of switch I and switch II. In the triphosphate-bound form, two hydrogen bonds from the γ-phosphate oxygens to the main chain NH groups of invariant threonine and glycine residues position switch I and switch II, respectively. The glycine is part of the conserved DXXG motif, whereas the threonine is additionally involved in binding of magnesium via its side chain (Fig. 2C). The equivalent of Thr-35 in switch I of Ras is Asp-75 in HypB, whose carboxylate ion coincides with Thr-35-OH and binds magnesium directly and via water. Asp-75 also interacts with Lys-153 of the adjacent monomer that in turn binds to the γ-phosphate (Fig. 3, A and B). Asp-75 of HypB is located on helix α3 that starts with a sharp kink following Asp-69 (Fig. 5A). This helix is not located atop the nucleotide as switch I in Ras, leaving the nucleotide-binding site open to allow access of monomer B (rather than a GTPase-activating protein) to the active site (Fig. 5A, gray molecule). Because the position of the kink and the succeeding helix is determined by a close network of interactions between magnesium, its coordinating water, the γ-phosphate, and the second monomer, we would expect a structural change upon nucleotide hydrolysis and/or nucleotide release, similar to what happens in switch I of Ras-like G-proteins.

The position of switch II of Ras is determined by Gly-60 from the DXXG motif. In HypB this glycine is part of the fully conserved EN(V/I)GNL(V/I)CP motif located on an extended loop between sheet β4 and helix α5 (Fig. 5, A and B). Because Cys-127 from the motif is involved in nickel coordination and because adjacent residues mediate dimer formation, a link between nucleotide switching, dimer formation, and nickel coordination can be envisioned. Asn-121 stabilizes the loop structure by water-mediated H-bonds to the main chain CO of Phe-131 and Pro-128 and by direct interaction to Asp-37. Although Asn-124 is related to the catalytic residue Gln-61 of Ras in switch II, it is pushed aside by the adjacent monomer and points away from the nucleotide, excluding a similar function. From the number of interactions we would predict that the EN(V/I)GNL(V/I)CP motif is analogous to switch II of Ras-related proteins. It relays structural changes induced by nucleotide hydrolysis to the dimer interface and the dimer-mediated metal ion-binding site (Fig. 4, C and B).

Structural Comparison and Functional Implications—DALI searches (37) using the HypB structure revealed structural sim-
ilarity to several nucleotide-binding proteins, the most significant of which were SRP from *Thermus aquaticus* (PDB code 1ng1), the nitrogenase Fe protein subunit NifH from *Azotobacter vinelandii* (PDB code 1m34), and the bacterial chromosome segregation ATPase Soj with Z-scores of 13.3, 12.3, and 12.4 respectively. The structures of the monomeric SRP (or SR) and HypB have a root mean square deviation of 3.0 Å over 164 equivalent residues (Fig. 6A). Regions including the nucleotide-binding site and the protein core are very similar (Fig. 6A, green and blue). The most significant difference between the SRP-SR heterodimer and the HypB homodimer is that the two nucleotides are antiparallel in the former (32, 38) and parallel in the latter, thereby creating a totally different dimerization interface and a different mechanism of GTP hydrolysis (Fig. 6A, cyan and light blue).

The ATP-hydrolyzing enzymes nitrogenase (NifH) and the bacterial chromosome segregation protein Soj belong to the MinD family of SIMIBI class proteins (28). Although the homology score between monomeric HypB and NifH/Soj is lower than for SRP, they show a remarkable similarity in their parallel nucleotide arrangement and the localization of their dimerization interfaces (Fig. 6B). Similar to HypB, Soj undergoes a nucleotide-dependent dimerization (34), acting as a molecular switch to regulate binding to single-stranded DNA by associating with the bacterial promoter region and repressing transcription of early sporulation genes (39). Although the distance between the nucleotides is somewhat shorter because of interactions between the P-loops, Soj also employs ribose binding by the second monomer for ATP-dependent dimerization (Fig. 6, B and C). For Soj, dimerization is not sufficient for stimulation of hydrolysis and requires in addition the interacting protein Spo0J (34), similar to the MinD ATPase that requires MinE (40). Based on the striking similarities between HypB and those proteins and the very low rate of GTP hydrolysis even under conditions of dimer formation (0.02 min⁻¹), a similar missing factor can be envisioned for HypB. This is further supported by the inability of HypB to bind AlF₄⁻, which together with GDP/ADP is supposed to mimic the transition state of NTP hydrolysis (data not shown). Another feature of the MinD-like proteins is the presence of an additional lysine (Lys-15) in the P-loop, which makes contact with the α- and γ-phosphates of the other monomer (Fig. 6B). Its role in ATP hydrolysis was demonstrated for NifH (41, 42) and Soj (34), and the crystal structure of an ADP/AlF₄⁻-bound NifH homodimer verified its role in the stabilization of the transition state (43). Although Lys-153 of HypB is situated in a different portion of the polypeptide, its localization and 100% conservation argues for a similar role of Lys-153 (Fig. 3).

NifH is the iron protein part of the nitrogenase complex that catalyzes the ATP-dependent reduction of nitrogen. In this process NifH forms a transient complex with the MoFe protein transferring electrons from the NifH dimer to the MoFe protein via iron-sulfur clusters located in NifH. NifH alone does not catalyze ATP hydrolysis at appreciable rates. Bound to the MoFe protein, hydrolysis is accelerated (44) similar to the related protein Soj and its ATPase-accelerating protein Spo0J. The MoFe protein binds NifH ~17 Å away from the nucleotide near the iron-sulfur cluster site (42). An overlay of HypB and NifH (r.m.s.d. of 3.0 Å over 171 equivalent residues) shows strong similarities (Fig. 6D). The GTP-binding site of HypB and the ATP-binding site of NifH
superimpose well and show the 4Fe:4S cluster of NifH and the metal-binding site of HypB in close proximity. Cys-132 of NifH as a component of the 4Fe:4S cluster is part of the switch II loop, as is Cys-127 of HypB (Fig. 6D). The 4Fe:4S cluster is close to the MoFe protein-binding site (Fig. 6D) because electron transfer is performed between the 4Fe:4S cluster of NifH and the electron acceptor (8Fe:7S P-cluster) in the MoFe protein.
HypB and Metal Binding

Based on the close structural and functional similarity between NifH and HypB, we would propose that the function of HypB is to act as a GTPase-regulated, dimerization-dependent switch for the delivery of metal ions, most likely nickel, into hydrogenases. The switch-dependent delivery of metal ions is mediated by the EN(V/I)GNL(V/I)CP motif in switch II, which appears to be required for relaying the nucleotide state of the protein to the metal-binding site. The importance of residues Cys-95, Cys-127, and His-96 in this process is obvious, because mutation of one of these residues results in a block of metal binding in vitro and an inactive enzyme in vivo (17, 18). The structural similarity between switch II of NifH and HypB and the total invariance of the motif across species and kingdoms further identifies this part of the HypB dimer as crucial for its function.

Based on our structural model, the exact mechanism of nickel delivery can only be postulated. Although only the zinc-bound structure of HypB was solved, and HypB has a higher affinity for zinc as compared with nickel, the actual nickel delivery reaction is likely to involve additional elements such as HypA and SlyD which forms a heterodimer with HypB (45). Nickel delivery might also involve the transition between a monomeric HypB with one metal ion to a GTP-induced dimer where two metal ions form a bi-nuclear, asymmetric cluster similar to the one observed here. This cluster would now be ready to deliver one nickel ion to its target. From the superimposition with NifH, we would postulate a docking site for Fe protein subunit of nitrogenase (Fig. 6D). HypB would thus be responsible for direct GTP-dependent nickel delivery to hydrogenases. In a final step and in analogy to the ATPases mentioned above, interaction with hydrogenases might activate GTP hydrolysis in HypB and induce dissociation of the dimer.

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