Concerted Action of Two Novel Auxiliary Proteins in Assembly of the Active Site in a Membrane-bound [NiFe] Hydrogenase*[S]

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[FeFe] hydrogenases catalyze the reversible conversion of H₂ into protons and electrons. The reaction takes place at the active site, which is composed of a nickel and an iron atom and three diatomic ligands, two cyanides and one carbon monoxide, bound to the iron. The NiFe(CN)₂CO cofactor is synthesized by an intricate posttranslational maturation process, which is mediated by a set of at least six conserved Hyp proteins. Depending on the cellular location and the physiological function, additional auxiliary proteins are involved in hydrogenase biosynthesis. Here we present evidence that the auxiliary proteins HoxL and HoxV assist in assembly of the Fe(CN⁻)₂CO moiety. This unit was identified as a cofactor intermediate of the oxygen-tolerant membrane-bound [NiFe] hydrogenase (MBH) in the β-proteobacterium Ralstonia eutropha H16. Both HoxL and HoxV proved to be essential for H₂-oxidizing activity and MBH-driven growth on H₂. Copurification studies revealed that HoxL and HoxV directly interact with the hydrogenase apoprotein. HoxV forms complexes with HoxL and HypC, a HoxL paralogue that is essential for cofactor assembly. These observations suggest that HoxL acts as a specific chaperone assisting the transfer of the Fe(CN⁻)₂CO cofactor intermediate from the Hyp machinery to the MBH. This shuttle also involves the scaffold protein HoxV. Indeed, infrared spectroscopy and metal analysis identified for the first time a non-redox-active Fe(CN⁻)₂CO intermediate coordinated to HoxV.

Depending on the physiological conditions, hydrogenases catalyze either heterolytic cleavage of H₂ into protons and electrons or the reduction of protons, yielding dihydrogen. Three convergently evolved groups of hydrogenases are distributed in nature. They have been classified according to their metal content in di-iron [FeFe], nickel-iron [NiFe], and monon-iron [Fe] hydrogenases. This study focuses on one member of the group of [NiFe] hydrogenases, which consists of a Ni-Fe active site-containing large subunit, a Fe-S cluster-accommodating electron transferring small subunit, and a membrane-spanning cytochrome b. The Ni-Fe cofactor is coordinated to the protein via thiol groups originating from four invariant cysteine residues, two of which form a bridge between nickel and iron. The iron is additionally equipped with two CN⁻ groups and one CO ligand, which confer a low spin state on the iron.

Biosynthesis of intricate metal cofactors usually involves protein-assisted maturation processes (5–7). Maturation of [NiFe] hydrogenases underlies posttranslational reactions that implicate a set of at least six conserved auxiliary proteins (HypA, -B, -C, -D, -E, and -F), which mediate assembly and insertion of the Ni-Fe cofactor (5, 8, 9). According to the current model, predominantly based on studies with hydrogenase 3 from Escherichia coli, a carbamoyl group is transferred from carbamoylphosphate via HypF to the thiol group of the C-terminal cysteine in HypE. Upon dehydration, a HypE-thiocyanate complex is formed, and subsequently the CN⁻ ligand is transmitted to an iron exposed on a HypC-D complex (10–13). It has been proven experimentally that carbamoylphosphate is the substrate of the CN⁻ ligands, whereas the origin of the CO ligand remains an open question (14–17).

Once the Fe(CN⁻)₂CO moiety is inserted, nickel is incorporated into the hydrogenase precursor mediated by HypA and HypB (18, 19). Folding and oligomerization of the catalytic subunit are triggered by removal of a C-terminal peptide catalyzed by a specific endopeptidase and dissociation of the chaperone HypC (20).

The basic module of the membrane-bound hydrogenase (MBH) of Ralstonia eutropha H16 (Re) is composed of the large subunit HoxG (67.1 kDa) containing the Ni-Fe active site and a small subunit HoxK (34.6 kDa) harboring three Fe-S clusters (21–24). Physiologically active MBH is exposed to the periplasm and anchored to the membrane via a hydrophobic C terminus of HoxK and the b-type cytochrome HoxZ (25). The entire gene cluster required for active MBH expression (Fig. 1) resides on megaplasmid pHG1 in Re and encompasses genes for the MBH subunits (HoxK, -G, and -Z), a specific endopeptidase

*This work was supported by Deutsche Forschungsgemeinschaft Grant Sfb498 and the Cluster of Excellence “Unifying Concepts in Catalysis” and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The abbreviations used are: MBH, membrane-bound hydrogenase; FTIR, Fourier transform infrared; ICP-OES, inductively coupled plasma with optical emission spectroscopy; Re, Ralstonia eutropha H16.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.
Fe(CN$^-$)$_2$CO Precursor of the Hydrogenase Active Site

(HoxM), a set of metal center assembly proteins (HypA1, -B1, -F1, -C, -D, -E, and -X), four proteins involved in transcriptional control (HoxA, -B, -C, and -J), and a cluster of additional accessory proteins (HoxL, -O, -Q, -R, -T, and -V) (Fig. 1) (23, 26–30), which are typical for this subgroup of membrane-bound hydrogenases. Recently, it was shown that HoxO and HoxQ are involved in the maturation of the small subunit precursor pre-HoxK. A dual chaperone function was assigned to these two proteins: protection of the Fe-S clusters against oxidative damage and avoidance of premature MBH export, which is mediated by the twin-arginine-translocation system (31).

The present study focuses on the role of HoxL and HoxV in the process of MBH maturation. HoxL is a HypC paralogue (23), of which multiple copies exist in a number of organisms (32, 33). HoxV and orthologous proteins display sequence similarity to the large subunit of [NiFe] hydrogenases (34).

In this study, experimental evidence is presented that HoxL and HoxV participate specifically in the maturation of the large subunit of the Re MBH. Biochemical and spectroscopic evidence supports the notion that HoxV functions as a scaffold delivering the Fe(CN$^-$)$_2$CO moiety to the precursor of the MBH large subunit pre-HoxG, whereas copurification experiments indicate that HoxL plays a role as a chaperone dedicated to pre-HoxG.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2, respectively. *E. coli* JM109 (35) was used as host in standard cloning procedures, and *E. coli* S17-1 (36) served as a donor in conjugal transfers. *R. eutropha* H16 is the wild-type strain harboring mepasin/hg1; strains carrying the letters HF are derivatives of *R. eutropha* H16.

For purification of maturation intermediates, a *StrepTag II* coding sequence was fused at the 5′-end of hoxV and at the 3′-end of hoxL and hoxG genes, respectively. The *StrepTag II* sequence was fused to the 3′-end of hoxG by PCR with primers CtermStrep-hoxG_RsrII-rev and CtermStrep-hoxG_RsrII-fwd with pCH1235 as template, which is a religated 4.20-kbp BsiWI fragment of pCH1229 (carrying parts of *hoxG* and *hoxZ*), resulting in a 4.24-kbp fragment. The PCR product was digested with RsrII and religated, yielding pCH1238. A 1.10-kbp BsaBI-AsiSI fragment was transferred from pCH1238 into pCH1351, yielding pCH1239. Then a 9.06-kbp SpeI-Ecl136II fragment from pCH1239 was inserted into pCH785, resulting in pCH1240, and a 21.60-kbp SpeI-XbaI fragment was transferred from pCH1240 into pEDY309, yielding pGE609. The mobilizable overexpression plasmid was transferred from *E. coli* S17–1 to *R. eutropha* HF631, yielding strain HF686. A *StrepTag II* fusion to hoxL was constructed as follows. Using primers CtermStrep-hoxL_Nhel-fwd and CtermStrep-hoxL_Nhel-rev, the *StrepTag II* sequence was fused to the 3′-end of hoxL by inverse PCR with pCH1136 as template, a pBluescriptII KS+ derivative harboring a 2-kbp Ecl136II-MfeI fragment of pCH462 in Ecl136II-EcoRI sites. The resulting PCR product was cut with Nhel and religated, resulting in pCH1137, from which a 1.4-kbp Xhol fragment was transferred to Sall-digested pLO2, yielding pCH1138. Finally, pCH1138 was transferred to *R. eutropha* HF632 by conjugation, and the mutation was introduced by allele exchange (37), resulting in strain HF656. The *StrepTag II* fusion of HoxV was constructed by inverse PCR with primers NtermStrep-hoxV_Nhel-fwd and NtermStrep-hoxV_Nhel-rev with pCH305 as template. The Nhel-digested PCR product was religated, yielding pCH1287. A 0.7-kbp Sphl-Sall fragment from pCH1287 was transferred to pLO2, yielding pCH1288. Finally, pCH1288 was transferred into HF632, and the mutation was introduced by allele exchange (37), yielding HF714.

Amino acid exchanges were introduced into *hoxV* using PCR on a subcloned *hoxV* sequence; a 4.16-kbp Sall fragment carrying the *hoxTV* hoxA/B/1/FIC region from pCH785 was subcloned into pBluescript KS+, resulting in pCH1355. A 199-bp Fsel-MscI fragment from the PCR product with primers hoxV_Fsel-fwd, hoxV_MscI-rev, and hoxV_C525-rev, with pCH785 as template, was transferred into pCH1355, yielding pCH1356. BspEl-Ecl136II fragments (159 bp) from PCR products with primers hoxV_BspEI-fwd, hypA1_SacI-rev, and hoxV_C366S-fwd or hoxV_Y369C-fwd with pCH785 as template were transferred into pCH1351, yielding pCH1357 and pCH1358, respectively. SpeI-Ecl136II fragments (9.02 kbp) from pCH1357 and pCH1358 were transferred to pCH785, yielding pCH1359 and pCH1360, respectively. Sall fragments (4.16 kbp) from pCH1356, pCH1359, and pCH1360 were transferred into pLO2, yielding pCH1361, pCH1362, and pCH1363, respectively. The respective mutations were introduced into *R. eutropha* by conjugal transfer of pCH1361, pCH1362, and pCH1363 into HF388 and allele exchange (37), resulting in HF742, HF743, and HF744, respectively. A 3.78-kbp Fsel (Klenow-treated)-Sall fragment of pCH1359 was cloned into EcoRV-Sall-digested pLO2, yielding pCH1400. A 455-bp Fsel-Ncol fragment of pCH1356 was transferred into pCH1287, yielding pCH1401, and a 1.31-kbp EcoRV-Ecl136II fragment of pCH1401 was cloned into pLO2, resulting in pCH1402. The respective mutations (*hoxV*C525 and C366S) were introduced into *R. eutropha* by conjugal transfer of pCH1402 and pCH1400 into HF752 and allele exchange (37), resulting in HF761 and HF762, respectively.

In frame deletions in *hoxG*, *hoxM*, *hoxL*, *hoxV*, *hoxD*, and *hoxP* were introduced in the respective strains by allele exchange (37) using plasmids pCH424, pCH411, pCH464, pCH430, pCH4130, and pCH547, respectively. A 1.11-kbp PstI-MscI fragment from pCH1375 cloned into PstI-EcoRV-digested pLO2 yielded pCH1304.

**Media and Growth Conditions**—Strains of *Re* were grown in Luria broth medium containing 0.25% (w/v) sodium chloride (LSLB) or in mineral salts medium (38). Sucrose-resistant segregants of *sacB*-harboring strains were selected on LSLB plates containing 15% (w/v) sucrose (37). Strains of *E. coli* were grown in Luria broth medium (39). Solid media contained 1.5% (w/v) agar. Antibiotics for *Re* were used at the following concentrations: kanamycin, 350 μg ml$^{-1}$; tetracycline, 15 μg ml$^{-1}$; Antibiotics for *E. coli* were used at the following concentrations: kanamycin, 25 μg ml$^{-1}$; tetracycline, 15 μg ml$^{-1}$; ampicillin, 100 μg ml$^{-1}$.

When cultivated on a small scale (0.1–1 liter culture volume), cells were grown under hydrogenase-depressing conditions in mineral salts medium containing 0.2% fructose and 0.2% glycerol at continuous shaking at 120 rpm (40). For large-
TABLE 1
Bacterial strains and plasmids used in this study

| Strains or plasmids | Relevant characteristic(s) | Source or reference |
|---------------------|----------------------------|---------------------|
| **R. eutropha strains** |                           |                     |
| H16                 | Wild type                  | DSM 428, ATCC 17699  |
| H1631               | pHG1.1, Δnor(R2A282);Δ[hoxF-lacZ] | Ref. 47             |
| HF80                | pLO6 in H1631             | Ref. 47             |
| HF388               | SH− (ΔhoxH)               | Ref. 46             |
| HF351               | Derivative of HF388, ΔhoxG | Ref. 46             |
| HF462               | Derivative of HF388, ΔhoxL | Ref. 46             |
| HF451               | Derivative of HF388, ΔhoxV | Ref. 46             |
| HF742               | Derivative of HF388, hoxVC52S | This study        |
| HF743               | Derivative of HF388, hoxVC3665 | This study      |
| HF744               | Derivative of HF388, hoxVY390C | This study      |
| HF692               | Derivative of HF632, ΔhoxL | This study         |
| HF697               | Derivative of HF632, ΔhoxV | This study         |
| HF742               | Derivative of HF632, ΔhoxG | This study         |
| HF686               | pGE609 in HF631, hoxG-C-terminal StrepTag II | This study  |
| HF687               | Derivative of HF686, ΔhoxM | This study         |
| HF719               | Derivative of HF687, ΔhoxP | This study         |
| HF748               | Derivative of HF687, ΔhoxL | This study         |
| HF749               | Derivative of HF748, ΔhoxD | This study         |
| HF656               | Derivative of HF632, hoxG-C-terminal StrepTag II | This study  |
| HF669               | Derivative of HF656, ΔhoxM | This study         |
| HF718               | Derivative of HF699, ΔhoxP | This study         |
| HF714               | Derivative of HF632, hoxVN-terminal StrepTag II | This study  |
| HF751               | Derivative of HF714, ΔhoxP | This study         |
| HF752               | Derivative of HF714, ΔhoxG | This study         |
| HF761               | Derivative of HF752, hoxVC52S | This study  |
| HF762               | Derivative of HF752, hoxVC3665 | This study  |
| **E. coli strains** |                           |                     |
| JM109               | F- traD36 lacZΔ(lacZΔM15 proA+ B–/cI857 (McrA− Δlac-proAB) thi gyrA96 (Nalr) endA1 | Ref. 35           |
|                     | hsdR17 (Km−)ΔM15 recA1 supE44 recA1 in pBluescript KS | Ref. 36          |
| S17-1               | Tra– recA pro thi hsdR, ccr:RP4-2 | Ref. 36          |
| **Plasmids**        |                           |                     |
| Litmus28            | Ap′ lacZ′, ColE1 ori       | New England Biolabs |
| pBluescriptSK/KS+   | Ap′ lacZ′, T7 gene 10 promoter, fl ori | Stragatgene Cloning Systems |
| pBluescript II K5+  | Ap′ lacZ′, T7 gene 10 promoter, ori | Stragatgene Cloning Systems |
| pL01/pL02           | Km′, sacB, RP4 oriT, ColE1 ori | Ref. 37         |
| pEDY309             | RK2 ori, Tet, Mob′ | Ref. 38         |
| pCH3151             | 8.96-kbp pUT·EclI36G6 fragment containing hoxG2MLQRTV in Litmus28 (−KpnI) | Ref. 31         |
| pCH785              | 21.56-kbp SpeI·XbaI fragment containing the H16 MBH operon in Litmus28 (−SacI) | Ref. 47         |
| pLO6                | MBH overexpression plasmid (pEDY309 derivative containing the MBH operon) | Ref. 46         |
| pH1219              | 4.54-kbp Mhel (Klenow-treated)·PstI fragment of pH1351 in EclI36H-PstI-digested Litmus28 | This study |
| pH1235              | 4.20-kbp BsiWI fragment containing parts of hoxG and hoxZ of pH1219 digested | This study |
| pH1238              | 4.24-kbp RsrII fragment from PCR (with primers CernStrep-hoxG, RsrII-rev and CernStrep-hoxG, RsrII-fwd) | This study |
| pH1239              | 1.10-kbp BsaBI·AsiSI fragment from pH1238 in pH1351 | This study |
| pH1240              | 9.06-kbp SpeI·EclI36G6 fragment from pH1239 in pH1351 | This study |
| pCO609              | 21.60-kbp SpeI·XbaI fragment containing the CH1340 in pEDY309 | This study |
| pH1351              | 2.7-kbp EcoRV fragment containing hoxMLQORQ in pMAL | This study |
| pH1361              | 2.0-kbp EclI136H-Mhel fragment from pH1362 in EclI36H-Mhel-digested pBluescript KS+ | This study |
| pH1377              | 5.60-kbp Nhel fragment from PCR (with primers CernStrep-hoxL, Nhel-fwd and CernStrep-hoxl, Nhel-rev on pH1316 digested) | This study |
| pH1378              | 1.4-kbp Xhol fragment from pH1137 in pH2O | This study |
| pH1385              | 1.52-kbp EcoRE·PstI fragment containing hoxTV·pwtA1 in pBluescript SK+ | This study |
| pH1387              | 4.47-kbp Nhel fragment from PCR (with primers NtermStrep-hoxV, Nhel-fwd and NtermStrep-hoxV, Nhel-rev on pH1305 digested) | This study |
| pH1388              | 0.7-kbp Pshl·SalI fragment of pH1287 in pH2O | This study |
| pH1394              | 2.2-kbp Sall·Msal fragment containing hoxG in pLOI | Ref. 46 |
| pH1464              | 0.9-kbp ScoI·Ssal fragment containing hoxL in pLOI | Ref. 46 |
| pH2430              | 1.1-kbp Xbal·EcoRV fragment containing hoxL in pLOI | Ref. 46 |
| pH1411              | 2.0-kbp Sphl fragment containing hoxM in pLOI | Ref. 46 |
| pH1375              | 1.2-kbp Sall·Xhol fragment containing hoxP in pLOI | Ref. 27 |
| pH13100             | 1.11-kbp PstI·MscI fragment containing ΔhoxD from pH375 in PstI·EcoRV-digested pLO2 | This study |
| pH1354              | pLO1 derivative containing ΔhoxP | Ref. 30 |
| pH1355              | 4.16-kbp Sall fragment containing hoxTV·pwtA1·PstI·F from pH1375 in pBluescript KS+ | This study |
| pH1356              | 199·bp FseI·MscI fragment from PCR (with primers hoxV·FseI-fwd, hoxV·MscI-rev and hoxV·C52S-rev on pH785) in pH1355 | This study |
| pH1357              | 159·bp BspE·EclI36I fragment from PCR (with primers hoxV·BspE·fwd, hoxA1·SalI-rev and hoxV·C366S-fwd on pH785) in pH1351 | This study |
| pH1358              | 159·bp BspE·EclI36I fragment from PCR (with primers hoxV·BspE·fwd, hoxA1·SalI-rev and hoxV·Y369C-fwd on pH785) in pH1351 | This study |
| pH1359              | 9.02·bp SpeI·EclI36G6 fragment from pH1358 in pH785 | This study |
| pH1360              | 9.02·bp SpeI·EclI36G6 fragment from pH1358 in pH785 | This study |
| pH1361              | 4.16·bp Sall fragment of pH1356 in pLO2 (hoxV·C32S, TGC→TCT) | This study |
| pH1362              | 4.16·bp Sall fragment of pH1359 in pLO2 (hoxV·C366S, TGC→AGC) | This study |
| pH1363              | 4.16·bp Sall fragment of pH1360 in pLO2 (hoxV·Y390C, TAT→TGT) | This study |
| pH1364              | 3.78·bp FseI·Klenow-treated·SalI fragment of pH1359 in pLO2 (EcoRV·Sall) (hoxV·C366S, TGC→AGC) | This study |
| pH1400              | 4·55·bp FseI·NcoI fragment of pH1356 in pH1287 | This study |
| pH1401              | 1.31·bp EcoRV·EclI36I fragment of pH1401 in pLO2 (EcoRV·C52S, TGC→TCT, with 3′ fused StrepTag coding sequence) | This study |
scale purifications, cells were grown in a fermenter (Biostat MD; Braun) at continuous stirring at 400 rpm. Cells were harvested (centrifugation at 5000 × g at 4 °C for 20 min) after 48 h of cultivation at 30 °C on an A<sub>426</sub> of about 10.

Preparation of Membrane Fraction and Soluble Extract—The cell pellets resulting from 100-ml cultures were resuspended in 2 ml of K-PO<sub>4</sub> buffer (50 mM, K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.0), DNase I was added (20 μg). The cells were disrupted using a French pressure cell (SLM Aminco) and centrifuged (4000 × g, 4 °C, 20 min). The supernatant was then ultracentrifuged at 100,000 × g at 4 °C for 45 min to separate the membrane fraction from soluble extract. The membrane fraction was homogenized in a appropriate volume of 50 mM K-PO<sub>4</sub> buffer (pH 7.0), ultracentrifuged again at 100,000 × g at 4 °C for 30 min, and homogenized in 200 μl 50 mM K-PO<sub>4</sub> buffer (pH 7.0).

Protein Purification—For purification of StrepTag II-tagged proteins, the cells were resuspended in 1 ml of 100 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl (washing buffer) per 1 g of cell material, and protease inhibitor mixture (Complete EDTA-free protease inhibitor mixture; Roche Applied Science) was added. The cells were disrupted using a French pressure cell (SLM Aminco), and the crude extract was ultracentrifuged (100,000 × g, 4 °C, 45 min). The supernatant was then loaded on StrepTactin Superflow columns (2.5-ml columns, 1-ml bed volume; MoBiTec) equilibrated with washing buffer (maximum 25 ml of solubilized membrane extract per column). In the case of large scale preparations (60 g of cell material), the StrepTactin matrix (4 ml) was added to the soluble extract. After stirring for 1 h on ice, the suspension was transferred to two 10-ml columns (MoBiTec). The columns were washed with 12 column volumes of washing buffer and eluted with elution buffer (washing buffer, 5 mM dithiothreitol). The protein-containing fractions were pooled and concentrated using a centrifugal filter device (Amicon Ultra-15 (PL-10 or PL-30); Millipore). For Fourier transform infrared (FTIR) analysis, the protein solution was further concentrated using Amicon Microcon (YM-30) (Millipore).

Metal and Cyanide Determination—Iron was determined colorimetrically via complex formation with ferene S (45). In addition, both the iron and nickel content were determined using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 2100 DV; PerkinElmer Life Sciences). The cyanide content was determined chemically according to the protocol described by Ref. 44.

RESULTS

Enzyme Assays—Hydrogenase activity in the membrane fraction was measured as described previously (21) in 50 mM K-PO<sub>4</sub> buffer at pH 7.0. Methylene blue was used as an artificial electron acceptor, and its absorption at 570 nm was measured spectrophotometrically.

Western Immunoblot Analysis—Proteins were resolved using denaturing polyacrylamide gel electrophoresis (42). Western immunoblot analysis was performed according to a standard protocol (43). For immunological detection of hydrogenase-related proteins, antisera were applied in the following dilutions: anti-HoxG serum (1:10,000), anti-HypC serum (1:500 dilution), and anti-HypD serum (1:500 dilution). Antibodies directed against the MBH accessory proteins HoxL and HoxV were produced by rabbit immunization with the respective proteins, purified under denaturing conditions.

Infrared and EPR Spectroscopy—Infrared spectra were recorded on a Bruker Tensor 27 spectrometer equipped with a liquid nitrogen-cooled MCT detector at a spectral resolution of 2 cm<sup>−1</sup>. The sample compartment was purged with dried air, and the sample (0.66–1.83 mM protein) was held in a temperature-controlled (10 °C) gas-tight liquid cell (volume ~7 μl, path length = 50 μm) with CaF<sub>2</sub> windows. Spectra were baseline-corrected by using a spline function implemented within OPUS 4.2 software supplied by Bruker. The spectra shown in this work were normalized with respect to the protein concentration. Attempts to reduce the samples were carried out with excess dithionite under anaerobic conditions. EPR spectroscopy was performed as described in Ref. 44.

Metal and Cyanide Determination—Iron was determined colorimetrically via complex formation with ferene S (45). In addition, both the iron and nickel content were determined using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 2100 DV; PerkinElmer Life Sciences). The cyanide content was determined chemically according to the protocol described by Ref. 44.

The Accessory Gene Products HoxL and HoxV Are Essential for MBH Activity—The MBH-specific accessory genes hox<sub>L</sub>, -<sub>M</sub>, -<sub>O</sub>, -<sub>Q</sub>, -<sub>R</sub>, -<sub>T</sub>, and -<sub>V</sub> are constituents of the MBH operon, which encompasses 21 genes (Fig. 1). Mutants carrying in-frame deletions in each of the seven accessory hox genes are defective in autotrophic growth on hydrogen (46). To access the specific function of HoxL and HoxV on the protein level, we took advantage of plasmid pLO6, which allows overexpression
HoxL and HoxV Copurify with HoxG

As shown in Fig. 2a, pre-HoxG, HoxL, and HoxV were efficiently enriched from soluble extracts by affinity chromatography. In order to obtain appropriate amounts of pre-HoxG, the maturation pathway was interrupted by deletion of the gene for the specific endoprotease, which cleaves off the C-terminal extension of pre-HoxG after active site assembly. Identity of the eluted proteins was verified by immunological analysis using polyclonal antibodies against HoxG, HoxV, HoxL, and HypC (Fig. 2b). As already evident from Coomassie staining, both HoxL and HoxV copurified with accompanying proteins (Fig. 2a). HoxG and HoxV were identified in the eluate of the HoxL purification. The eluate of the HoxV purification revealed a strong HypC signal in addition to weak bands assigned to HoxG (Fig. 2b). HoxK was not observed as a copurification product (data not shown), indicating that neither HoxL and HoxV nor pre-HoxG form stable complexes with the MBH small subunit. On the other hand, HoxG was detected in all three eluates, pointing to an interaction between the HoxL homologue HypC and HoxV. The amount of HoxG found in the HoxV eluate, however, was substantially lower than that in the HoxL fraction (Fig. 2b).

Previous studies have shown that HypC of Re interacts with the hydrogenase large subunits of the NAD−-reducing [NiFe] hydrogenase and the H2 sensor (28, 48). Surprisingly, HypC did not copurify with StrepTag II-tagged pre-HoxG. Instead, HypC was observed in considerable amounts in the eluate of the HoxV purification (Fig. 2b). This result suggests that the chaperone HypC does not communicate with pre-HoxG but interacts stably with the accessory protein HoxV.

Interaction of HoxL and HoxV in the Absence of hyp Genes—The observation that HoxV interacts with HoxL as well as with the HoxL homologue HypC raised the question of whether the Hyp maturation machinery has any effect on protein-protein interaction between HoxV, HoxL, and pre-HoxG. Thus, the three proteins were purified from cells lacking the entire hyp gene region (∆hypA, -B1, -F1, -C, -D, -E, -F, and -X). The resulting elution profiles, shown in Fig. 3a, resembled essentially those obtained in the previous experiment (Fig. 2a). However, the band intensity of the copurified proteins was considerably higher in both Coomassie staining and immunological assays (Fig. 3b). This result can be interpreted by an accumulation of HoxV-HoxL and HoxL-pre-HoxG complexes the moment the Hyp machinery is blocked. The interaction between pre-HoxG and HoxL was particularly strong in the ∆hyp background (Fig. 3c). The data suggest that HoxL and HoxV are involved in the Hyp-determined maturation pathway.

HoxV Sequesters the Fe(CN−)2CO Moiety—The fact that both HoxL and HypC copurified with HoxV suggests a transmitter function of HoxV in transfer of the Fe(CN−)2CO moiety.
To investigate if iron and diatomic ligands can be detected in HoxV, the protein was subjected to spectroscopic and chemical analyses.

FTIR spectroscopy has been successfully used for in depth analysis of the NiFe(CN\(^-\))\(_2\)CO cofactor of various hydrogenases (49). Thus, HoxV samples were purified from three different strain backgrounds and subsequently subjected to FTIR spectroscopy. The resulting spectra are shown in Fig. 4. The spectrum obtained for HoxV isolated from the wild-type background (HF714 HoxG\(^+\)/Hyp\(^+\)) revealed two broad peaks of low intensity with maxima at 2078 cm\(^{-1}\) (CN\(^-\) stretching) and 1972 cm\(^{-1}\) (CO stretching). The signal intensity of these two bands was considerably higher in a HoxV sample obtained from cells lacking the MBH large subunit HoxG (HF752 ΔhoxG/Hyp\(^+\)). These data allow the interpretation that a cofactor intermediate is temporarily sequestered on HoxV and finally transferred to HoxG. The absence of any signals attributable to cyanide and carbonyl ligands in HoxV purified from cells deleted for the hyp genes (HF751 HoxG\(^+\) ΔhoxG) clearly showed that cofactor assembly in HoxV depends on the Hyp machinery (Fig. 4).

The FTIR spectra strongly suggest the existence of iron-bound CN\(^-\) and CO ligands. For determination of the metal content, HoxV was isolated from the ΔhoxG background (HF752 ΔhoxG/Hyp\(^+\)) by affinity chromatography and subsequent size exclusion chromatography, yielding a protein sample with a content of ~70% HoxV, according to SDS-PAGE and Coomassie staining. Colorimetric iron determination with ferene S (45) yielded 1.1 mol of iron/mol of HoxV. A somewhat higher iron content (1.4–1.5 mol of iron/mol of HoxV) was determined by ICP-OES.

Standard [NiFe] hydrogenases as well as the mature MBH of Re contain one CO group and two CN\(^-\) ligands bound to iron (49, 50). This prompted us to more closely inspect the asymmetric band shape of the peak at 2078 cm\(^{-1}\) in the CN\(^-\) stretching region of the HoxV spectrum (see Figs. S1 and Fig. S2). Indeed, a distinct shoulder at 2071 cm\(^{-1}\) suggests two CN\(^-\) ligands in the HoxV-bound cofactor. This assumption was corroborated by the chemical determination of the CN\(^-\) content in HoxV, which revealed 1.5–2.0 mol of CN\(^-\)/mol of iron.

Attempts to reduce the cofactor in HoxV by the addition of excess dithionite did not result in a shift of the CN\(^-\) and CO bands to lower wave numbers (Fig. 4). EPR spectroscopy did not reveal any signals in the "as isolated" HoxV protein as well as in the dithionite-treated sample (data not shown), suggesting that the iron is maintained in the non-redox-active Fe(II) state. This result is consistent with the observation that HoxV is devoid of hydrogenase activity, as determined by H\(_2\)-dependent methylene blue reduction (data not shown). Moreover, according to ICP-OES, nickel was found to be absent in samples of HoxV.

**Significance of Conserved Cysteines in HoxV**—Alignment of proteins belonging to the HoxV family and the Ni-Fe active
The HoxV protein was stable in all mutant strains, whereas the content of MBH in the membrane fraction of mutant HF742 (HoxV C52S) was significantly decreased (data not shown) which is compatible with the low MBH activity observed in this strain.

FTIR spectra of the purified C52S and C366S derivatives of HoxV did not show signals in the CN\(^{-}\) and CO region (data not shown), indicating that the mutant proteins were not able to coordinate the Fe(CN\(^{-}\))\(_2\)CO moiety. This was rather unexpected, especially in the case of the C366S derivative, which displayed 40\% residual MBH activity in the membrane fraction and was able to grow on H\(_2\) (Table 3). This discrepancy indicates that the conversion of Cys-366 to Ser destabilizes the binding of Fe(CN\(^{-}\))\(_2\)CO so that the cofactor presumably dissociated during the purification procedure.

**DISCUSSION**

Synthesis of hydrogenases is one of the most intriguing processes of metalloprotein biochemistry, requiring in addition to a complex biosynthetic apparatus for Fe-S cluster assembly (reviewed by Johnson et al. (6) and Fontecave et al. (52)) sets of components specifically designed for assembly of the dinuclear metal centers. In organisms containing active [FeFe] hydrogenases, the three accessory genes, hydE, hydF, and hydG are well conserved (53). Recent studies have documented that these gene products are essential for acquiring catalytic competency of [FeFe] hydrogenases, demonstrating their involvement in the formation of the active site (54, 55).

The maturation process of [NiFe] hydrogenases is even more complex than that of [FeFe] hydrogenases. Of the six Hyp proteins abundant in all organisms containing this type of hydrogenase, HypC, HypD, HypE, and HypF play an essential role in synthesis and transfer of the Fe(CN\(^{-}\))\(_2\)CO moiety to the hydrogenase large subunit, whereby HypC usually plays a dual role. First, HypC participates in cofactor assembly in a complex with HypD; second, it acts as a chaperone, keeping the hydrogenase large subunit in an open, cofactor-susceptible conformation (5). An N-terminal invariant cysteine residue has been shown to be essential for both functions of HypC (13, 56). However, paralogues of HypC have been found in several organisms. In *E. coli*, HypC interacts specifically with hydrogenase 3, whereas its parologue HybG serves as a chaperone for hydrogenases 1 and 2. Despite their target specificity in chaperone activity, both variants are able to form functional complexes with HypD (32). For *Thiocapsa roseopersicina*, two separate HypC proteins have been shown to be involved in the maturation of three [NiFe] hydrogenases in this phototroph (33). The so-called stable hydrogenase was shown to interact specifically with HypC\(_2\) (57).
HypC of Re is required for the activity of all three [NiFe] hydrogenases resident in this bacterium (27, 30). Deletion of hoxL, the hypC parologue in Re, however, solely disrupts the MBH activity, while leaving the NAD⁺-reducing hydrogenase unaffected (46). Protein-protein interaction studies revealed that HypC forms complexes with both the large subunit precursors of the SH (NAD⁺-reducing, soluble hydrogenase) and the H₂ sensor but not with pre-HoxG of the MBH (28, 48). In the present study, we show that HoxL copurifies with precursor forms of HoxG even in the absence of the Hyp proteins, indicating that HoxL fulfills the chaperone function of HypC in the case of the MBH. Thus, in contrast to the situation of the two hydrogenase counterparts in Re, the function of HypC within the MBH maturation process appears to be restricted to the assembly of the Fe(CN⁻)₂CO cofactor intermediate. The fact that HypC copurifies with HoxV suggests direct interaction of the two components in cofactor transmission. Indeed, chemical analyses of purified HoxV revealed approximately one iron atom and two CN⁻ groups per protein molecule.

The presence of the diatomic ligands in HoxV was confirmed by FTIR spectroscopy, uncovering two major peaks attributable to the stretching modes of the CN⁻ and CO ligands, as observed in [NiFe] hydrogenases (49). The band broadening for both types of diatomic ligands can be explained by structural flexibility of the protein environment. Assuming a function as a scaffold for the Fe(CN⁻)₂CO, the cofactor should be less tightly bound to HoxV. This may also be the reason for the similar amplitude of the CO and the CN⁻ peaks, which is rather unusual compared with mature MBH and especially to model complexes (50, 58). Both the band broadening and the peak intensities may also reflect spectral contributions of partially assembled complexes and/or variations in iron coordination. Variations in the frequencies of CO and CN bands have been observed by comparing the [NiFe] hydrogenases from Desulfovibrio gigas, Allochromatium vinosum, and Desulfovibrio vulgaris in the same redox state as well as for hydrogenase derivatives from Desulfovibrio fructosovorans with amino acid exchanges near the active site (59, 60). These variations most likely result from slightly different spatial and electronic structures of the active site environment in these enzymes. Especially, the CO stretching in HoxV showed an unusual high frequency at 1972 cm⁻¹ (compared with 1950–1960 cm⁻¹ for the most oxidized states in hydrogenases). A probable explanation for this upshift is the absence of a nickel atom, as suggested previously for the isolated large subunit of the regulatory hydrogenase of Re, which was only partially loaded with nickel (61).

HoxV represents the first example of a nonhydrogenase protein that binds an intermediate form of the Ni-Fe cofactor. Loading of HoxV with the Fe(CN⁻)₂CO moiety relies on an active Hyp protein machinery. Mutants of Re encoding HoxV variants with exchanges in the two conserved cysteine residues are severely affected in MBH activity. Accordingly, analysis of FTIR spectra of these HoxV mutant proteins revealed the lack of signals typical of CN⁻ and CO ligands.

**FIGURE 6.** Model of Fe(CN⁻)₂CO transfer into the large subunit of [NiFe] hydrogenases. a, model of the Fe(CN⁻)₂CO transfer from a proposed HypCD complex into the precursor of the large subunit (preLSU) of [NiFe] hydrogenases (5). b, model of the Fe(CN⁻)₂CO transfer into the precursor of the MBH large subunit (preHoxG) based on the results of this study.

HoxV is devoid of H₂-oxidizing activity, and the cofactor is not reducible by dithionite and hence catalytically inactive. From these data and the copurification experiments, we conclude that HoxV-Fe(CN⁻)₂CO constitutes an obligate intermediate in the maturation pathway of the MBH, as shown in Fig. 6. Preliminary attempts to demonstrate a direct transfer of the Fe(CN⁻)₂CO moiety from HoxV to pre-HoxG in vitro using radioactively labeled iron or FTIR spectroscopy have not yet yielded conclusive results. However, experiments are underway to improve the assay conditions and the isolation of stable intermediates.

It is interesting to note that HoxV/HupK homologues are found in α-, β-, and γ-proteobacteria that harbor cytochrome b-linked, membrane-bound [NiFe] hydrogenases. The question arises why the maturation of this particular subclass of hydrogenases requires the additional function of HoxV and HoxL. The hoxLV-containing microorganisms include nitrogen-fixing bacteria, phototrophs, and facultatively chemolithoautotrophic “Knallgas” bacteria (62). The latter produce hydrogenases in the presence of molecular oxygen. HoxV may enable the Fe(CN⁻)₂CO cofactor assembly in an oxic environment. However, HoxV homologues are also functional in bacteria, such as T. roseopersicina, which synthesize hydrogenases only under strictly anaerobic conditions (63), indicating that O₂ pro-
tection may not be the primary function of HoxV. This assumption is supported by the fact that the maturation pathways of the O₂-tolerant NAD⁺-reducing hydrogenase as well as the O₂-resistant H₂ sensor of *Re* are completely independent of HoxV and HoxL.

In this study, we have demonstrated that HypC, which is supposed to act in concert with HypD in the transfer of the Fe(CN⁻)₂CO moiety to hydrogenase, is not able to interact stably with the MBH subunit HoxG. Our studies do not exclude the possibility that HypC is loosely attached to the HoxG precursor. It is important to note, however, that in contrast to HypC, HoxL forms stable intermediates with the MBH large subunit. Obviously, pre-HoxG and its cytochrome b-linked homologues require the specific scaffold HoxV and the HypC parologue HoxL for cofactor assembly. It is not known yet whether the CO ligand is a constituent of the cofactor intermediate bound by the HypCD complex (5). Theoretically, attachment of the carbonyl ligand could occur after incorporation of the Fe(CN⁻)₂ fragment into the large subunit. In the case of MBH-like hydrogenases, direct CO attachment into pre-HoxG might be hindered for structural reasons. Involvement of the HoxV scaffold could overcome this deficiency. Finally, HoxV may have a storage/seat-function for Fe(CN⁻)₂CO units similar to some H ypB proteins that are known to sequester nickel (64, 65).

Although the exact function of HovX/HoxL is not clear yet, the results presented here demonstrate unambiguously that biosynthesis of [NiFe] hydrogenases proceeds on diverse routes. The highest level of complexity is obviously associated with cytochrome b-linked membrane-bound hydrogenases. The involvement of a scaffold protein that carries a precursor form of the cofactor represents a common principle that is found in an increasing number of maturation pathways. In this regard, [NiFe] hydrogenase maturation can be added to the well known biosynthetic pathways of iron-sulfur cluster assembly and synthesis of the iron-molybdenum cofactor of nitrogense (66, 67).

Acknowledgments—We thank E. Krause for identification of proteins using mass spectrometry and F. Lendzian for valuable discussions concerning EPR spectroscopy.

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