Maturation of *Rhizobium leguminosarum* Hydrogenase in the Presence of Oxygen Requires the Interaction of the Chaperone HypC and the Scaffolding Protein HupK*

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**Background:** [NiFe] hydrogenase biosynthesis requires the interaction among multiple accessory proteins for metal cofactor assembly.

**Results:** Combined bioinformatic protein modeling and mutant analysis on HupK and HypC proteins identify key residues required for hydrogenase maturation.

**Conclusion:** HypC-HupK interaction is a relevant step for hydrogenase biosynthesis in the presence of oxygen.

**Significance:** The results expand our knowledge on the mechanism of hydrogenase biosynthesis in aerobic bacteria.

[NiFe] hydrogenases are key enzymes for the energy and redox metabolisms of different microorganisms. Synthesis of these metalloenzymes involves a complex series of biochemical reactions catalyzed by a plethora of accessory proteins, many of them required to synthesize and insert the unique NiFe(CN)2CO cofactor. HypC is an accessory protein conserved in all [NiFe] hydrogenase systems and involved in the synthesis and transfer of the Fe(CN)2CO cofactor precursor. Hydrogenase accessory proteins from bacteria-synthesizing hydrogenase in the presence of oxygen include HupK, a scaffolding protein with a moderate sequence similarity to the hydrogenase large subunit and proposed to participate as an intermediate chaperone in the synthesis of the NiFe cofactor. The endosymbiotic bacterium *Rhizobium leguminosarum* contains a single hydrogenase system that can be expressed under two different physiological conditions: free-living microaerobic cells (≈12 μM O2) and bacteroids from legume nodules (≈10–100 nM O2). We have used bioinformatic tools to model HupK structure and interaction of this protein with HypC. Site-directed mutagenesis at positions predicted as critical by the structural analysis have allowed the identification of HupK and HypC residues relevant for the maturation of hydrogenase. Mutant proteins altered in some of these residues show a different phenotype depending on the physiological condition tested. Modeling of HypC also predicts the existence of a stable HypC dimer whose presence was also demonstrated by immunoblot analysis. This study widens our understanding on the mechanisms for metalloenzyme biosynthesis in the presence of oxygen.

Hydrogenases catalyze the reversible oxidation of dihydrogen according to the reaction H2 ↔ 2H+ + 2 e−. These enzymes play an important role in the energy and redox metabolisms of many microorganisms by enabling them to use hydrogen as a fuel or as a sink for reducing equivalents in fermentative processes (1). There are three types of hydrogenases according to the composition of their catalytic sites: [Fe] (or FeS cluster-free), [FeFe], and [NiFe] hydrogenases. The [FeFe] and [NiFe] hydrogenases are the most common in nature. H2 production is frequently associated to fermentative processes in anaerobic habitats, and most [Fe-Fe] proteins are extremely sensitive to O2, which causes an irreversible degradation of the active center (2). Whereas in most cases [NiFe] hydrogenases exhibit reversible inactivation by O2, some of the enzymes from this group (such as those from *Ralstonia eutropha*, *Aquifex aeolicus*, and *Rhizobium leguminosarum*) are oxygen-tolerant and maintain the activity even under fully aerobic conditions (3). Bacteria expressing O2-tolerant [NiFe] hydrogenases have developed strategies that allow synthesis of the enzyme in the presence of O2 (3, 4). Given current biotechnological perspectives on the use of hydrogen as an energy vector, knowledge of molecular strategies of biological oxidation of H2 in the presence of O2 is acquiring a particular interest (5).

[NiFe] hydrogenases are dimeric enzymes with a large subunit of ~65 kDa and a small subunit of ~35 kDa. The large subunit contains the catalytic site of the enzyme on a heterobimetallic metal cluster [NiFe(CN)2CO] with one nickel and one iron atom coordinated to the protein by four cysteine residues.
In addition, the iron atom is further coordinated to two cyano ligands (Cys-2 and His-51 in the HypC protein residue of HypC (Cys-2) is essential for the interaction of this protein with HypC and identifies residues in these two proteins that are essential for maturation of hydrogenase.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—Strains and plasmids used in this study are listed in Table 1. *R. leguminosarum* strains were routinely grown at 28 °C in yeast mannitol broth (YMB),

4. Tryptone-yeast extract, or *Rhizobium* minimal media (24). *E. coli* DH5α was used for standard cloning procedures, and *E. coli* S17.1 was used as the donor for conjugative plasmid transfer between *E. coli* and *R. leguminosarum*. Antibiotic concentrations used were 100 μg ml⁻¹ ampicillin, 50 μg ml⁻¹ kanamycin, 5 μg ml⁻¹ tetracycline, and 5 μg ml⁻¹ (for *R. leguminosarum*) or 10 μg ml⁻¹ (for *E. coli*).

Hydrogenase Activity Assays—Hydrogenase activity in bacteriochrome and in free-living microaerobic cells was measured by an amperometric method using a Clark-type hydrogen microelectrode (Unisense) with oxygen as electron acceptor (29). Hydrogenase activity in vegetative cells was induced in bacterial cultures grown under microoxic conditions (1% O₂) as previously described (27). Protein contents of vegetative cell cultures and bacterioid suspensions were determined by the bicinchoninic acid method (30) after alkaline digestion of cells at 90 °C in NaOH for 10 min using bovine serum albumin as the standard.

**DNA Manipulation Techniques and Mutant Construction**—DNA manipulations, including purification, restriction, ligation, agarose gel electrophoresis, PCR amplification, and transformation into *E. coli* cells were carried out by standard methods (31). Oligonucleotides used as primers are listed in Table 2.

To generate the HypC::Strep-tagII (HypC<sub>strep</sub>) fusion protein, a modification of the Datsenko and Wanner deletion system (32), was used. The modification consisted of the insertion of the sequence coding for the Strep-tag II peptide (WSHPQFEK) at the 5’-end of the antibiotic resistance gene of the pKD3 plasmid (28), resulting in plasmid pPM71. This plasmid was used as template for in-frame fusing of the Strep-tag II sequence to the
3'-end of hypC from the pALPF1 plasmid using primers TAGYC1-TAGYC2. The resulting plasmid (pALPF36) harbors a hydrogenase gene cluster encoding a hypC::Strep-tag II (hypCST) gene fusion.

To express hypCST gene in microaeroically grown cultures of R. leguminosarum in a way compatible with Hup expression from pALPF1 derivatives, a pBBR1MCS derivative plasmid (pPM1350) harboring hypCST was constructed. To this end we amplified hypCST using plasmid pALPF36 as template and YCNDE-MAYD3 primers (Table 2). The amplified fragment was cloned (NdeI-XbaI) in pPM1350 plasmid (28). This plasmid harbors the PfixN promoter from pALPF1 plasmid using primers HUPK.F54C2/HUPK.F54C1 for hypK and HUPK.R30L1/HUPK.Q52L2 for hypC. Substitution of target nucleotides as well as the absence of additional mutations was verified by DpnI digestion to eliminate the native template. Substitution of target nucleotides with adequate centered nucleotide substitutions (Table 2) and DpnI digestion to eliminate the native template. Substitution of target nucleotides as well as the absence of additional mutations was verified by DpnI digestion to eliminate the native template.

### Purification of Fusion Proteins

Purification of Fusion Proteins—Protein purification was carried out from 3 liters of R. leguminosarum cultures routinely induced for hydrogenase activity under microaerobic conditions (1% O2). A portion of the culture was assayed for hydrogenase activity as described before. Preparation of cell extracts and purification of proteins by Strep-Tactin affinity chromatography was performed as described (27).

### Western Immunoblot Analysis

Western Immunoblot Analysis—Immunological detection of proteins was carried out by Western blot assays as described previously (33) using antisera raised against R. leguminosarum HupL (1:400 dilution), HupK (1:100 dilution), and HypB.
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(1:2000 dilution). For identification of Strept-tag fusion proteins, AP-conjugated Strept–Tactin (1:2500; IBA, Göttingen, Germany) was used. Immunoblot analyses were performed with 60 and 20 μg of total protein from vegetative cells and bacteroid crude extracts, respectively, for HupL or with 10 μg for HypB detection. For analysis of interactions with HypC protein, immunoblot analysis was performed using 4 μg of protein from pooled eluate fractions and 60 μg of protein from soluble fraction samples.

Plant Tests—Pea (Pisum sativum L. cv. Frisson) surface-sterilized, pregerminated seeds were sown in Leonard jar-type assemblies and inoculated with R. leguminosarum bv. viciae strains as previously described (29). Plants were grown for 21 days in a greenhouse under bacteriologically controlled conditions at 18/25 °C (night/day) temperatures. Plants were irrigated with a nitrogen-free nutrient solution (33) supplemented with 17,612 water molecules added according to the TIP3P model (46) together with Na+ and Cl− ions added to counter the total negative electric charge of the system while providing 0.150 M salt concentration. The particle mesh Ewald summation method (47) was used for long range electrostatics, and a 10 Å cutoff was set for short range non-bonded interactions. The geometry of the HupK-HypC complex was optimized performing the following cycles of calculation: 1) 20,000 conjugate gradient minimization steps; 2) equilibration of water with all protein atoms fixed for 100 ps at 2-fs time steps (50,000 energy calculation steps) at 298 K and 1 atm; 3) a new set of 30,000 conjugate gradient minimization steps for the whole proteins-water-ions system.

Interactions and identification of amino acid residues in the protein-protein interface at the optimized geometry of HupK-HypC-cofactor complex were analyzed with the PISA software (48, 49) using the EBI-PISA server.

The model structure of dimeric HypC was obtained upon generation of models from dimers predicted by PISA and ClusPro 2.0 for 2Z1C together with the dimer predicted by ClusPro 2.0 for the homology-modeled structure of HypC. The model dimer with the best QMEAN quality estimate was selected to perform a geometry optimization in vacuo with NAMD 2.9 and the CHARMM 3.1 force field. The Poisson-Boltzmann electrostatic potential was calculated for the final model structure of dimeric HypC with APBS 1.3 (50) using AMBER99 (51) charges and radii assigned with PDB2PQR 1.7 (52). The nonlinear Poisson-Boltzmann equation was solved in sequential focusing multigrid calculations in three-dimensional meshes composed of 1293 points (spatial grids with step size about 0.5 Å) at 298.15 K and 0.150 M ionic concentration. Dielectric constants of 4 for proteins and 78.54 for water were used. The numerical output in scalar OpenDX format was mapped onto protein molecular surfaces and rendered with PyMOL (53). Poisson-Boltzmann electrostatic potential values are given in units of kT per unit charge (k, Boltzmann’s constant; T, absolute temperature).

RESULTS

HupK Is Essential for Hydrogenase Activity in R. leguminosarum Free-living Cells and in Bacteroids—Previous R. leguminosarum hupK-deficient mutants were obtained through transposon insertion, thus leading to polar effects on downstream genes (54). To test the essentiality of hupK gene for the biosynthesis of hydrogenase in R. leguminosarum, an in-frame hupK deletion mutant was generated and tested for hydrogenase activity in microaerobic (1% O2) free living cells and in bacteroids induced in pea plants (Table 3). Deletion of hupK gene resulted in the suppression of hydrogenase activity in microaerobic free-living cells. Interestingly, the ΔhupK mutant retained low but significant levels of hydrogenase activity under symbiotic conditions, where the bacteroid is maintained under ultra low oxygen tensions to protect nitrogenase activity (10–
Hydrogenase activity of *R. leguminosarum* strains in microaerobic cultures (1%O<sub>2</sub>) and in pea bacteroids

| Strain             | Genotype       | Hydrogenase activity<sup>a</sup> | Bacteroids |
|--------------------|----------------|----------------------------------|------------|
| UPM1155 (pALPF1)   | WT             | 13,610 ± 1,930                   | 6,100 ± 1,000 |
| UPM1155 (pALPF14)  | ΔhypC          | <200                             | <200        |
| UPM1155 (pALPF10)  | ΔhypK          | 12,730 ± 1,830                   | 5,080 ± 870  |
| UPM1155 (pALPF14/pPM502) | ΔhypC/hypC<sub>ST</sub> | 18,360 ± 1,650                   | 5,970 ± 740  |
| UPM1155 (pALPF10/pPM165) | ΔhypK/hypK<sub>ST</sub> | 15,760 ± 1,300                   | 6,920 ± 340  |
| UPM1155 (pALPF1/pPM502) | WT/hypC<sub>ST</sub> | 20,880 ± 560                    | 6,390 ± 770  |
| UPM1155 (pALPF1/pPM501) | WT/hypK<sub>ST</sub> | 20,140 ± 1,040                   | 7,080 ± 370  |
| UPM1155 (pALPF1/pPM165) | WT/hypK<sub>ST</sub> | 1,650 ± 870                      | 1,040 ± 740  |

<sup>a</sup> Values, expressed in nmol of H<sub>2</sub>/(mg of protein)<sup>−1</sup> hr<sup>−1</sup>, are the average of three independent assays.

100 nm, (55)). In contrast, a ΔhypC mutant exhibited a clearly Hup<sup>−</sup> phenotype under both physiological conditions. These data indicate that HupK is essential for hydrogenase synthesis in free-living cells and also suggest that a low level of hupK-independent hydrogenase synthesis occurs under symbiotic conditions.

The hydrogenase-deficient phenotypes of ΔhypC and ΔhupK mutant strains were complemented by plasmids pPM502 (P<sub>fixN</sub>/hypC<sub>ST</sub>) and pPM165 (P<sub>fixN</sub>/hupK<sub>ST</sub>), respectively, under both free living and symbiotic conditions, indicating that the addition of the Strep-tag did not affect the functionality of these proteins. In these experiments we observed that, under microaerobic free-living conditions, the ΔhupK mutant was complemented for hydrogenase activity at levels 34% higher than those in the wild-type strain. These data suggest that under such conditions (1% O<sub>2</sub>), the level of HupK might limit the biosynthesis of hydrogenase in *R. leguminosarum*. To test this hypothesis, we determined the effect of extra copies of hypC, hupK, and hupF genes on the hydrogenase activity induced in microaerobic cultures and in pea bacteroids of the wild-type strain *R. leguminosarum* UPM1155(pALPF1). Strains carrying extra copies of either hupF<sub>ST</sub> or hupK<sub>ST</sub> genes, but not those carrying additional hypC<sub>ST</sub> copies, showed increments of ~50% on the level of hydrogenase activity under free living conditions. In contrast, pea bacteroids induced by these strains showed activity levels only slightly higher than those in the wild-type and similar to the levels exhibited by the strain carrying extra hypC<sub>ST</sub> copies (Table 3). These data indicate that the requirements of HupF and HupK are higher under free-living conditions, suggesting that these proteins might be involved in an adaptation of the system to the presence of oxygen.

**HypC Interacts with HupK during Hydrogenase Biosynthesis**—To identify potential interactions of *R. leguminosarum* HypC with HupK and HupL proteins, as described in the *R. eutropha* system (26), co-purification experiments were carried out using a HypC protein variant incorporating a Strep-tag II affinity tail at the C-terminal end (HypC<sub>ST</sub>). To “freeze” potential complexes formed during the biosynthetic process, HypC<sub>ST</sub> was expressed in a background deficient for the HupL-specific protease HupD. Soluble extracts of microaerobic cultures expressing HypC<sub>ST</sub> from plasmid pPM502 were loaded into a Strep-Tactin column, and desthiobiotin-eluted proteins were separated by SDS-PAGE and identified immunologically using the corresponding antisera (Fig. 1). A hupK-deficient background was also used as a negative control. As expected, immunoblot membranes developed with a Strep-Tactin-AP conjugate showed the presence of a strong band of the expected molecular size for HypC<sub>ST</sub> (~8 kDa). In these experiments we observed that the levels of HypC<sub>ST</sub> recovered were similar in the ΔhupD and ΔhupK mutants.

Similar immunoblots were developed using antisera raised against HupL and HupK (Fig. 1). Analysis of the eluates obtained from the hupD-deficient background revealed the presence of an antiHupL-immunoreactive band of a size corresponding to the unprocessed form of HupL (~66 kDa). This band was also detected in the soluble fraction. In the case of the
anti-HupK antiserum, several reactive bands were detected, but only one of them showed the expected size of HupK (37 kDa) and was absent in the eluate of a hupK mutant. Interestingly, very low levels of antiHupL-immunoreactive protein co-puriﬁed with HypCST in eluates from the hupK-deﬁcient strain (Fig. 1) even though HypCST was fully stable in this strain and pre-HupL was present in the soluble extract. A HypC-HupK complex was also detected in a hupL-deﬁcient background (data not shown). These data indicate that HypC participates in complexes with HupL and HupK in which the interaction of HypC with HupL is probably indirect, via HupK.

Modeling of Protein-Protein Interactions in the HupK-HypC Complex—The lack of experimentally available structures for HypC and HupK proteins from R. leguminosarum led us to undertake their modeling as explained under “Experimental Procedures.” The resulting model structures generated with the R. leguminosarum sequences are shown in Fig. 2 superimposed to reference structures: chain B of HypC protein from Thermo-
coccus kodakarensis (37) and the large subunit of the [NiFe] hydrogenase from A. vinosum (56).

Initial and ﬁnal optimized geometries of the HupK-HypC-cofactor complex, obtained as explained under “Experimental Procedures,” show a striking difference in the location of the N-terminal tail of HypC protein before and after its interaction with HupK. This tail adopts an extended coil conformation that allows the conserved essential Cys-2 residue to interact with partner proteins in complexes and participate in the binding of Fe(CN)2CO cofactor (12, 16, 37, 57, 58). Although this tail, taken unchanged from the HypC structure in protein-protein docking setting the initial geometry, protrudes toward the cofactor binding site inside the HupK protein (Fig. 3A), it is well outside that site in the optimized geometry (Fig. 3B). Because the cofactor was inserted into HupK in the complex after docking, this result might be interpreted as a hint of structural stabilization of the cofactor in the HupK binding site in the complex without further participation of HypC.

The PISA analysis (48, 49) of the protein-protein interface in the complex optimized geometry allows identifying residues that might participate in the interaction between HupK and HypC. According to surface areas buried upon binding and inter-monomer contacts, residues represented in Fig. 3C play a major role in that interaction by forming hydrogen bonds. If one considers besides that some of these residues participate in more than one hydrogen bond (Table 4), the following amino acids appear to play a particularly relevant role in the interaction: Arg-30 and Gln-52 in HupK and Cys-2, His-46, and Glu-58 in HypC. Leu-33 residue of HypC is not included in the
list of amino acids in the protein-protein interface. However, it shows a particularly high exposure to solvent (see Fig. 3C) with a 143 Å² accessible surface area, indeed the largest value after that of Cys-2 in HypC protein. Because leucine side chain is hydrophobic, this feature led us to conjecture that Leu-33 could be relevant to the properties of the HupK-HypC complex regarding the interaction with other components of the hydrogenase biosynthetic process (see below).

The selection of residues in a neighborhood of 4 Å around the cofactor results in the following ones in HupK (Fig. 3D): Phe-54, Cys-57, Cys-292, Arg-294, Thr-318, Cys-357, and Phe-360. Although further calculations would be needed to optimize the geometry of this binding site at a detailed atomic level incorporating electron effects (i.e., quantum calculations, which are beyond the scope of the present work), the approximate geometry allows a reasonable selection of a number of residues likely to play a role in binding the cofactor. Thus, cysteines 57 and 357 should bind the iron atom, whereas phenylalanines 54 and 360 should stabilize the cofactor binding probably upon making C-X−π interactions between electronegative X = N, O atoms in CN−, and CO ligands and the π electron cloud of aromatic rings in phenylalanines.

Taking into account the preceding considerations, our molecular modeling study selects the following amino acids as residues likely playing a relevant role in both protein-protein interaction and cofactor binding in the HupK-HypC complex: HupK, Arg-30, Gln-52, Phe-54, Cys-57, Cys-357, and Phe-360; HypC, Cys-2, Leu-33, His-46, and Glu-58.

**Functional Analysis of HypC Residues Potentially Involved in the Interaction with HupK**—To study the functional role of HypC residues identified above, plasmids expressing HypCST variants altered in these residues were introduced into the hypC-deleted strain UPM1155(pALPF14), and transconjugants were analyzed for hydrogenase activity and processing. Only basal levels of hydrogenase activity were detected in microaerobic cultures expressing HypCST variants C2S and H46A (Table 5). Pea bacteroids induced by the strain expressing the HypCST C2S mutant exhibited no detectable hydrogenase activity, whereas the H46A derivative was associated to low but significant levels of the same activity (14% that in the wild-type, Table 5). Immunoblot analysis showed that crude extracts of these mutants accumulated the unprocessed form of the large structural subunit under both conditions (Fig. 4). These results indicate that HypC residues Cys-2 and His-46 are essential for hydrogenase synthesis under both free-living and symbiotic conditions.

We also analyzed the effect of the E58L exchange in HypC. In this case, the mutant variant complemented the hypC mutant to 70% that of the wild-type level of activity in microaerobic cells, whereas virtually full complementation was obtained in pea bacteroids (Table 5). Consistently, the level of processed HupL in crude extracts was reduced in free-living cultures, where some unprocessed form was still present (Fig. 4, top left panel). The level of HupL processing in bacteroids from this mutant was not significantly altered (Fig. 4, top right panel). Similar levels of HypB-immunoreactive bands were detected in all the extracts (Fig. 3, lower panels), indicating that hydrogenase induction was equally efficient for all strains in each treatment.

An L33A HypC variant was generated to check the potential relevance of this residue. Interestingly, the level of hydrogenase activity in this mutant was severely reduced in free-living cells as compared with the wild type. In contrast, the same protein variant was associated to wild-type levels of hydrogenase activity in pea bacteroids (Table 5). Consistently, analysis of the status of hydrogenase large subunit revealed that, whereas only the unprocessed form of HupL was detected in crude extracts from microaerobic cells of the L33A mutant, the processed form was present in bacteroids of this mutant at similar levels as in the wild type. These data suggest that Leu-33 might be necessary for hydrogenase biosynthesis in the presence of O₂ in *R. leguminosarum*.

To study whether the residue substitutions in HypC affected its ability to interact with HupK and HupL, pulldown experiments with soluble fractions of *R. leguminosarum* carrying HypCST variants were carried out. Immunoblot analysis using the Strep-Tactin-AP conjugate revealed that the mutation of these residues did not affect the stability of HypC (Fig. 5). Immunoblot analysis with the anti-HupK antiserum revealed that the E58L mutation led to a significantly reduced level of HupK co-purifying with HypC, suggesting a weaker interaction.
of HypC<sub>ST</sub>-E58L with HupK (Fig. 5). The other mutations tested had a stronger effect on the HupK-HypC interaction. Purified HypC<sub>ST</sub> C2S, L33A and H46A variants showed very low levels of co-purified HupK indicating that these residues are relevant for the interaction of HypC with HupK. The analysis with anti-HupL antiserum revealed that the mutant variants showed highly reduced levels of co-purified HupL (Fig. 5). This is consistent with the HupK-dependent HupL-HypC interaction described above.

Functional Analysis of HupK Residues Potentially Involved in Hydrogenase Biosynthesis—To study the significance of HupK residues identified in the modeling analysis, plasmids expressing HupK variants harboring mutants R30L, Q52L, F54C, C57A, C357A, and F360C were generated and transferred into the <i>hupK</i>-deleted strain UPM1155. Transconjugant strains were analyzed for hydrogenase activity and HupL biosynthesis and processing in microaerobic (1% O<sub>2</sub>) free-living cells and in bacteroids (Table 6). These experiments showed that mutations C57A and F360C were associated to hydrogenase levels on the same range as those present in the <i>hupK</i>-deleted mutant, used as negative controls, under both conditions assayed. Immunoblot analysis showed that crude extracts of these mutants only contained the unprocessed form of HupL in free living cells, whereas low levels of the processed form of HupL could be observed in bacteroid crude extracts of these two mutants (Fig. 6). Such levels were similar to the levels detected in the <i>hupK</i>-deleted strain. These results indicate that both Cys-57 and Phe-360 are essential for HupK function.

In the case of <i>hupK</i><sub>ST</sub> C357A mutant, the level of hydrogenase activity in microaerobic cultures was significantly reduced, but only a slight reduction in activity was detected in bacteroids (activity reductions of 82% versus 12%, Table 6). This result indicates that this residue is essential for hydrogenase synthesis under free-living conditions, whereas it might not be required under the ultralow oxygen tensions present in bacteroids.

<i>hupK</i><sub>ST</sub> variants carrying mutations R30L, Q52L, and F54C complemented the Δ<i>hupK</i> mutant to wild-type levels both for hydrogenase activity and HupL processing under free-living and symbiotic conditions. The results indicate that these residues are not critical for hydrogenase synthesis under the two conditions tested.
TABLE 6

Effect of amino acid substitutions in HupK on R. leguminosarum hydrogenase activity

Hydrogenase activities are expressed as percentages of hydrogenase activity associated with the wild-type strain UPM1155 (pALPF10/pPM165). The absolute values (100%) of hydrogenase activity (nmol of H₂/µg of protein⁻¹) for the wild-type strain were 12,866 ± 181 in microaerobic cultures and 4,048 ± 65 in pea bacteroids. Values are the average of three independent assays.

| Strain                  | Genotype          | Hydrogenase activity |
|-------------------------|-------------------|----------------------|
|                         |                  | % O₂      | Bacteroids |
| UPM1155 (pALPF10)       | ΔhupK             | 2 ± 1    | 10 ± 2     |
| UPM1155 (pALPF10/pPM165)| ΔhupK/hupKc₅₇A  | 100 ± 1  | 100 ± 2    |
| UPM1155 (pALPF10/pPM165.R30L)| ΔhupK/hupKc₅₇A.R30L | 119 ± 7 | 101 ± 3   |
| UPM1155 (pALPF10/pPM165.Q52L)| ΔhupK/hupKc₅₇A.Q52L | 84 ± 3  | 106 ± 14  |
| UPM1155 (pALPF10/pPM165.F₅₄C)| ΔhupK/hupKc₅₇A.F₅₄C | 101 ± 9 | 96 ± 8    |
| UPM1155 (pALPF10/pPM165.C₅₇A)| ΔhupK/hupKc₅₇A.C₅₇A | 4 ± 1    | 12 ± 2    |
| UPM1155 (pALPF10/pPM165.C₃₅₇A)| ΔhupK/hupKc₅₇A.C₃₅₇A | 18 ± 6 | 88 ± 1    |
| UPM1155 (pALPF10/pPM165.F₃₆₀C)| ΔhupK/hupKc₅₇A.F₃₆₀C | 5 ± 2    | 17 ± 1    |

FIGURE 6. Effect of amino acid substitutions in HupK on R. leguminosarum HupL processing. Immunodetection of HupL (upper panels) and HypB (lower panels) was carried out in crude extracts from vegetative cells induced for hydrogenase activity (1% O₂) and in bacteroid extracts. Strains: R. leguminosarum UPM1155 derivatives carrying plasmids pALPF10 (ΔhupK), pALPF10/pPM165 (hupKc₅₇A), pALPF10/pPM165.C₅₇A (hupKc₅₇A.C₅₇A), pALPF10/pPM165.F₅₄C (hupKc₅₇A.F₅₄C), pALPF10/pPM165.F₃₆₀C (hupKc₅₇A.F₃₆₀C), pALPF10/pPM165.R30L (hupKc₅₇A.R30L), and pALPF10/pPM165.Q52L (hupKc₅₇A.Q52L). Proteins were resolved in 9% (HupL) or 12% (HypB) acrylamide SDS-PAGE gels. Numbers in the right margin of the panels indicate the position of the molecular mass standards in kDa.

In Silico and Functional Evidence for HypC Dimerization—In immunoblot analysis of the eluates obtained from HypCST-expressing strains with AP-conjugated Strept–Tactin revealed the existence of an additional reactive band of ~16 kDa (Fig. 7A) in addition to the expected 8-kDa band corresponding to HypCST. This additional band does not likely correspond to an unspecified Strept–Tactin reactive protein, as it was absent in the eluate of a strain expressing HupPST (Fig. 7A). MALDI-TOF analysis of this band demonstrated that it contained exclusively sequences found within HypC protein (Fig. 7B) suggesting the existence of a HypC dimer. The in silico model of this HypC dimer revealed a highly stable structure, as demonstrated by the large number of intermonomer contacts computed with PISA (48, 49), the protein-protein interaction energy ~14.8 kcal/mol estimated with the DComplex approach (59), and the remarkable electrostatic complementarity between monomers (Fig. 7C). The existence of a double hydrogen bond between backbone nitrogen of Cys-2 and the two oxygen atoms of Glu-58 residues in both monomer chains with short interatomic distances of 2.58 and 2.63 Å (Fig. 7C) is noteworthy. This high stability suggests that the dimer might not be fully resolved by SDS treatment even in the absence of intermolecular covalent bonds.

DISCUSSION

In this work we have demonstrated that HupK is essential for the biosynthesis of hydrogenase in R. leguminosarum. This result is consistent with previous data obtained in other [NiFe] hydrogenase systems such as those from R. eutropha and Thio- capsas roseopersicina (26, 60). Recent experimental evidence showed that HupF, also an accessory protein linked to hydrogenase systems from aerobic bacteria (27). Our laboratory had previously shown that the R. leguminosarum hydrogenase system is expressed under symbiotic conditions in a NifA-dependent way that links its expression to that of nitrogenase (23, 61). It is thus conceivable that the levels of expression of HupK and HupF had been adjusted to the needs of a virtually anaerobic habitat such as the legume nodule (55) and might become limiting when the system is expressed under higher oxygen tensions.

The data presented here indicate a direct interaction between HypC and HupK. These data fit with the model proposed for R. eutropha (62) and T. roseopersicina (63), where the existence of a HypC–HupK complex was demonstrated by co-purification experiments. Here we also describe evidence of the existence of a physical association between HypC and HupL but only in the presence of HupK. In the cases of R. eutropha and T. roseopersicina no interaction between HypC and HupL was identified (26, 63, 64), although complexes involving HypC and
other hydrogenase large subunits were found for *T. roseopersicina* (63). It is possible that the *R. leguminosarum* HypC<sub>ST</sub> construct allows more stable associations than in the case of the N-terminal fusion proteins used in the other bacteria.

We recently used our model structure of HypC protein to study the binding of the Fe(CN)<sub>2</sub>CO cofactor to the isolated protein. The stability of the HypC-cofactor complex was addressed by means of molecular dynamics calculations, and residues involved in binding the cofactor in the isolated protein and in the HypCD complex were also identified (16). Cys-2 and His-46 residues in HypC were shown to participate in that binding. As shown in the present work, these two residues are also involved in relevant interactions occurring in the protein-protein interface of the HupK-HypC complex. Functional analysis with site-specific mutants revealed that HypC residues Cys-2 and His-46 are essential for hydrogenase synthesis and also for the interaction of HypC with HupK. Previous studies in *E. coli* had shown that Cys-2 is essential for interaction of HypC with HypD and HycE (11). *E. coli* HypC residues Cys-2 and His-51 (equivalent to His-46 in *R. leguminosarum*) are required for coordination of iron and CO<sub>2</sub> to the protein, probably direct precursors of the Fe(CN)<sub>2</sub>CO cofactor (15).

Our work has also allowed the identification of residues relevant for the ability to synthesize hydrogenase in the presence of oxygen. The phenotype associated to HypC L33A mutant indicates that this residue is relevant for hydrogenase synthesis under these conditions. It is tempting to speculate that this surface-exposed hydrophobic residue is involved in an interaction with another protein(s) during the biosynthetic process. HupL and HupF are candidate proteins for such interaction, which could take place at the late stages of the transfer of cofactor precursor into HupL.

The potential relevance of HypC Glu-58 residue was deduced from the modeling of HupK-HypC interaction. Although this residue is conserved in HypC from different hydrogenase systems (37), its functional relevance had not been studied before. *R. leguminosarum* HypC E58L mutant variant was associated to a significant reduction of hydrogenase activity in vegetative cells under 1% O<sub>2</sub>, but not in bacteroids, and also to a slight reduction in its ability to bind to HupK. These data correlate well with the *in silico* model for HupK-HypC interaction, in which hydrogen bonds of this residue with HupK residues Gly-6 and Ala-7 might contribute to complex stabilization. The mild phenotype of the mutant indicates, however, that these bonds might have a slight, additive effect on the stability of the interaction. According to the available data, the stability of the complex would be more relevant under free-living conditions.

15% acrylamide SDS-PAGE gels, and membranes were developed with AP-Strep-Tactin. The arrow indicates the band corresponding to the potential HypC<sub>ST</sub> dimer. Numbers in the left margin of the panels indicate the position of the molecular mass standards (kDa). B, amino acid sequences (bold letters) identified by peptide mass fingerprinting analysis of the band indicated by the arrow in panel A. C, predicted structure of HypC dimer. Shown is a ribbon diagram of the predicted structure of HypC dimer (upper, left), intermonomer hydrogen bonds between Cys-2 and Glu-58 residues (upper, right), and different views of the Poisson-Boltzmann electrostatic potential mapped onto the molecular surface (middle and lower) showing the electrostatic complementarity between monomers.
We present here the first in silico developed protein model for a member of the HupK family of hydrogenase accessory proteins. This model proposes a HupK structure compatible with that of a HupL-like chaperone protein able to bind a precursor form of the [NiFe] cofactor. In fact, structural comparisons between the model structure of HupK and a number of HupL structures from different bacteria show a remarkably good superposition of most of the architecture of HupK and the helical core of HupL proteins (Fig. 2). Of the 370 total residues of HupK, the structural superposition aligns the following number of residues (r.m.s.d. backbone atoms in parentheses) with HupL proteins from the following bacteria: 312 (1.107 Å) E. coli, 317 (1.186 Å) R. leguminosarum, 315 (1.148 Å) R. etutropha, 318 (1.196 Å) A. vinosum, and 321 (1.021 Å) D. vulgaris. The residues of HupK not structurally aligned with HupL segments are mainly located in loops and unstructured parts (Fig. 2). These structural superposition data suggest an evident resemblance between the HupK structure and the core architecture of HupL proteins without significant differences with regard to any particular species.

Binding of HoxV, the R. etutropha HupK homolog, to the Fe(CN)$_2$CO precursor was previously shown by FTIR analysis (26), establishing an interesting mechanistic parallelism with the biosynthesis of the cofactor of another complex metalloenzyme, the [FeMo] nitrogenase, whose [FeMo] cofactor is assembled in the NifEN chaperone proteins and then transferred to NifDK (65). In that case, however, the chaperone proteins are catalytically involved in the incorporation of several moieties of the cofactor for nitrogenase. In the case of R. leguminosarum hydrogenase, HupK would participate by just protecting the cofactor from the presence of oxygen, as nickel incorporation is carried out once the cofactor is transferred into HupL. Further investigation is required to gain insight on the mechanism through which HupK exerts this proposed protective action. The identified residues whose alteration has a differential role in microaerobic versus symbiotic conditions will be useful tools to elucidate such mechanism.

In the present study bacteroids of the hupK-deleted mutant exhibited low, but significant, levels of hydrogenase activity. Similar results were obtained in our laboratory with bacteroids of the hupF-deleted mutant (27). These data led us to initially hypothesize that under symbiotic conditions the cofactor transfer from HypC to pre-HupL via HupK and HupF might coexist with an alternative HupK/HupF-independent pathway. However, this is likely not the case, as a double deletion mutant ΔhupFΔhupK had a phenotype equivalent to those of hupF and hupK individual mutants under both microaerobic and symbiotic conditions (data not shown).

According to the protein model presented here, HupK residues Cys-57 and Cys-357 should bind the iron atom in the cofactor precursor, whereas Phe-54 and Phe-360 might participate in the interaction with other atoms of the cofactor. Comparative analysis of the phenotypes of mutants obtained in the hydrogenase systems from R. leguminosarum, R. etutropha, and T. roseopersicina (26, 60) indicate some similarities but also interesting differences. Mutations on Cys-57 or equivalent residues led to a drastic decrease of hydrogenase activity in all three systems, thus stressing the key nature of this residue for binding the precursor cofactor. This similar behavior of mutants across systems extends to Phe-54, for which mutations into cysteine showed no effect on phenotype in both R. leguminosarum and T. roseopersicina systems. For Cys-357, replacement by alanine in the R. leguminosarum protein had a strong effect on free-living cells but not on symbiotic cells. A similar mutation on the T. roseopersicina protein had no apparent effect and only a moderate effect in the case of R. etutropha. These results suggest that the role of Cys-357 is less relevant and likely linked to the protection of precursor cofactor in the presence of oxygen. For Phe-360, conversion of this residue into a cysteine was critical in the case of R. leguminosarum, whereas equivalent mutations had no significant effect on hydrogenase activity in T. roseopersicina (60) or in R. etutropha (26). Taken together these data indicate that the general role of HupK as a scaffolding protein for preserving Fe(CN)$_2$CO cofactor from oxygen is carried out in subtly different ways for different bacteria.

Evidence from immunoblot analysis and molecular modeling indicates that HypC can exist as a stable dimer. This dimerization capability might be a general feature for HypC, as SDS-PAGE-resistant homodimers involving HypC and HybG paralogs have been recently described also in E. coli (15). The dimeric structure predicted here for R. leguminosarum HypC would occlude residues Cys-2 and Glu-58, likely making the dimeric protein inactive. The existence of homo-oligomers is recognized as a form of allostery to regulate protein function (66), and the dimeric form of HypC protein could be a way to keep this protein in a stable conformation when low levels of ligands are present. Inspection of the immunoblot analysis of these mutants reveals the presence of this band in all cases (Fig. 5, middle panel), suggesting that other intermonomer contacts and global electrostatic and hydrophobic interactions are sufficient for dimer stabilization.

The available data suggest that the activities of HupK and HupF proteins are critical for incorporation of the cofactor precursor, thus allowing hydrogenase synthesis in the presence of oxygen. Additional experimentation is required to elucidate the precise mechanism that allows the transfer of this oxygen-sensitive metal cofactor under aerobic conditions, an aspect of obvious biotechnological interest.

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