The [NiFe]-hydrogenase accessory chaperones HypC and HybG of *Escherichia coli* are iron- and carbon dioxide-binding proteins

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**Article info**

Article history:
Received 5 June 2013
Revised 27 June 2013
Accepted 27 June 2013
Available online 10 July 2013

Edited by Peter Brzezinski

**Keywords:**
Carbon dioxide
Hydrogenase
Iron
Infrared spectroscopy
Metalloprotein
Maturation

**Abstract**

[NiFe]-hydrogenase accessory proteins HypC and HypD form a complex that binds a Fe–(CN)\(_2\)CO moiety and CO\(_2\). In this study two Hyp homologues from *Escherichia coli* were purified under strictly anaerobic conditions and both contained sub-stoichiometric amounts of iron (approx. 0.3 mol Fe/mol HypC). Infrared spectroscopic analysis identified a signature at 2337 cm\(^{-1}\) indicating bound CO\(_2\). Aerobically isolated HypC lacked both Fe and CO\(_2\). Exchange of either of the highly conserved amino acid residues Cys2 or His51 abolished both Fe- and CO\(_2\)-binding. Our results suggest that HypC delivers CO\(_2\) bound directly to Fe for reduction to CO by HypD.

**Structured summary of protein interactions:**
HypC and HypC bind by comigration in sds page (View interaction)
HybG and HybG bind by comigration in sds page (View interaction)

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**1. Introduction**

[NiFe]-hydrogenases catalyze both H\(_2\) evolution as well as H\(_2\) oxidation [1]. The catalytic subunit of the enzyme harbours a NiFe–(CN)\(_2\)CO cofactor and its synthesis requires the coordinated activities of a number of highly conserved Hyp accessory proteins [2,3]. The cyanide ligands (CN\(^-\)) are derived from carbamoylphosphate [4,5], while the metabolic origin of the carbonyl ligand (CO) is still unresolved.

Recent studies have revealed that biosynthesis of the Fe–(CN)\(_2\)CO moiety is likely to occur on an assembly platform comprising minimally the HypC, HypD and HypE proteins, with HypD forming the key scaffold [6–8]. Fourier-transform infrared (FT-IR) analysis of an anaerobically isolated complex of HypC and HypD (HypCD) revealed signatures for CO and two CN\(^-\) ligands [6,7]. Moreover, an additional contribution at 2337 cm\(^{-1}\) was identified, which is consistent with the asymmetrical stretch vibration of CO\(_2\) [6]. The peak is characteristic of bound CO\(_2\) and can be clearly distinguished from CO\(_2\) dissolved in water (2342 cm\(^{-1}\)) and gaseous (2349 cm\(^{-1}\)) CO\(_2\) [9,10].

Once the Fe–(CN)\(_2\)CO group is synthesized, it is proposed that the moiety is transferred by HypC to a precursor form of the hydrogenase large subunit [3,11]. This proposition is based on the fact that as well as forming a complex with HypD [11,12], HypC also has been isolated in complex with the hydrogenase large subunit [13]. The combined results of these studies have led to a formulation of the HypC cycle in which the 10 kDa protein acts as a chaperone shuttling between the iron–sulfur protein HypD and the hydrogenase large subunit apo-protein [11].

The HypC/HupF superfamily comprises proteins of approximately 90 amino acid residues [14–16]. Early mutagenesis studies revealed that HypC has a highly conserved cysteinyl residue at amino acid position 2, which is essential for complex formation with HypD and for maturation activity [11,17]. The crystal structure of HypC [18] revealed that this N-terminal cysteinyl residue is located in an OB-fold and is in proximity to a conserved histidine residue (His51 in *Escherichia coli*). It has been suggested that these two residuescoordinate the iron ion of the Fe–(CN)\(_2\)CO cofactor [19].

Many organisms that synthesize multiple hydrogenases have more than one HypC homolog, e.g., *E. coli* synthesizes the homologs...
HypC and HybG [20]. Mutants lacking hypC fail to complete hydrogenase 3 (Hyd-3) maturation and consequently lack an active hydrogen-evolving formate hydrogenlyase (FHL) complex [2,20]. The hybG gene, in contrast, is located within the hyb operon encoding both structural and accessory components of Hyd-2 [21]; HybG is required for biosynthesis of both Hyd-1 and Hyd-2, and interacts with both apo-large subunits [20,22].

The combined action of the carbamoyl transferase HypF and the ATP-dependent dehydratase HypE supplies the cytochrome ligands and HypD is the enzyme that assembles the Fe–(CN)₆²⁻ CO moiety [8]. The metabolic origin and route of delivery of the cytochrome ligands are both unclear. Moreover, little is known about how the iron ion in the Fe–(CN)₆²⁻ CO moiety is supplied to HypD, as recent evidence suggests that the route is independent of the FeS biosynthetic machinery [23]. Here we present evidence that the HypC superfamily represents a new class of Fe- and CO₂-binding proteins, suggesting that HypC delivers Fe along with CO₂ to the reductase-active HypD scaffold protein where reduction to CO probably takes place.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The E. coli strains used included MC4100 [24], DHB-G (ΔhybG) [20], DHP-C (ΔhypC) [2], BEF314 (ΔhypB-E) [2], DHP-H2 (ΔhypF) [25] and BL21(DE3) [26]. The plasmids used included phypDHis [8], pT-hypDEFStrep [12], pASK-hybG encoding C-terminally Strep-tagged HybGStrep, and pC176 encoding C-terminally His-tagged HypCHis (Novagen) to generate the plasmid phypC. To generate plasmids of different combinations we used either immediately or stored at −20°C until use.

2.2. Protein purification

Unless stated otherwise, all steps were carried out under anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, USA). Hi- and Strep-tagged proteins and protein complexes were purified exactly as described previously [6,8].

2.3. FTIR spectroscopy

Fourier-transform infrared (FT-IR) spectroscopy was performed exactly as described [8] using on a Tensor27 (Bruker Optik, Ettlingen, Germany) equipped with a three-reflection silicon crystal attenuated total reflection (ATR) cell (Smith Detection, Warrington, USA). Protein samples (typically 1 μg ml⁻¹ of HypC or HybG) were dried on top of the ATR crystal under pure N₂ or air by help of home made gas mixers. HypC was isolated from B. soboh transformed with pBEF314 (ΔhybBCDE) and samples were incubated with up to 20 mM EDTA in buffer W for 5 min prior to ATR analysis of the mixture. Oxidation of HypC or HybG by H₂O₂ was performed on wetted films [8]. All spectra were recorded at room temperature.

2.4. Non-heme Fe determination

Iron content was determined by inductively coupled plasma mass spectrometry (ICP-MS) exactly as described [6]. For ICP-MS analysis 0.1 mg of purified HypC or HybG (1 mg ml⁻¹) was used and samples were analyzed for iron, nickel, zinc, and copper.

2.5. Other methods

SDS-PAGE was performed using 15% (w/v) polyacrylamide as described [27] and polypeptides were transferred to nitrocellulose membranes as described [28]. Determination of protein concentration was done as described [29].

UV-vis spectroscopy was performed as described [6]. The protein concentration of HypC or HybG variants was 5 mg ml⁻¹.

3. Results and discussion

3.1. Oxygen-labile coordination of Fe by HypC and HybG

The HypC100 protein was readily purified from crude extracts of BL21(DE3) and migrated as an approximately 10 kDa polypeptide in SDS-PAGE (Fig. 1A). If cell disruption and affinity chromatographic steps on Co²⁺-NTA Sepharose were performed aerobically, enriched HypC100 was colorless and had no associated cofactors. If oxygen was excluded from all steps, however, the resulting HypC protein was brown in color. The Fe content of purified HypC was determined by inductively coupled plasma-emission mass spectrometry (ICP-MS) to be 0.32 ± 0.08 mol Fe per mol protein. To rule out that Fe was non-specifically bound to the C-terminal His-tag, a version of HypC that was constructed with a Strep-tag in the same position. After over-production in BL21(DE3) and anaerobic isolation on Strepactin–Sepharose HypCStrep had on average 0.31 ± 0.06 mol Fe associated with the protein. Isolation of the tagged proteins in air resulted in a ten-fold decrease in Fe concentration (<0.03 mol).

Fusion of the His-tag with Cys2 at the N-terminus of HypC resulted in a protein that failed to restore hydrogenase 3 activity to an E. coli hypC mutant (not shown) and when isolated by anaerobic purification, HypC was essentially colorless and devoid of Fe (<0.01 mol) as determined by ICP-MS. This result indicated that, despite the Cys residue being present, no stable Fe bound to the protein presumably because the amino terminus of the cysteiny1 residue was blocked by the His-tag.

To determine whether Fe-binding was a general feature of HypC proteins, we isolated both C-terminally His- and Strep-tagged
variants of the E. coli homolog, HybG (Fig. 1A). It was noted that purified HybGStrep revealed both a 10 kDa and a 20 kDa polypeptide upon SDS–PAGE analysis (Fig. 1A). Western blot analysis with anti-Strep antiserum confirmed that the 20 kDa band contains Strep-tagged polypeptide and mass spectrometry identified the band as HybG. A similar observation was made for HypCHis, suggesting that both HypC and HybG are capable of forming minimally homodimers; however, why they were resistant to denaturation in SDS buffer remains to be resolved. Attempts to determine the oligomeric state of HypC or HybG by anaerobic gel filtration or native PAGE revealed no discrete peak but only multiple oligomeric species suggesting that native HypC readily forms higher-order structures.

Metal analysis revealed a similar Fe ratio for HybGStrep and HypCHis (0.3 ± 0.01 mol Fe/mol protein and 0.34 ± 0.01 mol Fe/mol protein, respectively). UV–visible spectroscopy of HybGStrep revealed an absorbance peak at 420 nm (Fig. 1B). Together, these findings suggest that HypC might supply the Fe ion for biosynthesis of the Fe–(CN)2CO moiety. This finding would be consistent with recent results[23], which indicate that the Fe ion for cofactor biosynthesis is not supplied by the Isc or Suf iron–sulfur cluster biogenesis pathways.

3.2. Anaerobically isolated HypC and HybG reveal FT-IR signatures of bound CO2

Recent studies revealed that in addition to absorption bands at 2095 cm⁻¹, 2073 cm⁻¹ and 1955 cm⁻¹ characteristic of metal-bound CO and CN⁻ ligands the anaerobically isolated HypCD complex also exhibited a signature at 2337 cm⁻¹, which was assigned to the asymmetrical stretch vibration (ν₃) of CO₂ [6] (see also Fig. 2A, spectrum b). Purified HypD shows signatures of CO and CN⁻ exclusively [8] (see Fig. 2A, spectrum a). Infrared analysis of anaerobically isolated HypC and HybG proteins treated with 15% (v/v) H₂O₂. This serves as a measure of Fe concentration and is plotted against cEDTA (sigmoidal fit with R = 0.98). Both Fe and CO₂ are lost upon increasing the EDTA concentration. BSA serves as a negative control (triangles).

To rule out that HypD or any of the other Hyp accessory proteins, was required for iron- and CO₂ binding HybGStrep was purified anaerobically from strain BEF314 (ΔhypB-E) [2]. The protein...
had an iron content of approximately 0.3 (determined with two independent preparations) and it revealed a sharp peak at 2337 cm⁻¹ (Fig. 2A, spectrum f). Similarly, results were obtained when HybGStrep was purified from DHP-F2 (ΔhypF) (data not shown).

3.3. Cys2Ala and His51Arg variants do not show the CO₂ signature

A Cys2Ala variant of HybGStrep lacked iron (<0.01 mol Fe) and FT-IR analysis of the anaerobically isolated protein showed no absorbance band at 2337 cm⁻¹ (spectrum similar to d in Fig. 2A). Similarly, a His51Arg variant of HybGStrep also lacked the absorbance band at the 2337 cm⁻¹ wavenumber (not shown) and ICP-MS analysis showed that His51Arg-substituted HybGStrep does not bind iron (<0.01 mol Fe). In a previous study it was reported that a His51Arg variant of HybG lacked hydrogen gas production [31]. Together, these data indicate that Cys2 and His51 are required for coordination of both Fe and CO₂.

4.2. Strong correlation between Fe- and CO₂-binding suggested from reactions with EDTA and H₂O₂

Treatment of either HybGStrep or HybGStrep with EDTA resulted in concomitant loss of bound Fe and the CO₂ signature at 2337 cm⁻¹. To probe the loss of Fe spectroscopically, samples of HybGStrep were treated with up to 20 mM EDTA and a H₂O₂/water vapor mixture as reported earlier [8]. In a process commonly referred to as Fenton chemistry, H₂O₂ is reduced by Fe²⁺ to produce OH⁻ radicals, which rapidly oxidize the amino acid environment of the metal site [32]. The CO₂ released in this reaction is detected as a transient off-gas unspecifically bound to the protein film [33,34]. A new peak forms at 2337 cm⁻¹ whose intensity is a direct measure of Fe²⁺ concentration. As the ν3 mode is vibrationally insensitive to the ligand of CO₂ [10,35], ‘natural’ and Fenton CO₂ both appear at the same position. It is important to note that CO₂ was detected in as-isolated samples only under strictly reducing conditions. Thus, contamination of the natural signal with Fenton CO₂ is highly unlikely. Fig. 2B shows the decrease of Fenton CO₂ (circles) as a function of EDTA concentration in comparison to the loss of ‘natural’ CO₂ (squares) in as-isolated HybGStrep. Both natural and Fenton-generated CO₂ were sequentially measured on the same sample. Bovine serum albumin (BSA, triangles) was used as a negative control to ensure that the CO₂ released in the H₂O₂ assay depended exclusively on Fe and was not directly affected by incubation with EDTA. The bending mode of gaseous CO₂ in the far-IR (667 cm⁻¹) is more suitable to analyze the chemical nature of the binding partner [10,36]; however, we failed to detect the ν2 absorption band in HybC and HybG due to low signal intensity.

Together, these results are consistent with the proposal [37] that metabolic CO₂ could be the origin of the CO ligand. This is also in accord with ¹³C-labeling studies in which exogenously supplied CO₂ failed to result in a red-shift in the absorption band of CO at 1955 cm⁻¹ [38]. Despite being highly characteristic, the asymmetric stretch vibration of CO₂ is not informative with regard to ligand partners. Mascetti and Tranquile reported that CO₂ binds to transition metals like Fe via the carbon atom, leaving the ν3 vibration largely unaffected [35]. Both the observation of a concerted loss of Fe and CO₂ upon EDTA treatment plus the precise overlap of natural and Fenton CO₂ at 2337 cm⁻¹ in Fig. 2B supports this coordination model.

4. Conclusions

The findings presented in this study demonstrate that HybC/HybG binds both Fe and CO₂ and this is independent of the other

Hyp accessory proteins. Residues Cys2 and His51 of HypC are both essential for binding of CO₂ and Fe. Together with our recent demonstration that HypD is the scaffold on which the Fe–(CN)₂CO moiety is assembled [8], the findings presented here strongly suggest that HypC proteins deliver an Fe with bound CO₂ to the redox-active HypD where reduction to CO occurs. Future studies will be required to prove that both the Fe ion and CO₂ attached to HypC are the direct precursors of the Fe–(CN)₂CO moiety.

Acknowledgements

The authors are indebted to Denise Hübner for help with protein purification and to Chris Pickett and Wolfgang Weigand for discussion. The work conducted in the authors’ laboratories was supported by EFRE funds of the EU and the DFG (Grant SA 494/3–1, 494/3–2) to R.G.S. and by the BMBF (H₂ design cells) to J.H.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.055.

References


