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# [NiFe]-hydrogenase maturation: Isolation of a HypC–HypD complex carrying diatomic CO and $CN^-$ ligands

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ABSTRACT

**The HypC and HypD maturases are required for the biosynthesis of the $Fe(CN)_2CO$ cofactor in the large subunit of [NiFe]-hydrogenases. Using infrared spectroscopy we demonstrate that an anaerobically purified, Strep-tagged HypCD complex from *Escherichia coli* exhibits absorption bands characteristic of diatomic CO and $CN^-$ ligands as well as $CO_2$. Metal and sulphide analyses revealed that along with the $[4Fe–4S]^{2+}$ cluster in HypD, the complex has two additional oxygen-labile Fe ions. We prove that HypD cysteine 41 is required for the coordination of all three ligands. These findings suggest that the HypCD complex carries minimally the $Fe(CN)_2CO$ cofactor.**


## 1. Introduction

[NiFe]-hydrogenases are heteromeric enzymes that catalyze the reversible activation of dihydrogen. The large subunit of these enzymes harbours a $NiFe(CN)_2CO$ cofactor, which is responsible for catalysis [1,2]. The cofactor is coordinated through four conserved cysteinyl residues in the large subunit of the enzyme and the Fe ion has one CO and two $CN^-$ ligands [3,4]. Synthesis of a functionally active hydrogenase enzyme requires the coordinated activities of a number of highly conserved accessory enzymes amongst which the Hyp proteins have a prominent role [5]. Current evidence clearly indicates that insertion of the $Fe(CN)_2CO$ cofactor into a precursor form of the large subunit occurs prior to insertion of the nickel ion [5,6]. Thus, synthesis of the $Fe(CN)_2CO$ cofactor is possibly completed on a maturase protein complex before being inserted into its destination substrate, the large subunit of hydrogenase. The components of this maturase complex are likely to be the HypC, HypD, HypE and HypF proteins, which are required for the synthesis of the $Fe(CN)_2CO$ cofactor of all [NiFe]-hydrogenases [5,7,8]. Consequently, mutants lacking any one of the genes encoding these enzymes have a hydrogenase-negative phenotype [9].

Studies carried out with the Knallgas bacterium *Ralstonia eutropha* and the enterobacterium *Escherichia coli* have demonstrated that the $CN^-$ ligand is made from carbamoylphosphate [5], while the origin of the CO is different [10–12]. In an ATP-dependent reaction the carbamoyltransferase HypF transfers a carbamoyl moiety to the C-terminal cysteinyl residue of HypE [7,8,13,14], which then catalyses the ATP-dependent dehydration of the thiocarboxamide to a thiocyanate group. In vitro studies have demonstrated that the $CN^-$ moiety can be transferred to the HypCD complex, provided that it is isolated anaerobically; however, neither component individually can accept the $CN^-$ group, suggesting the proteins share coordination of the cofactor [15]. Based on the chemical properties of the HypCD–CN complex [15] it has been proposed that the $CN^-$ is coordinated either to a mononuclear Fe species or to one of the Fe ions of the $[4Fe–4S]^{2+}$ cluster of HypD. However, as HypD is the only Hyp protein that has a low-potential $[4Fe–4S]^{2+}$ cluster [5,15,16], and a two-electron reduction step is presumably required for the attachment of each of the ligands to the iron, it is unlikely that the $CN^-$ and CO groups are attached to one of the Fe ions of the [4Fe–4S] cluster. Instead, it has been proposed that one of two groups of highly conserved cysteinyl residues play an important role in coordinating the iron atom of the $Fe(CN)_2CO$ cofactor [17]. In particular, Cys41 of HypD has been shown to be important for enzyme function [17].

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The form in which the $CN^-$ group is bound to the HypCD complex, as well as the steps subsequent to its transfer to the complex, are also unclear and nothing is really known about how and when the CO ligand is introduced into this complex.

Due to the fact that the HypC protein of *E. coli* has been shown to interact with the precursor form of the large subunit of hydrogenase 3 (Hyd-3) [18], this has led to the proposal that HypC proteins deliver the $Fe(CN)_2CO$ group directly to the hydrogenase active site, suggesting that the HypCD complex must be a carrier of, or generate, the cofactor [5]. An exception in this regard is the HoxV maturase of *R. eutropha,* which has been shown to carry a $Fe(CN)_2CO$ cofactor and it has been proposed to deliver this cofactor to a specific class of membrane-bound hydrogenases that are cytochrome *b*-linked and found in certain proteobacteria [19].

As with Cys41 of HypD, the highly conserved and essential Cys2 of HypC has been shown to be essential for interaction with both the HycE large subunit precursor of *E. coli* and HypD [20], suggesting that this residue too might be involved in metal centre coordination. Nevertheless, no direct evidence for the proposed coordination of the $Fe(CN)_2CO$ group on the HypCD complex has been provided. In this study we demonstrate that the HypCD complex carries CO, $CN^-$ and $CO_2$ ligands and that the presence of these correlates strongly with two labile iron atoms in the complex. The highly conserved Cys41 of HypD is essential for coordination of all three ligands.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and growth conditions

The strains used during this study included MC4100 [21], DHP-D (Δ*hypD*) [9] and BL21(DE3) [22]. Plasmid pT-hypDEFCStrep [15] was used as the source of $HypC_{Strep}$–HypD and was also used as a template for site-directed mutagenesis (using the QuickChange procedure of Stratagene) of codon 41 of *hypD* to encode Ala instead of Cys using oligonucleotides 5′-CGGATTATGGAAGTGGCGGGCGGT CATACCCAC-3′ and 5′-GTGGGTATGACCGCCCGCCACTTCCATAATC CG-3′, which resulted in plasmid pT-hypDEFCStrep[C41A].

For overproduction of the $HypC_{Strep}$–HypD complex, *E. coli* strain BL21(DE3) transformed with pT-hypDEFCStrep or pT-hypDEFCStrep[C41A] was grown in modified TB medium [23], containing 100 μg/ml of ampicillin, on a rotary shaker at 37 °C until an optical density of 0.4 at 600 nm was reached. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at that point to a final concentration of 0.3 mM to induce gene expression. After induction, the culture was incubated at 30 °C for a further 3 to 5 h. Cells were harvested ($OD_{600\ nm}$ of 1.0) and cell pellets were used either immediately or stored at −20 °C until use.

For preparation of crude extracts to analyse hydrogenase enzyme activity *E. coli* cells were cultivated anaerobically at 37 °C in buffered TYEP medium [24] supplemented with 0.8% (w/v) glucose.

### 2.2. Preparation of crude extracts and protein purification

All steps were carried out under anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, USA). Wet cell paste was resuspended at a ratio of 1 g per 3 ml buffer W (100 mM Tris–HCl, 150 mM NaCl, pH 8.0) including 2 mM sodium dithionite, 5 μg DNase/ml and 0.2 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and debris were removed by centrifugation for 30 min at 50 000*g* at 4 °C. The supernatant derived from 10 g wet weight of cells was used for anaerobic purification of the $HypC_{Strep}$–D complex. Proteins were isolated using an 8-ml gravity-flow Strep-Tactin-Sepharose column, (IBA, Göttingen, Germany). Unbound proteins were removed by washing with 5 column volumes of buffer W. Recombinant $HypC_{Strep}$–HypD complex was eluted with buffer W including 5 mM desthiobiotin. Desthiobiotin was subsequently removed by passage through a series of Hi-Prep PD10 desalting columns (GE Healthcare) connected to an ÄKTA apparatus (GE Healthcare). Proteins were concentrated by centrifugation at 7500*g* using centrifugal filters (Amicon Ultra, 50 K, Millipore, Eschborn, Germany). About 1.5 mg of $HypC_{Strep}$–D complex could be purified from 1 g of wet-weight of cells.

To determine the effect of EDTA on the $HypC_{Strep}$–HypD complex approximately 2 mg of anaerobically purified $HypC_{Strep}$–D complex was treated with buffer W containing 5 mM EDTA for 30 min. To remove excess EDTA, the mixture was subsequently desalted anaerobically using a Hi-Prep PD10 desalting column equilibrated with buffer W and used for metal analysis. For analysis of the $HypC_{Strep}$–HypD complex by UV–Vis spectroscopy after EDTA treatment, the same procedure was used except the desalting step was omitted.

### 2.3. Polyacrylamide gel electrophoresis and protein determination

Non-denaturing polyacrylamide gel electrophoresis (PAGE) using 7.5% (w/v) polyacrylamide with subsequent staining for hydrogenase enzyme activity was performed exactly as described[25]. SDS–PAGE was performed using 15% (w/v) polyacrylamide as described [26]. Determination of protein concentration was done as described [27].

### 2.4. UV–visible spectroscopy

UV–Vis spectroscopy was performed exactly as described in [28]. The protein concentration of $HypC_{Strep}$–HypD complex was 11 mg $ml^{-1}$ and that of $HypC_{Strep}$–$HypD_{C41A}$ complex was 9.5 mg $ml^{-1}$.

### 2.5. FTIR spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was conducted on a Tensor27 (Bruker Optik, Ettlingen, Germany) equipped with a three-reflection silicon crystal attenuated total reflection (ATR) cell (Smith Detection, Warrington, USA). The spectrometer was permanently run under dry $N_2$ in an air-tight glove box (Coy Laboratories, Grass Lake, USA) with less than 10 ppm $O_2$ and no $H_2$. Protein samples (typically 1 μl of 10 mg $ml^{-1}$ $HypC_{Strep}$–HypD) were dried on top of the ATR crystal under pure $N_2$ or air by help of home-build gas mixers. All spectra were recorded at room temperature.

### 2.6. Non-heme iron and acid-labile sulfide determination

Iron and acid-labile sulfide were determined as described previously [29,30]. Iron content was confirmed by inductively coupled plasma mass spectrometry (ICP-MS) [28]. For ICP-MS analysis 0.1 mg of purified $HypC_{Strep}$–D complex (1 mg $ml^{-1}$) was used.

## 3. Results and discussion

### 3.1. Identification of $CN^-$ and CO ligands in the HypCD complex

Previous studies demonstrated that the HypC and HypD maturases form a complex and that $^{14}CN^-$ could be transferred to this complex only if the HypE and HypF proteins were supplied [15]. No transfer occurred to either HypC or to HypD alone and the

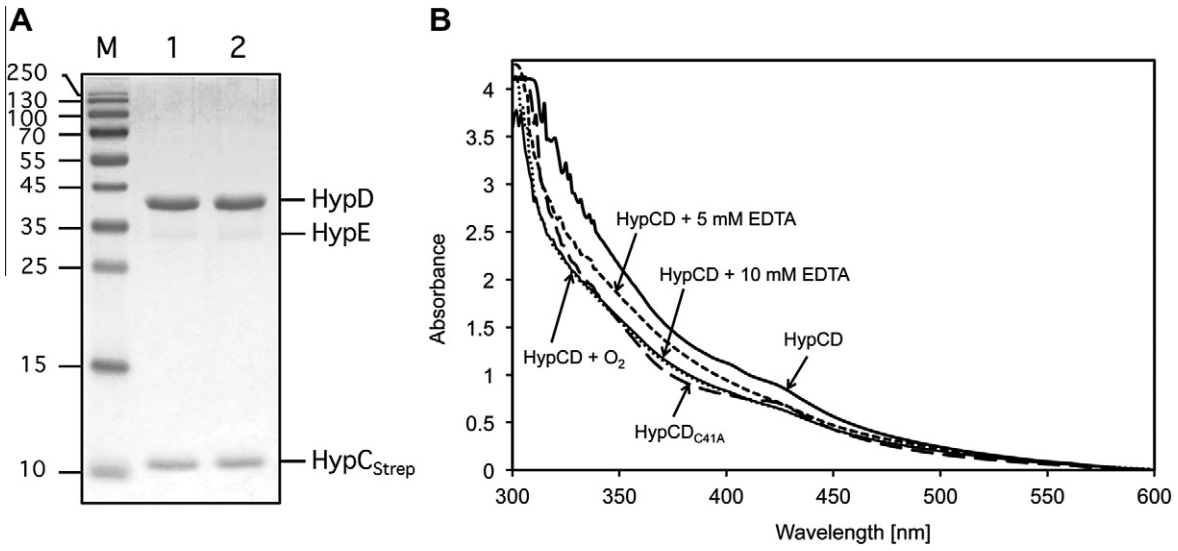


**Fig. 1.** (A) Analysis of purified HypC$_{Strep}$–HypD complexes. Purified HypC$_{Strep}$–HypD complexes (5 μg of each) were separated by SDS–PAGE (15% w/v polyacrylamide) and stained with Coomassie Brilliant Blue. Lane M, PageRuler-Plus prestained molecular mass marker in kDa (Fermentas); lane 1, HypC$_{Strep}$–HypD; lane 2, HypC$_{Strep}$–HypD$_{C41A}$. (B) UV–Vis absorption spectra of anaerobically purified HypC$_{Strep}$–HypD complex (full line; 11 mg ml$^{-1}$), HypC$_{Strep}$–HypD$_{C41A}$ complex (broad dotted line; 9.5 mg ml$^{-1}$), aerobically purified HypC$_{Strep}$–HypD complex (fine dotted line; 9.0 mg ml$^{-1}$) and anaerobically isolated HypC$_{Strep}$–HypD complex (11 mg ml$^{-1}$) treated with 5 mM (dotted line) or 10 mM EDTA (full line) were recorded between 300 and 600 nm. In the interest of clarity the HypC$_{Strep}$–HypD complexes are designated HypCD with the indicated treatment.

transfer of the labelled $CN^-$ group was inhibited by oxygen. Together, these findings indicated that the HypCD complex is the acceptor of the $CN^-$ product derived from the activity of the HypF–HypE carbamoyltransferase-dehydratase complex and that an iron atom in the complex coordinated it [15]. To provide evidence in support of this proposal we undertook to analyse the HypC$_{Strep}$–HypD complex by Fourier-transform infrared spectroscopy. The HypC$_{Strep}$–HypD complex was purified using affinity and gel-filtration chromatographies under strictly anaerobic conditions from *E. coli* BL21(DE3) transformed with plasmid pT-hypDEFCStrep [15]. As shown in Fig. 1A, the complex was close to homogenous and, as reported earlier [15], reproducibly contained sub-stoichiometric amounts of HypE. Henceforth, this ternary complex will be referred to as HypC$_{Strep}$–HypD.

Anaerobic UV–Vis spectroscopy of the complex revealed a broad maximum around 420 nm (Fig. 1B), consistent with the presence of the $[4Fe–4S]^{2+}$ cluster previously identified in HypD [15]. Additional features between 350–410 nm were observed, which were not present in isolated HypD. The nature of these features remains unclear but suggests the presence of a chromophore in addition to the Fe–S cluster.

The anaerobically isolated complex contained six Fe and approximately four acid-labile S per mol complex as determined colorimetrically (Table 1). Metal analysis determined by inductively-coupled plasma mass spectrometry (ICP-MS) confirmed that the as-isolated HypC$_{Strep}$–HypD complex contained six Fe. Notably, the amount of Ni, Cu, Co, and Zn was below the detectable limit. Analysis of aerobically isolated HypC$_{Strep}$–HypD complex identified only four Fe and approximately three S per mol of complex (Table 1), suggesting that these formed the $[4Fe–4S]^{2+}$ cluster. Sulfides in $[4Fe–4S]^{2+}$ clusters are readily oxidized [30] possibly accounting for the slightly lower content determined in the aerobically isolated HypC$_{Strep}$–HypD complex. UV–Vis spectroscopic analysis of aerobically isolated HypC$_{Strep}$–HypD complex revealed a reduction in the intensity of the features between 350–410 nm (Fig. 1B). Together, these findings strongly suggest that the additional two Fe ions in the complex are labile and were lost during aerobic purification. To provide further support for this contention, the anaerobically isolated HypC$_{Strep}$–HypD complex was incubated with EDTA (see Section 2) and, after anaerobic gel filtration chromatography, metal analysis revealed that only four mol Fe per mol complex were detectable (Table 1). This indicates that the two additional Fe atoms were accessible to chelator while those of the $[4Fe–4S]^{2+}$ cluster were not. UV–Vis spectroscopic analysis of HypC$_{Strep}$–HypD complex treated with 5 mM or 10 mM EDTA revealed a progressive decrease in the intensity of the absorption features in the 350–410 nm range and treatment of the complex with 10 mM EDTA resulted in a spectrum similar to that of the aerobically isolated HypC$_{Strep}$–HypD complex (Fig. 1B). Two highly conserved motifs (motif 1: $C^{41}$-G-X-$H^{44}$-X-H and motif 2: G-P-G-$C^{69}$-$P^{70}$-$V^{71}$-$C^{72}$-X-X-$P^{75}$) within HypD have been shown to be essential for hydrogenase maturation [17] and it is conceivable that the two additional Fe ions are coordinated by these motifs.

Analysis of the as-isolated HypC$_{Strep}$–HypD complex by FTIR revealed strong absorption bands at 2096 cm$^{-1}$, 2073 cm$^{-1}$ and 1955 cm$^{-1}$ (Fig. 2), which correspond to vibrational frequencies of the $CN^-$ and CO diatomic ligands found in [NiFe]- and [FeFe]-hydrogenases [4,31] and which have been observed in Hyd-2 of *E. coli* [11]. An additional band centred at 2337 cm$^{-1}$ can be assigned to the asymmetrical stretch vibration of $CO_2$ [32]. The $(CN)_2CO$ site on HypC$_{Strep}$–HypD gives rise to atypically broad peaks (Fig. 2). This reflects heterogeneity in complex formation and a coordination sphere less well-defined than for functional hydrogenases [4,11,31]. In this respect HypCD differs from other maturases too [33]. The aerobically isolated HypC$_{Strep}$–HypD complex showed no absorption bands when analysed by FTIR (data not shown), suggesting that the CO and $CN^-$ ligands might be coordinated by one of the labile Fe atoms.

### *3.2. Cys-41 of HypD is required for $CN^-$, CO and $CO_2$ ligation but not for HypC–HypD complex formation*

Previous studies have shown that the cysteinyl residue at amino acid position 41 in *E. coli* HypD is essential for maturase activity [9,17] and because the $CN^-$ ligand from HypE can be transferred to the HypCD complex [15], it is therefore conceivable that Cys41

**Table 1**
Determination of iron and sulfide content of the $HypC_{Strep}$–HypD preparations.

| Protein sample | Non-heme Fe (mol per mol of protein) | Sulfide (mol per mol of protein) |
|---|---|---|
| *Colorimetric*[a] | | |
| $HypC_{Strep}$–HypD (anaerobic) | 5.9 ± 0.45 | 3.8 ± 0.24 |
| $HypC_{Strep}$–HypD (aerobic) | 4.1 ± 0.3 | 3.1 ± 0.22 |
| [b]$HypC_{Strep}$–HypD (anaerobic) (5 mM EDTA) | 3.9 ± 0.35 | 2.9 ± 0.39 |
| $HypC_{Strep}$–$HypD_{C41A}$ (anaerobic) | 4.8 ± 0.4 | 3.8 ± 0.22 |
| *ICP-MS*[a] | | |
| $HypC_{Strep}$–HypD (anaerobic) | 6.04 ± 0.01 | n.d[c] |
| $HypC_{Strep}$–HypD (aerobic) | 4.24 ± 0.08 | n.d |
| $HypC_{Strep}$–HypD (5 mM EDTA) | 4.05 ± 0.03 | n.d |
| $HypC_{Strep}$–$HypD_{C41A}$ (anaerobic) | 4.88 ± 0.02 | n.d |

[a] Method used for iron determination. Molar ratios are calculated based on the molecular mass of a stoichiometric $HypC_{Strep}$–HypD complex. Data are the average of at least two independent determinations ± standard error.
[b] Anaerobically purified $HypC_{Strep}$–HypD complex was treated with 5 mM EDTA under anaerobic conditions.
[c] n.d. not determined.

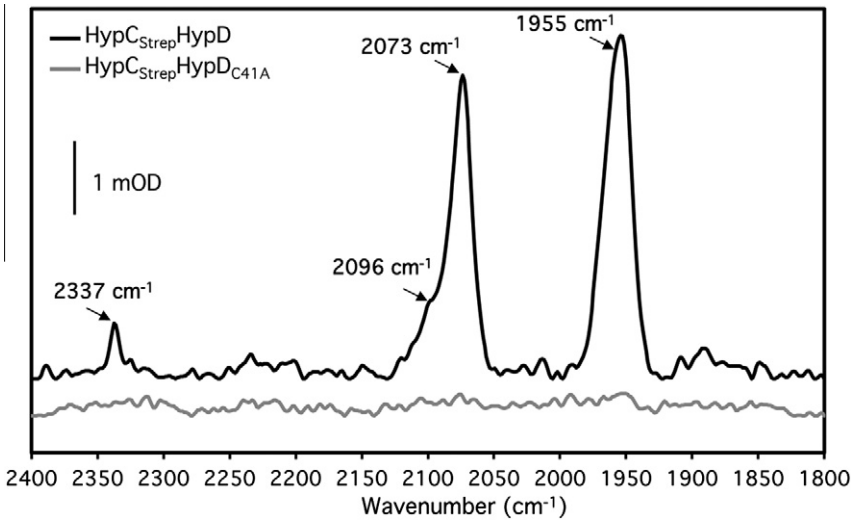


**Fig. 2.** FTIR spectra of purified $HypC_{Strep}$–HypD complexes. Protein samples were dried under pure $N_2$ on a three-reflection silicon ATR crystal. The displayed spectra are the average of 1280 scans recorded at a spectral resolution of 4 $cm^{-1}$. The absorptions spectra of the CO and $CN^-$ ligands are overlapped by the broad 'waging mode' of water, which was substracted in the displayed range by a broad spline function. Bands in the IR spectrum of $HypC_{Strep}$–HypD (black) were fitted to mixed Lorentz-Gauss functions with maxima at 2096, 2073 and 1955 $cm^{-1}$, respectively. Another Gauss was fitted to 2337 $cm^{-1}$. No specific absorption in this region could be detected for the variant $HypC_{Strep}$–$HypD_{C41A}$ film (grey).

is involved in coordinating the $CN^-$ and CO ligands. In order to test this hypothesis, codon 41 in the *hypD* gene on plasmid pT-hypDEFCStrep was mutated to one coding for alanine. To demonstrate that the mutation caused inactivation of HypD, the resulting plasmid, pT-hypDEFCStrep[C41A] was transformed into the *hypD* mutant DHP-D [9] and the ability of $HypD_{C41A}$ to restore Hyd-1 and Hyd-2 enzyme activity to the mutant was monitored by enzyme-specific activity staining (Fig. 3) [25]. It should be noted that in MC4100 the *hyp* genes on pT-hypDEFCStrep are expressed due to inherent promoter activity on the plasmid backbone. Whilst plasmid pT-hypDEFCStrep restored activity of both enzymes to the mutant, pT-hypDEFCStrep[C41A] failed to restore the activity of either enzyme.

Next, plasmid pT-hypDEFCStrep[C41A] was transformed into BL21(DE3) and the $HypC_{Strep}$–$HypD_{C41A}$ complex was purified exactly as described for the wild-type complex (see Section 2). The purified complex was indistinguishable from that containing native HypD and even included sub-stoichiometric amounts of HypE. This contrasts with previous findings [17] in which the C41A variant of HypD could not be isolated complexed with $HypC_{Strep}$. In the former study, however, the *hypC* and *hypD* genes were expressed from separate plasmids and our findings have indicated that a stable complex containing stoichiometric amounts of $HypC_{Strep}$ and HypD can only be obtained when both genes are co-expressed from a plasmid that includes the *hypE* and *hypF* genes. This, together with slight modifications in the isolation procedure, probably explains the discrepancy between the findings presented here and those reported previously [17].

The UV–Vis spectrum of $HypC_{Strep}$–$HypD_{C41A}$ revealed the flat, broad absorption maximum at 420 nm due to the $[4Fe–4S]^{2+}$ cluster (Fig. 1B), however, the additional features at the lower wavelengths were clearly less intense. A metal analysis revealed approximately 5 Fe and 4 S per mol of the complex (Table 1). FTIR analysis of $HypC_{Strep}$–$HypD_{C41A}$ did not reveal any absorption bands corresponding to the wavenumbers characteristic of the CO and $CN^-$ diatomic ligands observed for the native complex (Fig. 2). Moreover, the absorption band corresponding to $CO_2$ was also absent from the $HypC_{Strep}$–$HypD_{C41A}$ complex.

Taken together, these results demonstrate that the complex containing $HypD_{C41A}$ retains the $[4Fe–4S]^{2+}$ cluster but has less iron than the native complex. It also lacks both the $CN^-$ and CO absorption bands strongly suggesting that it can no longer coordinate the

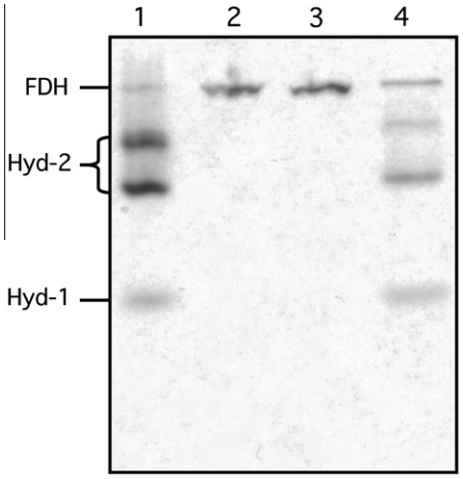


**Fig. 3.** In-gel hydrogenase activity analysis. Crude extracts (75 μg of protein) derived from strain DHP-D (Δ*hypD*) transformed with plasmid pT-hypDEFCStrep or pT-hypDEFCStrep[C41A] were analysed for Hyd-1 and Hyd-2 enzyme activity by separation in native PAGE (7.5% w/v) under non-denaturing conditions followed by staining for hydrogenase activity [25]. The stained bands corresponding to active Hyd-1 and Hyd-2 are indicated. A weak hydrogenase-independent activity due to formate dehydrogenase is indicated as Fdh. Lane 1, MC4100 (wild-type); Lane 2, DHP-D (*ΔhypD*); lane 3, DHP-D + pT-hypDEFCStrep[C41A] (encoding $HypD_{C41A}$); lane 4, DHP-D + pT-hypDEFCStrep.

$Fe(CN)_2CO$ cofactor. These results also indicate that Cys41 of HypD is required to coordinate the $Fe(CN)_2CO$ cofactor and is necessary to visualize the $CO_2$ absorption band; however, it is not essential for complex formation of HypD with HypC.

## 4. Conclusions

It was previously suggested [5,15,17] that a nucleophilic iron acts as the acceptor for the cyanide ligand generated by the HypFE maturases. Here we have demonstrated that the anaerobically purified $HypC_{Strep}$–HypD complex carries not only two $CN^-$ ligands but also coordinates a single CO. The similar patterns of $\nu$(CN) and $\nu$(CO) vibrations in the HypCD complex to those in the active site of hydrogenases [4,31], together with the strong correlation between the FTIR bands and the presence of additional iron atoms provide compelling evidence for coordination of the diatomic ligands by one of these Fe ions. Cys41 of HypD presumably ligates this cofactor to HypD and we assume that Cys2 of HypC, which is essential for HypC activity [20], also is involved in coordinating the cluster.

The oxygen-labile nature of this new $Fe(CN)_2CO$ cofactor coordination to HypCD also provides a possible explanation for the observed instability towards oxygen of an in vitro hydrogenase maturation system [34]. Future studies must now focus on the role and nature of the second additional Fe ion in HypD, which presumably is coordinated by the conserved and essential cysteinyl residues 69 and 72 [17], as well as the function of the $[4Fe–4S]^{2+}$ cluster in either generating the CO ligand or modulating the oxidation state of the second Fe ion. The finding of $CO_2$ bound to HypD and the strong correlation with the presence of the additional Fe ions, along with the $CN^-$ and CO ligands, leads to the exciting possibility that this might be the source of the CO. Moreover, the fact that a more stable HypCD complex is isolated when the *hypCDEF* genes are co-expressed strongly suggests that these four proteins form a complex in vivo, confirming earlier findings [15], and this is presumably necessary to orchestrate $Fe(CN)_2CO$ cofactor biosynthesis and insertion into the precursor of the large subunit. These studies further highlight the importance of sulphur chemistry in biosynthesis of the active site cofactor of [NiFe]-hydrogenases.

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