The iron–sulfur-containing HypC-HypD scaffold complex of the [NiFe]-hydrogenase maturation machinery is an ATPase

Kerstin Nutschan1, Ralph P. Golbik2 and R. Gary Sawers1

1 Institute for Biology/Microbiology, Martin-Luther University Halle-Wittenberg, Germany
2 Institute of Biochemistry and Biotechnology, Martin-Luther University Halle-Wittenberg, Germany

Keywords
[NiFe]-hydrogenase; HybG; HypC chaperone; HypD; iron–sulfur cluster; metalloenzyme maturation

Correspondence
R. G. Sawers, Institute for Biology/Microbiology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, Halle (Saale) 06120, Germany
Fax: +49 345 5527010
Tel: +49 345 5526350
E-mail: gary.sawers@mikrobiologie.uni-halle.de

(Received 9 September 2019, revised 26 September 2019, accepted 14 October 2019)
doi:10.1002/2211-5463.12743

HypD and HypC, or its paralogue HybG in Escherichia coli, form the core of the scaffold complex that synthesizes the Fe(CN)2CO component of the bimetallic NiFe-cofactor of [NiFe]-hydrogenase. We show here that purified HypC-HypD and HybG-HypD complexes catalyse hydrolysis of ATP to ADP (kcat ≈ 0.85/s); the ATPase activity of the individual proteins was between 5- and 10-fold lower than that of the complex. Pre-incubation of HypD with ATP was necessary to restore full activity upon addition of HybG. The conserved Cys41 residue on HypD was essential for full ATPase activity of the complex. Together, our data suggest that HypD undergoes ATP-dependent conformational activation to facilitate complex assembly in preparation for substrate reduction.

The catalytically active large subunit of [NiFe]-hydrogenases (Hyd) harbours a NiFe(CN)2CO-cofactor [1]. Synthesis of this cofactor requires the activities of six Hyp proteins, but not all steps catalysed by these proteins are understood. While it is known that the CN− ligands are synthesized from carbamoylphosphate through the combined actions of the HypEF maturation proteins [2,3], the metabolic origin(s) of the CO ligand remains unresolved. It is likely that two other maturation proteins, HypC and HypD, are directly involved in CO ligand synthesis. This is suggested because these proteins form a complex that has been shown to coordinate the Fe(CN)2CO moiety of the cofactor via conserved amino acid residues Cys 41 on HypD (Escherichia coli numbering) and Cys2 on HypC [4–6]. Furthermore, HypD has a [4Fe–4S] cluster and is the only one of the Hyp proteins that is redox-active. It has been hypothesized [7,8] that electron transfer is a prerequisite for generation of CO if it originates from endogenously produced CO2 through the activity of a decarboxylase, as well as for cyanide transfer from the thiocyanate generated on HypE [9].

Structural analysis of the HypC-HypD complex [10,11] identified a conserved thioredoxin-like fold linking the [4Fe–4S] cluster with Cys41, and results of a recent study have confirmed that HypD undergoes thiol-disulphide exchange, which would be commensurate with a proposed 2-electron and 2-proton reduction/dehydration of CO2 to CO [1,12]. Further circumstantial evidence supporting CO2 as a potential source of CO

Abbreviations
CO, carbon monoxide; CO2, carbon dioxide; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hyd, [NiFe]-hydrogenase.
for diatomic ligand biosynthesis was the finding that anaerobically isolated HypC and its paralogue, HybG in *E. coli*, showed a signal in the infrared region of the spectrum characteristic of Fe-bound CO₂ [8]. The appearance of this signal was oxygen-sensitive and was dependent on the conserved Cys2 residue, which is essential for maturation activity of HypC.

As well as these unresolved issues, a further barrier that would need to be overcome whether intracellularly produced CO₂ is indeed the substrate for CO ligand synthesis is that the recently determined redox potential of the [4Fe-4S] in isolated HypD is too positive (E⁰ = −260 mV [12]) to drive the reduction of CO₂, which requires a substantially more negative redox potential (E⁰ = −530 mV). Such a thermodynamic barrier could, however, be overcome whether the electron transfer reaction within HypD was coupled to ATP hydrolysis, as for example in analogy to the role of the iron protein of nitrogenase [13]. HypD is structurally unlike any other protein in the pdb database [11], and no classical ATP-binding motif is discernible in the protein. HypD does have a Rossmann fold-like sequence (Fig. 1A), but this is not located on the same face of the protein as the conserved and essential Cys41 residue, based on the crystal structure [10]. In this study, we demonstrate not only that HypD has a low intrinsic ATPase activity, but surprisingly that HypC and its paralogue HybG also have a similar activity. Notably, however, complex formation of HypD with either HypC or HybG significantly stimulates the ATPase activity of the heterodimer, but only after ATP was first incubated with HypD.

**Materials and methods**

**Bacterial strains, plasmids and growth conditions**

The *E. coli* strains used included MC4100 [14], and its isogenic derivative BEF314 (*ΔhypB-E*) [15]. The plasmids used included pT-hypDEFhybG<sub>Strep</sub> pIBA3hybGW<sub>T</sub>, pIBA3hybG<sub>C2A</sub>, pIBA3hypC<sub>2A</sub> [8], pT7-7hypC<sub>2A</sub> [16], pIBA5-hypD<sub>WT</sub>, pIBA5hypD<sub>C41A</sub>, pT7-7hypD<sub>C41A</sub>EFhypC<sub>Strep</sub> and pT7-7hypD<sub>WT</sub>-hypC<sub>Strep</sub> [17]. pT7-7hypD<sub>C41A</sub>EFhybG<sub>Strep</sub> was constructed by converting the DNA sequence at codon 41 in the hypD of plasmid pT7-7hypDEFhybG<sub>Strep</sub> from that coding for Cys to Ala using PCR-based mutagenesis (Q5<sup>®</sup> Site-Directed Mutagenesis Kit procedure of New England Biolabs, Ipswich, MA, USA) and the oligonucleotides fwd: 5'-CCG ATT ATG GAA GTG GCG GGT CAT ACC CAC-3' and rev: 5'-GTG GGT ATG ACC GCC CGC CAC TTC CAT ATT CCG-3'. *Escherichia coli* strains were transformed with the appropriate plasmid and were grown in modified TB medium [8], containing 100 µg·mL⁻¹ of ampicillin at 37 °C on a rotary shaker until an optical density of 0.4 at 600 nm was reached. Note that when BEF314 was used, the growth medium included 25 µg·mL⁻¹ of chloramphenicol. Gene expression was induced by the addition of 0.2 µg·mL⁻¹ anhydrotetracycline followed by incubation with gentle shaking at 30 °C for 3–5 h. Cells were harvested (OD<sub>600nm</sub> of 1.0) by centrifugation for 15 min at 50 000 g at 4 °C, and cell pellets were used either immediately or stored at −20 °C until use.

**Protein purification**

All protein purification steps were carried out under anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA). The HypC<sub>strep</sub>-HypD complex, HypC<sub>strep</sub>-HybG<sub>strep</sub>, and HypD<sub>strep</sub> were purified from BEF314, while HybG<sub>strep</sub>-HypD and the HypC<sub>strep</sub>-HypD complexes were purified from MC4100. Henceforth, the ‘strep’ subscript will be omitted when referring to these proteins and complexes. Strep-tagged proteins and protein complexes were purified exactly as described [8]. Purified proteins were buffer-exchanged into 50 mM Tris/HCl pH 8 containing 150 mM NaCl, concentrated by use of Vivaspin ultra-filters (Sartorius AG, Göttingen, Germany) with 5, 10 or 30 kDa cut-off, as appropriate for the protein being concentrated. Purified proteins were stored at −80 °C. Determination of protein concentration was done as described [18].

**Determination of ATP hydrolysis**

The ATPase activity of Hyp proteins was determined by an HPLC-based assay. For the assay, a ZIC<sup>®</sup>-HILIC column (PEEK 150 × 4.6 mm, 5 µm, 200 A; Merck, Darmstadt, Germany) was used attached to a Hitachi Elite LaChrome HPLC system. Proteins (typically 50 µm of each) were equilibrated in 50 mM HEPES, 2 mM MgCl₂, pH 7.4 and incubated with 500 µM ATP in a final volume of 0.4 mL at 37 °C for 30 min. In ATP-dependent activation reactions, 50 µm of either protein was incubated with 500 µM of ATP for 15 min at 37 °C and the second protein (also at 50 µm) was added and the incubation was continued for a further 15 min. After 30 min, 0.6 mL of 65% acetonitrile and 35% 100 mM ammonium acetate pH 4.5 were added and the mixture was centrifuged for 10 min at 15 000 g in a desk-top centrifuge at RT. The supernatant was filtered through a 0.2 µm filter (Sartorius), and ATP and ADP were separated using an isocratic gradient (flow rate 0.8 mL min⁻¹; 20 min; detection at 254 nm). Peak areas were integrated using the program EZChrom Elite. The amount of ADP formed was determined using a calibration curve prepared using an ADP solution of defined concentration, and the activity is presented as specific activity (µmol ATP hydrolysed min⁻¹mg of protein⁻¹). Kinetic data were investigated according to Michaelis–Menten and Eadie using the program KaleidaGraph (SYNERGY Software, Dubai, UAE).
All experiments reported in this study were performed using minimally using three biological replicates, unless otherwise stated.

Results

HypB-HypD and HypC-HypD complexes have ATPase activity

Performing BLAST searches with the ExPASy web tool (web.expasy.org) using HypD, we identified short peptide sequences exhibiting amino acid sequence similarity to UbiD-type decarboxylase, carbamoylphosphate synthetase and acyl-CoA reductase from different microorganisms (Fig. 1A; see legend for % similarities and the bacterial source of each sequence). HypD also showed some amino acid similarity with kinases and an N-terminal stretch of approximately 60 amino acids in length bore significant similarity to a Rossmann fold, as has been previously described for HypD [10,11]. Surprisingly, HypC and its parologue HybG both exhibited amino acid sequence similarity to histidine kinases and other ATP-dependent enzymes (Fig. 1B,C). Together, these observations are consistent with our contention that the HypC-HypD complex might use ATP as a substrate. To test this, we analysed the ability of the isolated HypC-HypD complex to catalyse the hydrolysis of ATP (Fig. 2). Anaerobically purified HypD-HypC and HypD-HybG complexes hydrolysed ATP to ADP at a rate of approximately 1 μmol min⁻¹ mg⁻¹ (kcat = 0.85 s⁻¹). Small amounts of HypE, which also has an ATPase activity [9], occasionally co-purified with the HybG-HypD or HypC-HypD complexes [17] when they were isolated from strain MC4100. To exclude that the ATPase activity of the complex was due to contamination with HypE, the HypC-HypD complex was purified from strain BEF314 (DhypBCDE) [15] carrying plasmid pT7-7hypD-hypCStrep [17] and a similar activity was measured to that shown in Fig. 2, thus excluding that the ATPase activity determined for the complex was due to HypE contamination.

HypD purified after over-production in strain BEF314, and independently of HypC or HybG, had...
an ATP-hydrolysing activity that was only approximately 20% that of the complex (Fig. 2). Surprisingly, both HypC and HybG also showed weak, intrinsic ATPase activities that were in the range of 0.1–0.14 μmol⋅min\(^{-1}\)⋅mg\(^{-1}\) (Fig. 2). It should be noted that no chemical hydrolysis of ATP during the period of the assay was observed, nor did BSA cause hydrolysis of ATP (Fig. 3), indicating that the ATP hydrolysis catalysed by HypC, HypD and HybG was specific. Moreover, AMP was never observed as a reaction product.

**Pre-incubation of HypD with ATP restores full ATPase activity when HypC is added**

Mixing separately purified HypD with an equimolar amount of HybG, and adding ATP at the same time, failed to restore full ATPase activity to the complex during the 30-min assay (Fig. 4). A similar observation was made when the experiment was performed with HypC instead of HybG. Pre-incubation of HypD and HypC together for 15 min with subsequent addition of ATP also did not restore full ATPase activity, delivering an activity similar to that determined when all three components were mixed simultaneously (data not shown). However, pre-incubation of HypD with ATP for 15 min prior to addition of HybG resulted in nearly full recovery of ATPase activity to a level similar to that measured for the as-isolated complex (compare Figs 2 and 4). Performing this experiment by pre-incubating HybG or HypC with ATP and then adding HypD after a 15-min incubation failed to restore full ATPase activity to the complex (Fig. 4). Note that during the 15 min pre-incubation, maximally 0.6% of the ATP was hydrolysed, and therefore, ATP was not limiting in this experiment.
The conserved Cys41 residue of HypD is essential for ATPase activity of the heterodimer

The conserved residues Cys41 of HypD and Cys2 of HypC (and HybG) have been shown to be essential for hydrogenase maturation activity [8,17,19] and have been proposed to coordinate the Fe(CN)2CO moiety on the HypC-HypD scaffold complex [4–6]. A Cys41Ala variant of HypD (HypDC41A) was anaerobically purified and tested for its ability to hydrolyse ATP (Fig. 5). The results clearly show that after incubation with ATP, neither addition of HypC nor addition of HybG was capable of restoring full ATPase activity to HypDC41A. The HypDC41A variant on its own had an ATPase activity that was approximately 70% that of native HypD when the assay was performed in the absence of HypC (Fig. 2). Together, these data indicate that the conserved Cys41 residue is essential for full ATPase activity of the complex, but not for the low intrinsic ATPase activity of the HypD protein.

To test whether the conserved Cys2 residue on the HypC family of proteins is important for the full ATPase activity of the complex, variants HypC C2A and HybG C2A were analysed in the ATPase assay. Wild-type HypD was incubated with ATP, and then, either HybG C2A or HypC C2A was added and after a further 15-min incubation the ATPase activities were determined (Fig. 5). The results clearly show that the Cys2 residue could be exchanged to Ala without any significant effect on the ATPase activity of the ATP-activated complex.

Kinetics of ATP hydrolysis

Analysis of the kinetic data (Fig. 6) according to Michaelis–Menten yielded an apparent $K_m$ of HypD for ATP of 365 μM (Table 1). For comparison, we have included the analyses of the data according to Eadie and according to Hanes in Table 1, but will focus on the Michaelis–Menten data. While HypC showed a $K_m$ of 235 μM, HybG had a lower affinity for ATP of $K_m$ of 475 μM. The distribution of $V_{max}$ corresponded roughly with that of the $K_m$ values, whereby HybG had a $V_{max}$ that was more than double that of HypC (Table 1). The $K_m$ for ATP was reduced approximately 10-fold for the HybG-HypD complex compared with that for HypD alone, while that of the HypC-HypD complex was ~50% lower than that of HypD (Table 1). The $V_{max}$ values of both complexes were significantly higher than those of the individual proteins.

Incubation of the HybG-HypD complex with equimolar concentrations (500 μM) of ATP and GTP did not cause any inhibition of ATPase activity, ruling out that GTP is a substrate for the complex. Moreover, incubation of the HybG-HypD complex with a 50-fold molar excess of ADP over ATP also failed to result in inhibition of ATPase activity, indicating that ADP is not a competitive inhibitor of the reaction (data not shown).

Discussion

The discovery that the HypC-HypD complex has an ATP-hydrolysing activity extends to three, including...
those catalysed by HypF and HypE, the number of ATP-dependent steps during the biosynthesis of the Fe (CN)2CO moiety of the NiFe cofactor. The identification of an ATPase activity for the complex would be in accord with the hypothesized involvement of the complex in the reduction of CO2 to generate CO[7,8]. Furthermore, the fact that full ATPase activity was observed only by the complex underscores CO2 as a putative substrate for the reaction because HypC has been shown to carry, and thus likely deliver to HypD, iron and CO2 [8]. The amino acid sequence similarities identified between HypD and short peptide sequences of decarboxylases, carbamoylphosphate synthetase and a CoA reductase are also in accord with a proposed function of HypD in using CO2 as a substrate (as well as separately transferring a cyanyl group from HypE [1,9]) and catalysing its 2 e− + 2 H+ reduction (formally a dehydration) to CO. Hydrolysis of ATP might therefore provide the energy required to overcome the thermodynamic barrier presented by the redox potential of HypD’s [4Fe-4S] cluster, which is insufficiently negative to reduce CO2 spontaneously, if CO2 indeed proves to be the substrate for CO diatomic ligand synthesis. However, we have not yet observed any influence of CO2 addition on the ATP-hydrolysing activity of the HypC-HypD complex (data not shown), which might be due to the fact that CO2 is either bound to a protein or in an iron-bound form, as has been previously suggested [8]. Another possibility is that ATP hydrolysis might be important in establishing complex
formation with other components (e.g. HypE and HypF) of the maturation machinery. The fact that the HypC-HypD complex isolated from the BEF314 (ΔhypB-E) has the same ATPase activity as that isolated from a hyp\(^+\) wild-type strain suggests that ATP hydrolysis is probably not involved in transfer of the Fe(CN)\(_2\)CO group from the complex to the [NiFe]-hydrogenase large subunit. Nevertheless, future experiments may be needed to test these possibilities.

We can rule out that ATP triggers the disassembly of the HypC-HypD complex because we could never observe HypC being released from the ass-isolated complex upon incubation with ATP (data not shown). Rather, the observed ATP-dependent activation of HypD, which restored full ATPase activity when either HypC or HybG was added to the mixture, suggests that an ATP-dependent conformational activation of HypD might occur, which would promote interaction with HypC or HybG. Evidence that a conformational change in HypD occurs upon complex formation with HypC was provided by the structural analyses performed on HypD, HypC and the complex of the two proteins isolated from the archaeon *Thermococcus kodakaren*s [10,11].

The fact that the simultaneous mixing and incubation of HypD, HypC and ATP failed to manifest the full ATPase activity suggests that the presence of HypC in solution sterically hinders the binding of ATP to HypD. HypD lacks either Walker A or B motifs [19], or a PurM-like ATP-binding motif, which is present in HypE [10], and thus, it is unclear what the ATP-binding site in HypD looks like. Together with the N-terminal motif that includes the essential C41 residue, there are two other highly conserved motifs in HypD, namely the GPGCPVCxxP (amino acids 66–75) and GFETT (amino acids 146–150) motifs (Fig. 1A), which might be considered as candidates for ATP interaction. Apart from the C41A variant, which we show in this study to lack full ATPase activity, exchanges of the conserved C69 and C72 residues of HypD also abolish both maturation activity in vivo and interaction with HypC in vitro [20]. This suggests that the N-terminal region of HypD might be important for binding ATP. It was initially rather unexpected to discover that both HypC and HybG also have a low intrinsic ATPase activity. This is likely to be of relevance, however, for the ATP-binding site within the complex and perhaps suggests that the interface of the HypC and HypD proteins forms this site. Indeed, the structure of the HypC-HypD complex, as well as revealing considerable conformational restraint, also revealed the formation of a deep cleft along the interface, suggestive of a substrate-binding site [11].

Finally, it is also conceivable that ATP-binding and hydrolysis might influence the redox chemistry of the conserved [4Fe-4S] cluster in HypD, facilitating energization of electrons necessary for the otherwise endothermic substrate reduction to produce the diatomic CO ligand. As such, the HypC-HypD two-component ATPase is formally similar to a class of enzymes that performs ATP-driven redox reactions, in which the first component is an ATPase with an iron–sulfur cluster and the second component is a metalloenzyme, which is the electron acceptor and site of substrate reduction [21]. Due to the fact that the HypC protein (second component) has been shown to bind iron and CO\(_2\) [8], coupling the ATP-dependent conformational activation of HypD (first component) might trigger electron transfer and drive substrate reduction. Whether that substrate is indeed CO\(_2\) can now be addressed in future studies that incorporate ATP in the assay.

### Acknowledgements

We thank Constanze Pinske for discussion. This work was supported by the Deutsche Forschungsgemeinschaft as part of the research initiative ‘Iron-Sulfur for Life (SPP 1927; SA 494/7-1)’. We acknowledge the

---

**Table 1. Kinetic analysis of the enzyme data.**

| Protein       | Michaelis–Menten\(^a\) | Eadie\(^b\) | Hanes\(^b\) |
|---------------|-------------------------|-------------|-------------|
|               | \(V_m\) (µmol·min\(^{-1}\)·mg\(^{-1}\)) | \(K_m\) (µmol·L\(^{-1}\)) | \(V_m\) (µmol·min\(^{-1}\)·mg\(^{-1}\)) | \(K_m\) (µmol·L\(^{-1}\)) | \(V_m\) (µmol·min\(^{-1}\)·mg\(^{-1}\)) | \(K_m\) (µmol·L\(^{-1}\)) |
| HypC          | 0.234 ± 0.026           | 235.72 ± 71.15 | 0.167 ± 0.025 | 87.61 ± 23.32 | 0.223 | 181.52          |
| HypD          | 0.398 ± 0.046           | 364.48 ± 100.16 | 0.295 ± 0.035 | 172.31 ± 31.88 | 0.363 | 261.30          |
| HybG          | 0.560 ± 0.016           | 475.67 ± 29.91 | 0.541 ± 0.067 | 426.38 ± 73.62 | 0.577 | 510.36          |
| HypC-HypD     | 0.995 ± 0.119           | 143.48 ± 55.10 | 0.803 ± 0.090 | 64.57 ± 13.92 | 1.041 | 141.11          |
| HybG-HypD     | 1.423 ± 0.082           | 34.78 ± 8.77   | 1.365 ± 0.089 | 28.02 ± 4.65  | 1.561 | 56.62           |

\(^a\)Kinetic data of the v/S-plots were investigated using the Program KaleidaGraph\textsuperscript{\textregistered} (SYNERGY Software). Standard errors of the fitting routine are indicated for data investigation according to Michaelis–Menten and after linearization according to Eadie.

\(^b\)The standard errors of enzymatic parameters derived from data investigation after linearization according to Hanes by using the laws of error propagation would exceed reasonable values and are not indicated in the table.
financial support within the funding programme Open Access Publishing by the German Research Foundation (DFG).

**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

KN carried out the experiments. KN, RPG and GRS designed the experiments and analysed the data. GRS drafted the manuscript and conceived the study. All authors read and approved the final manuscript.

**References**

1. Böck A, King PW, Blokesch M and Posewitz MC (2006) Maturation of hydrogenases. *Adv Microbiol Physiol* 51, 1–71.
2. Paschos A, Bauer A, Zimmermann A, Zehelein E and Böck A (2002) HypF, a carbamoyl phosphate-converting enzyme involved in [NiFe] hydrogenase maturation. *J Biol Chem* 277, 49945–49951.
3. Reissmann S, Hochleitner E, Wang H, Paschos A, Lottspeich F, Glass RS and Böck A (2003) Taming of a poison: biosynthesis of the NiFe-hydrogenase cyanide ligands. *Science* 299, 1067–1070.
4. Soboh B, Stripp ST, Muhr E, Granich C, Braussemm M, Herzberg M, Heberle J and Sawers RG (2012) [NiFe]-hydrogenase maturation: isolation of a HypC-HypD complex carrying diatomic CO and CN- ligands. *FEBS Lett* 586, 3882–3887.
5. Stripp ST, Soboh B, Lindenstrauss U, Braussemm M, Herzberg M, Nies DH, Sawers RG and Heberle J (2013) HypD is the scaffold protein for Fe-(CN)2CO cofactor assembly in [NiFe]-hydrogenase maturation. *Biochemistry* 52, 3289–3296.
6. Bärstl I, Siebert E, Winter G, Hummel P, Zebger I, Friedrich B and Lenz O (2012) A universal scaffold for synthesis of the Fe(CN)2(CO) moiety of [NiFe] hydrogenase. *J Biol Chem* 287, 38845–38853.
7. Roseboom W, Blokesch M, Böck A and Albracht SP (2005) The biosynthetic routes for carbon monoxide and cyanide in the Ni-Fe active site of hydrogenases are different. *FEBS Lett* 579, 469–472.
8. Soboh B, Stripp ST, Bietak C, Lindenstraß U, Braussemm M, Javaid M, Hallensleben M, Granich C, Herzberg M, Heberle J *et al.* (2013) The [NiFe]-hydrogenase accessory chaperones HypC and HypG of *Escherichia coli* are iron- and carbon dioxide-binding proteins. *FEBS Lett* 587, 2512–2516.
9. Blokesch M, Paschos A, Bauer A, Reissmann S, Dralop N and Böck A (2004) Analysis of the transcarbamoylation-dehydration reaction catalyzed by the hydrogenase maturation proteins HypF and HypE. *Eur J Biochem* 271, 3428–3436.
10. Watanabe S, Matsumi R, Arai T, Atomi H, Imanaka T and Miki K (2007) Crystal structures of [NiFe] hydrogenase maturation proteins HypC, HypD and HypE: insights into cyanation reaction by thiol redox signaling. *Mol Cell* 27, 29–40.
11. Watanabe S, Matsumi R, Atomi H, Imanaka T and Miki K (2012) Crystal structures of the HypCD complex and the HypCDE ternary complex: transient intermediate complexes during [NiFe] hydrogenase maturation. *Structure* 20, 2124–2137.
12. Adamson H, Robinson M, Bond PS, Soboh B, Gillow K, Simonov AN, Elton DM, Bond AM, Sawers RG, Gavaghan DJ *et al.* (2017) Analysis of HypD disulfide redox chemistry via optimization of Fourier transformed ac voltammetric data. *Anal Chem* 89, 1565–1573.
13. Seefeldt LC, Yang Z-Y, Duval S and Dean DR (2013) Nitrogenase reduction of carbon containing compounds. *Biochim Biophys Acta* 1827, 1102–1111.
14. Casadaban MJ (1976) Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* 104, 541–555.
15. Jacobi A, Rossmann R and Böck A (1992) The hyp operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. *Arch Microbiol* 158, 444–451.
16. Dralop N and Böck A (1998) Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation. *Biochemistry* 37, 2941–2948.
17. Blokesch M, Albracht SPJ, Matzanke BF, Dralop NM, Jacobi A and Böck A (2004) The complex between hydrogenase-maturation proteins HypC and HypD is an intermediate in the supply of cyanide to the active site iron of [NiFe]-hydrogenases. *J Mol Biol* 344, 155–167.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265–275.
19. Ambudkar SV, Kim I-W, Xia D and Sauna ZE (2006) The A-loop, a novel conserved aromatic acid subdomain upstream of the Walker A motif in ABC transporters, is critical for ATP binding. *FEBS Lett* 580, 1049–1055.
20. Blokesch M and Böck A (2006) Properties of the [NiFe]-hydrogenase maturation protein HypD. *FEBS Lett* 580, 4065–4068.
21. Jeoung J-H and Dobek H (2018) ATP-dependent substrate reduction at an [Fe8S9] double-cubane cluster. *Proc Natl Acad Sci USA* 115, 2994–2999.