Biochemical and Biophysical Research Communications 424 (2012) 158–163

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evidence for an oxygen-sensitive iron–sulfur cluster in an immature large subunit species of *Escherichia coli* [NiFe]-hydrogenase 2

Basem Soboh [a], Martin Kuhns [a,1], Mario Braussemann [a], Mandy Waclawek [a,2], Enrico Muhr [a], Antonio J. Pierik [b], R. Gary Sawers [a,*]

[a] Institute for Biology/Microbiology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle (Saale), Germany
[b] Institute of Cytobiology and Pathology, Philipps University, Robert Koch Str. 6, 35037 Marburg, Germany

ARTICLE INFO

Article history:
Received 12 June 2012
Available online 23 June 2012

Keywords:
[NiFe]-hydrogenase
Large subunit
Maturation
Iron–sulfur cluster
Processing

ABSTRACT

Endoprotease-specific C-terminal processing is required to complete the maturation of the large subunit of [NiFe]-hydrogenases. This happens only after synthesis and insertion of the NiFe(CN)$_2$CO cofactor by the Hyp maturases has occurred. It is assumed that in the absence of maturation the unprocessed species of the large subunit lacks cofactors. In this study we isolated a variant of the hydrogenase 2 large subunit, HybC, containing a fused C-terminal pentapeptide. The polypeptide could not be processed and was unable to associate with the small subunit to deliver an active enzyme. The His$_6$-HybC variant protein isolated was brown and had sub-stoichiometric amounts of an oxygen-sensitive Iron–sulfur cluster, which could be chemically reconstituted to a [4Fe–4S] cluster. This cluster was coordinated by the conserved cysteinyl residues that normally ligate the NiFe(CN)$_2$CO cofactor. Our findings provide evidence for temporary promiscuity of cofactor-binding sites.

1. Introduction

[NiFe]-hydrogenases catalyse the reversible activation of dihydrogen. The enzymes generally comprise a large subunit harbouring a NiFe(CN)$_2$CO cofactor, which is responsible for catalysis. A second small subunit with Iron–sulfur (FeS) clusters is responsible for electron transfer to and from the active site in the large subunit [1,2]. Often these enzymes have a third, membrane-anchoring subunit for electron transport to the quinone pool.

The model bacterium *Escherichia coli* synthesizes three membrane-associated [NiFe]-hydrogenases (Hyd) [3]. Hyd-1 and Hyd-2 are respiratory enzymes that couple hydrogen oxidation to quinone reduction. Hyd-3 is part of the formate hydrogenlyase complex, which catalyses hydrogen production from formate at the cytoplasmic side of the inner membrane.

The Hyp maturases are involved in the biosynthesis and insertion of the NiFe(CN)$_2$CO cofactor into the hydrogenase large subunit [4]. Cofactor synthesis is initiated by HypF, which transfers the carbamoyl group of carbamoylphosphate to a C-terminal cysteinyl residue of HypE. HypE then catalyses the ATP-dependent dehydration of the thiocarboxamide group to thiocyanate. The presumptive subsequent transfer of the cyanide group to an iron atom in the HypCD complex and the generation of the CO ligand are less well understood. Experimental evidence indicates that once insertion of the Fe(CN)$_2$CO cofactor into the large subunit has occurred then the Ni ion is introduced by HypA, HypB and SlyD [4–6]. Only after completion of NiFe(CN)$_2$CO cofactor insertion does active site closure through a specific endoproteolytic cleavage of a short C-terminal peptide from the large subunit occur [4,7,8]. A mutation that prevents any one of the above steps results in an unprocessed large subunit, which lacks the cofactor and is generally assumed to lack any associated metal. In this study we demonstrate that the unprocessed large subunit, HybC, of Hyd-2 can be isolated with an oxygen-sensitive FeS cluster.

2. Materials and methods

2.1. Strains and growth conditions

The strains and plasmids used in this study were: MC4100 (F$^-$, *araD139*, Δ(*argF-lac*)U169, λ-, *rpsL150*, *relA1 deoC1*, *flhD5301*, Δ(*fruK-yeiR*)725(*fruA25*), *rbsR22*, Δ(*fimB-fimE*)632(::IS*1*)) [9]; FTD147 (like MC4100 but Δ*hyaB* Δ*hybC* Δ*hycE*) [10]; DHP-F2 (like MC4100 but Δ*hypF*) [11]; CP477 (like MC4100 but Δ*iscA*:Kan^R) [12]; pASK-hybC [13]; pCAN-hybC encoding HybC with an N-terminal hexa-histidine tag and a C-terminal pentapeptide (GLCGR)

* Corresponding author. Address: Institute for Microbiology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle (Saale), Germany. Fax: +49 345 5527010.
E-mail address: gary.sawers@mikrobiologie.uni-halle.de (R.G. Sawers).
[1] Present address: Institute of Molecular Bioscience, Goethe University of Frankfurt, Max-von-Laue-Str. 9, 60438 Franfurt, Germany.
[2] Present address: Institute of Plant Physiology, Martin-Luther University Halle-Wittenberg, Weinberg Weg 10, 06120 Halle (Saale), Germany.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.096

[14], which we refer to henceforth as His_6-$HybC_{GLCGR}$. *E. coli* strains were grown anaerobically at 37 °C in TGYEP medium [15]. Ampicillin and chloramphenicol were used at the final concentration of 100 μg and 12 μg per ml, respectively. Protein overproduction from strain FTD147 containing plasmid pCAN-hybC or pASK-hybC in cultures (10 l) was induced by the addition of 0.3 mM IPTG or anhydrotetracyline (0.2 μg ml^{-1}) followed by incubation at 30 °C for 3 to 5 h. Cells were harvested ($OD_{600\ nm}$ of 0.9) and cell pellets were used immediately or stored at −20 °C until use.

2.2. Preparation of crude extracts and protein purification

All steps were carried out under anaerobic conditions in a Coy™ chamber. Wet cell paste was resuspended at a ratio of 1 g per 3 ml in 50 mM MOPS pH 7 including 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 was used for anaerobic purification of His_6-$HybC_{GLCGR}$ exactly as described [16].

2.3. Mutagenesis of pCAN-hybC

Plasmid pCAN-hybC served as DNA template for the introduction of mutations into the *hybC* gene using the QuickChange (Stratagene) mutagenesis procedure exactly as described by the manufacturer. The oligonucleotide primers used to introduce a stop codon, generating pCAN-$hybC_{STOP}$ were 5′-CAGTGAAGGTTCTTTGACTATGCGGCCGC-3′ and 5′-GCGGCCGCATAGTCAAAGAACCTTCACTG-3′. For mutagenesis of the the Cys codons the following primers were used: for plasmid pCAN-hybC(C61A/C64A) with a Cys61Ala/Cys64Ala double exchange 5′-TGCAACGTATCGCTGGCGTAGCTACTACCACTC-3′ and 5′-GAGTGGTAGTAGCTACGCCAGCGATACGTTGCA-3′ were used; for pCAN-hybC(C546A/C549A) with a Cys546Ala/Cys549Ala double exchange 5′-CCTTTGACCCGGCCATGGCCGCTGCGGTACACG-3′ and 5′-CGTGTACCGCAGCGGCCATGGCCGGGTCAAAGG-3′ were used. The entire mutated *hybC* genes were verified by DNA sequencing.

2.4. 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 [13]. SDS–PAGE was performed using 10% (w/v) polyacrylamide as described [17]. Determination of protein concentration was done as described [18].

2.5. UV–visible and electron paramagnetic spectroscopy

UV–Vis and EPR spectroscopies were performed exactly as described in [19]. Samples of His_6-$HybC_{GLCGR}$ (50 mg ml^{-1}) were frozen in cold isopentane/methylcyclohexane (5:1, ∼120 K) and stored in liquid nitrogen.

2.6. Chemical reconstitution of a FeS cluster into His-HybC

To chemically reconstitute a FeS cluster, the different His_6-HybC variants (1 mg ml^{-1}) were incubated anaerobically in 50 mM Tris–HCl pH 8, containing 150 mM NaCl and 2 mM sodium dithionite for 1 h at 15 °C. The reconstitution was started by slow addition of 0.7 mM ferric ammonium citrate and 1 mM Li_2S and the mixture was incubated for 20 min at 15 °C. To remove non-bound iron and sulfide, the reconstituted His_6-HybC variants were passed through a Superdex-200 gel filtration column equilibrated with 25 mM Tris–HCl, pH 7.8 containing 200 mM NaCl, 5% (w/v) glycerol and 2 mM sodium dithionite. The assembly of a FeS cluster in the His_6-HybC variants was ascertained by non-heme iron and sulfide analyses.

2.7. Non-heme iron and acid-labile sulfide determination

Iron and acid-labile sulfide were determined as described previously [20,21]. Iron content was confirmed by inductively coupled plasma mass spectrometry (ICP-MS) [22]. For ICP-MS analysis 1.5 mg of purified the His_6-HybC variant (15 mg ml^{-1}) was mixed with 85 μl of 67% (w/v) nitric acid. The mixture was heated at 70 °C for 2 h, then diluted with distilled water to a final concentration of 2 % nitric acid and introduced into an X-Series 2 ICP-MS system (Thermo Fisher Scientific, Bremen) via a peristaltic pump. The sample was aspirated with humidified argon, passed into the plasma, and analysed for iron, nickel, zinc and copper. Commercial multi-element calibration standards were used.

3. Results

3.1. Addition of a C-terminal pentapeptide prevents endoproteolytic processing of His_6-HybC

The Keio collection of *E. coli* strains [14] includes a series of plasmids encoding N-terminally (hexa-histidine) His_6-tagged fusion proteins of each open reading frame (ORF) in the *E. coli* genome. As well as the N-terminal His_6-tag, each ORF in this series also carries a C-terminal extension of five amino acids (GLCGR) [14]. Keio collection plasmid pCAN-hybC thus encodes His_6-$HybC_{GLCGR}$, the large subunit of Hyd-2. Introduction of this plasmid into strain FTD147, which has chromosomal deletions in *hyaB*, *hybC* and *hycE*, encoding the large subunits of Hyd-1, 2 and 3, respectively [10], failed to restore Hyd-2 activity to the strain (Fig. 1A) as determined by specific staining for hydrogenase activity after separation of the enzymes by native-PAGE [13]. In contrast, plasmid pASK-hybC encoding HybC with a N-terminal Strep-tag but lacking a C-terminal pentapeptide extension complemented the *hybC* mutation and restored Hyd-2 enzyme activity (Fig. 1A). To determine whether the N-terminal His_6-tag or the C-terminal pentapeptide extension on His_6-$HybC_{GLCGR}$ prevented enzyme activity being restored, we reintroduced an opal (UGA) stop codon in plasmid pCAN-$hybC_{GLCGR}$ delivering plasmid pCAN-$hybC_{STOP}$. This removed the 5 C-terminal amino acids from His_6-$HybC_{GLCGR}$ and the gene product, referred to as His_6-HybC, had a C-terminus identical to the native protein. This derivative, when transformed into FTD147 complemented the *hybC* mutation and restored Hyd-2 enzyme activity (Fig. 1A), indicating that the C-terminal pentapeptide extension on His_6-$HybC_{GLCGR}$ was responsible for the lack of Hyd-2 enzyme activity.

3.2. Characterization of purified unprocessed His-tagged HybC

In order to determine why the pCAN-hybC plasmid could not deliver active Hyd-2 enzyme, the His_6-$HybC_{GLCGR}$ polypeptide encoded by pCAN-hybC was overproduced and purified by cobalt-NTA affinity chromatography performed under strictly anaerobic conditions (see Section 2). The protein purified from the cytoplasmic fraction showed a single polypeptide with apparent molecular mass of 67 kDa on SDS–PAGE (Fig. 1B). In comparison, when overproduced Strep-HybC was enriched, a double band was observed (Fig 1B), which is characteristic for a mixture of processed and unprocessed HybC polypeptide [13]. Moreover, the small subunit HybO was associated with Strep-HybC, while no accompanying small subunit co-purified with the unprocessed His_6-$HybC_{GLCGR}$ polypeptide (Fig. 1B). These data indicate that His_6-$HybC_{GLCGR}$ was inactive due to the lack of C-terminal processing of the polypeptide and the absence of an associated electron-transferring small subunit.

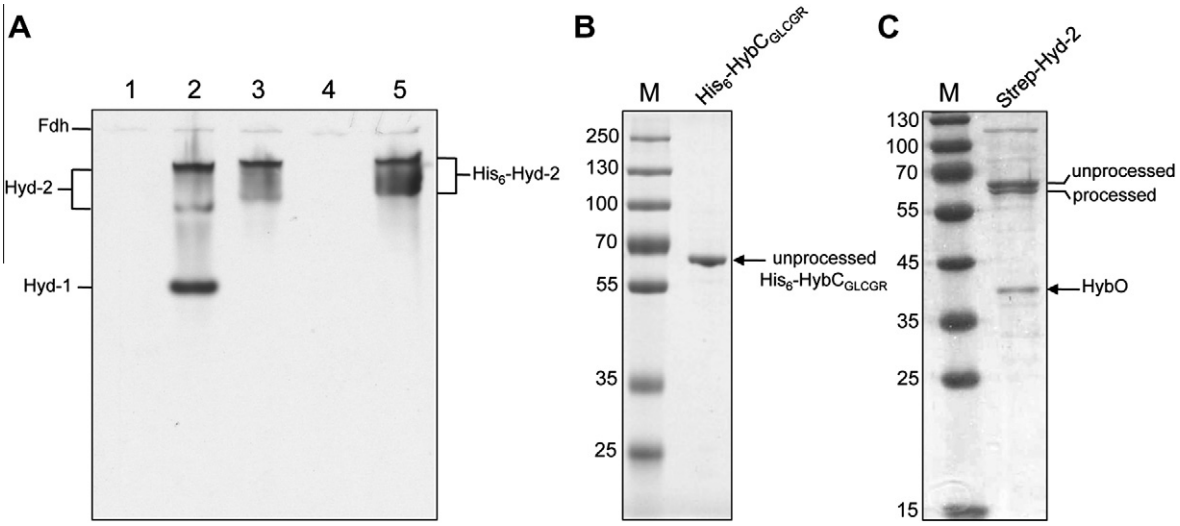

Fig. 1. His_6-$HybC_{GLCGR}$ is functionally inactive and unprocessed. A. Crude extracts with 25 μg total protein of strains MC4100 and DHP-F2 (Δ*hypF*) grown anaerobically in TGYEP, pH 6.5 were subjected to 7.5% (w/v polyacrylamide) non-denaturing PAGE and subsequently stained for hydrogenase enzyme activity. Lane 1, crude extract of FTD147 (Δ*hyaB* Δ*hybC* Δ*hycE*) as a negative control; lane 2, crude extract of MC4100 (wild-type); lane 3, crude extract of FTD147 + pASK-hybC (Strep-HybC); lane 4, crude extract of FTD147 + pCAN-hybC (His_6-$HybC_{GLCGR}$); lane 5, FTD147 + pCAN-$hybC_{STOP}$ (His_6-HybC, lacking the C-terminal pentapeptide). The activity band assignment of Hyd-1, Hyd-2 and His_6-Hyd-2 (Hyd-2 enzymes with His_6-tagged HybC) is indicated. A weak hydrogenase-independent activity due to formate dehydrogenase is indicated as Fdh and acts as a loading control. B. Purified His_6-$HybC_{GLCGR}$ (5 μg protein) was separated by SDS–PAGE (10% w/v polyacrylamide) and C. Strep-Hyd-2 (Hyd-2 enzyme with a Strep-tagged HybC; 5 μg protein) was separated by 12.5% SDS–PAGE. The gels were stained with Coomassie Brilliant Blue and in B the protein size marker (M) was PageRuler Plus prestained marker (Fermentas) and in C PageRuler prestained marker (Fermentas) was used. The location of the single, unprocessed His_6-$HybC_{GLCGR}$ polypeptide in part B and the unprocessed and processed forms of Strep-HybC, together with the associated HybO small subunit, in part C are indicated.

It was noted that concentrated His_6-$HybC_{GLCGR}$ had a brown colour. UV–Vis spectroscopic analysis of the protein sample revealed a spectrum characteristic of a FeS protein (Fig. 2A). The broad shoulder at 420 nm together with the approximate 50% reduction in signal intensity after treatment of the sample with 2 mM dithionite suggests the presence of a $[4Fe–4S]^{2+}$ cluster [19]. The weak features at 410 and 460 nm suggest, however, that some $[2Fe–2S]^{2+}$ is also present in the sample (see Fig. 2A). Metal analysis of the protein sample performed by both colorimetric and ICP-MS methods revealed approximately 0.2 mol Fe per mol His_6-$HybC_{GLCGR}$ (Table 1), showing partial occupancy of the FeS cluster in the protein. Less than 0.01 mol nickel per mol protein was detected and acid-labile sulfide was also below the detection limit.

Isolation of His-HybC under aerobic conditions resulted in a protein sample that was essentially colourless and which contained less than 0.01 mol Fe per mol protein.

3.3. His-HybC isolated from a hypF mutant retains the FeS cluster

To investigate the dependence on the Hyp maturases for introduction of the FeS cluster into His_6-$HybC_{GLCGR}$, the protein was purified under anaerobic conditions from an *E. coli* strain deleted for the *hypF* gene, encoding one of the key enzymes on the Ni-$Fe(CN)_2CO$ cofactor maturation pathway [11]. UV–Vis spectroscopic analysis of the purified protein revealed a spectrum similar to that for His_6-$HybC_{GLCGR}$ isolated from a Hyp^+ strain (Fig. 2B) and this strongly suggests that the FeS cluster was introduced independently of the Hyp protein machinery.

EPR analysis of a concentrated sample of as-isolated His_6-$HybC_{GLCGR}$ from FTD147 reduced with dithionite revealed *g*-value features consistent with the presence of a $[4Fe–4S]^{1+}$ cluster (Fig. 2C).

3.4. The FeS cluster in unprocessed His_6-$HybC_{GLCGR}$ is coordinated by the Cys residues that coordinate the Ni-$Fe(CN)_2CO$ cofactor

Two pairs of highly conserved Cys residues coordinate the Ni-$Fe(CN)_2CO$ cofactor in the hydrogenase large subunit [2]. In the large subunit of Hyd-2 these are the N-terminally located Cys61/Cys64 and the C-terminally located Cys546/Cys549 pairs [23]. In order to determine whether the labile FeS cluster in His_6-$HybC_{GLCGR}$ is coordinated by these Cys residues, two different His_6-$HybC_{GLCGR}$ variants were constructed in which either the N-terminal Cys pair was exchanged for Ala residues encoded on pCAN-hybC(C61A/C64A), or the C-terminal Cys pair was exchanged for Ala residues encoded on pCAN-hybC(C546A/C549A). Isolation of a soluble variant with the amino acid exchanges in the C-terminal pair was not possible due to inclusion body formation (data not shown). Overproduction and purification of the His_6-$HybC_{GLCGR}$ variant with the C61A/C64A exchanges was successful and the UV–Vis spectrum of the concentrated protein only revealed a weak contribution of stray light at 350–600 nm (Fig. 2B). Moreover, metal analysis revealed that non-heme iron in this protein was below the detection limit (<0.01 Fe/protein).

3.5. A [4Fe–4S] cluster can be chemically reconstituted into His-HybC

To provide further support that a [4Fe–4S] cluster can be bound by the four cysteinyl residues that bind the Ni-$Fe(CN)_2CO$ cofactor before complete maturation of HybC, we attempted chemical reconstitution of the FeS cluster in His_6-$HybC_{GLCGR}$ as described in the Methods section. Anaerobically isolated His_6-$HybC_{GLCGR}$ reconstituted with iron and sulfide was submitted to gel-filtration chromatography under anaerobic conditions to remove Fe/S aggregates and non-specifically bound iron. Reconstituted His_6-$HybC_{GLCGR}$ had approximately 4 mol Fe and 2.5 mol acid-labile sulfide per mol His-HybC (Table 1). Because the reconstitution reaction was performed in the presence of dithionite no UV–Vis spectrum of the sample could be taken. Therefore, to demonstrate that metal-binding was specific we performed the same reconstitution experiment with the His_6-$HybC_{GLCGR}$ variant with the amino acid exchanges C61A/C64A. In this case less than 1 mol Fe could be detected in the protein (Table 1), strongly suggesting that binding was specific and that the Cys residues 61 and 64 were required for optimal coordination of the cluster.

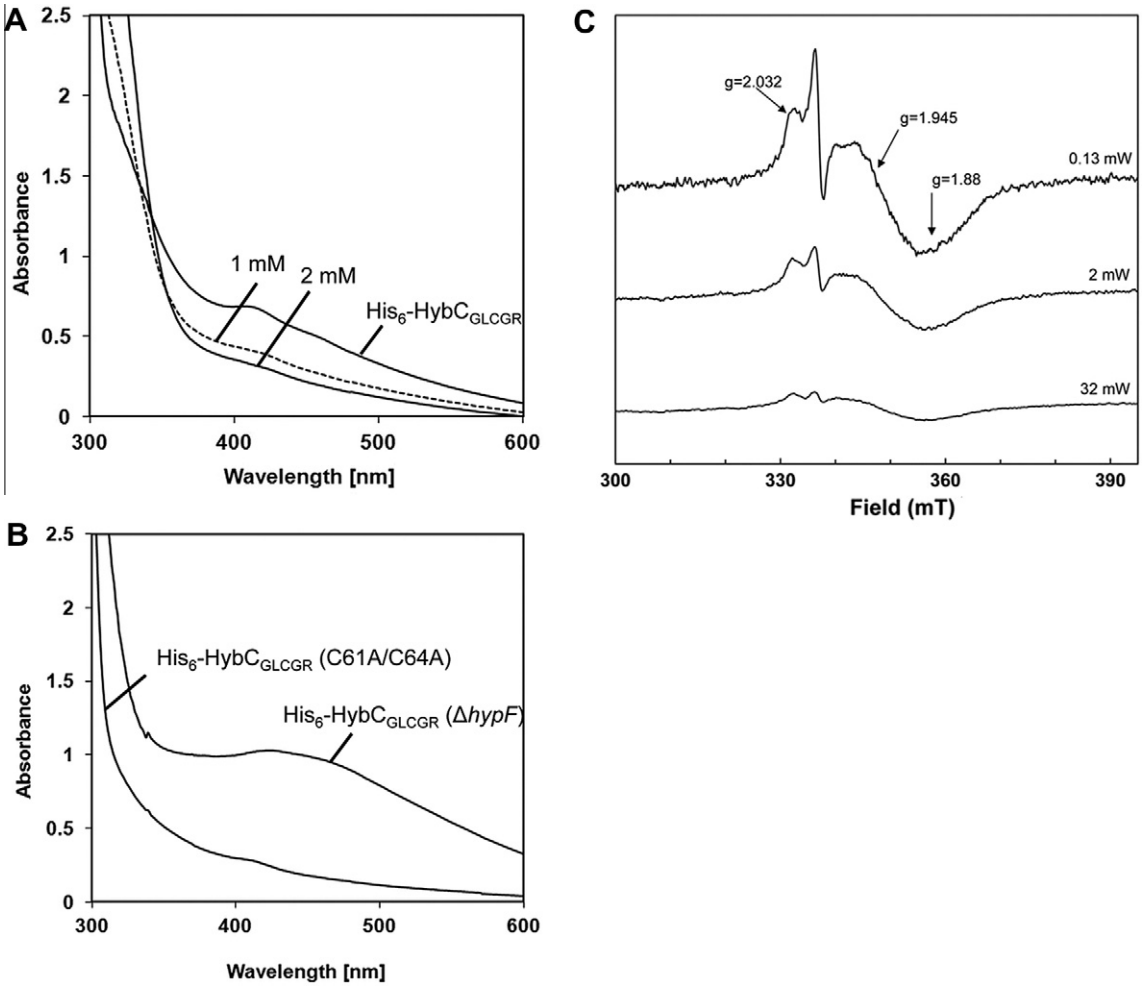

Fig. 2. Anaerobically isolated His_6-$HybC_{GLCGR}$ has an iron-sulphur cluster. (A) His_6-$HybC_{GLCGR}$ was incubated in anaerobic 50 mM Tris–HCl pH 8.0, 150 mM NaCl (5 mg ml^{-1}) (as-isolated) or with 1 mM or 2 mM sodium dithionite in an anaerobic chamber at room temperature for 10 min. UV–Vis spectra were recorded between 300 and 600 nm as indicated. (B) The UV–Vis spectra of His_6-$HybC_{GLCGR}$ (4 mg ml^{-1}) isolated from strain DHP-F2 (Δ*hypF*) and His-HybC(C61A/C64A) (4 mg ml^{-1}) are shown. (C) EPR spectra were taken with an anaerobically prepared sample of His_6-$HybC_{GLCGR}$ (50 mg ml^{-1}) and the conditions were 9.458 GHz, 10 K and the indicated microwave power.

Table 1
Non-heme iron and sulfide analyses.

Protein sample	Non-heme Fe (mol per mol of protein)	Sulfide (mol per mol of protein)
Colorimetric[a]		
His-HybC as-isolated	0.2 ± 0.02 (n = 4)[b]	Below detection limit[c]
His-HybC reconstituted	4.4 ± 0.25 (n = 4)	2.25 ± 0.27
His-HybC(C61A/C64A)	Below detection limit (n = 2)	n.d.[d]
His-HybC(C546A/C549A)	Below detection limit (n = 2)	n.d.
His-HybC(C61A/C64A) reconstituted	0.81 ± 0.05 (n = 4)	0.56 ± 0.07
ICP-MS[a]		
His-HybC as-isolated	0.16 ± 0.015 (n = 2)	n.d
His-HybC-iscA[e]	0.06 (n = 2)	n.d

[a] Method used for iron determination.
[b] n is the number of independent measurements
[c] lower limit of detection for sulfide ⩾ 1 nmol.
[d] n.d. not determined.
[e] His-HybC isolated from an *iscA* mutant with a defect in Iron–sulfur cluster biosynthesis.

3.6. Reduced levels of iron are present in His-HybC isolated from an *iscA* mutant

We have shown recently that the A-type carrier proteins IscA and ErpA are important for maturation of the FeS clusters in the small, electron-transfer subunits of hydrogen-oxidising hydrogenases of *E. coli* [24]. As IscA has an important function in transfer and delivery of FeS clusters to apo-protein substrates [25], we tested whether His_6-$HybC_{GLCGR}$ isolated from an *iscA* mutant had an altered non-heme iron content. Contrary to expression in wild-type or *hypF* mutant cells, the majority of the protein was found in inclusion bodies. Nevertheless, it was possible to isolate 1.3 mg of soluble His_6-$HybC_{GLCGR}$ from the soluble fraction derived from 1 g of wet cell paste of the *iscA* mutant (for comparison

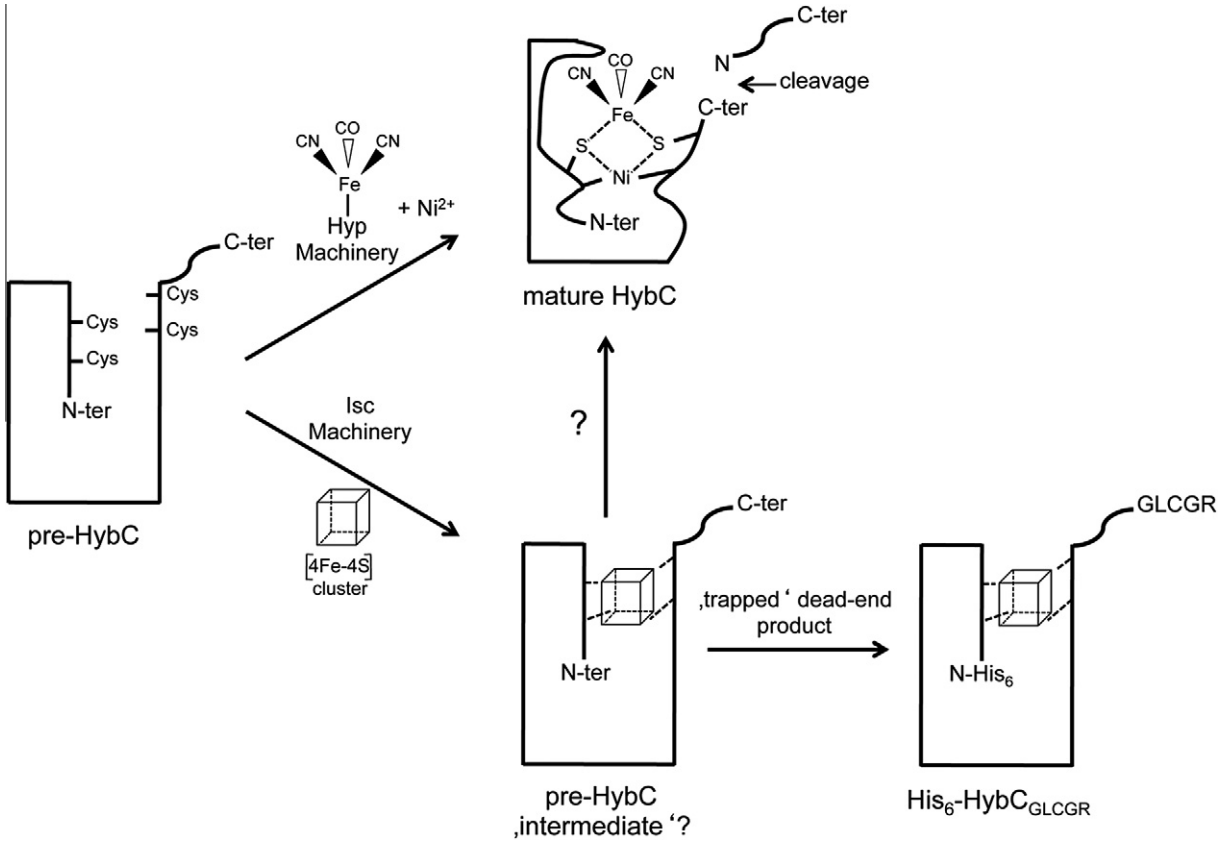

Fig. 3. A schematic model for the observed data is presented. The 4 Cys residues in pre-HybC that coordinate the $NiFe(CN)_2CO$ cofactor (upper part) also coordinate the FeS cluster, which is introduced by the Iron–sulfur cluster machinery (lower part). After $NiFe(CN)_2CO$ cofactor insertion by the Hyp maturases cleavage of the C-terminal 15 amino acids occurs with a conformational change and generation of the mature HybC. His_6-$HybC_{GLCGR}$ variant with the C-terminal pentapeptide extension is not a substrate for the endonuclease HybD and the FeS cluster is trapped as a dead-end product. The 'squiggle' signifies the C-terminal 15 amino acid peptide in the native protein that is cleaved after maturation. The question mark signifies that the pre-HybC with the FeS might be an obligatory intermediate.

FTD147 cells yield 7.5 mg of His_6-$HybC_{GLCGR}$ per g wet cell paste). Analysis of non-heme iron levels from two independent purifications revealed an iron content of only 0.06 mol per mol of His_6-$HybC_{GLCGR}$ (Table 1).

4. Discussion

After insertion of the $NiFe(CN)_2CO$ cofactor into the large subunit of [NiFe]-hydrogenases by the Hyp maturases has been completed, a highly specific endoproteolytic cleavage occurs, which is necessary to allow closure of the active site [4]. Each hydrogenase large subunit has its own specific protease. Only after this specific processing event has occurred can the hydrogenase small subunit form a complex with the large subunit, which ultimately results in the assembly of an active enzyme [4,13]. Thus, in mutants lacking any of the Hyp maturases, nickel-transport proteins or the endoprotease no C-terminal processing occurs, no enzyme activity results and the immature large subunit remains in the cytoplasm. The large subunits of the Hyd-1 and Hyd-2 hydrogen-oxidising enzymes of *E. coli* both undergo an endoproteolytic cleavage that results in removal of a 15 amino acid peptide [4,26], while the HycE large subunit of Hyd-3 loses a 32 amino acid C-terminal peptide [7]. In all three instances, however, cleavage occurs three amino acids C-terminal to the final Cys residue (Cys549 in HybC) that coordinates the $NiFe(CN)_2CO$ cofactor [4] (depicted schematically in Fig. 3). In this study we have shown that extension of the C-terminus of the Hyd-2 large subunit by only five amino acids is sufficient to prevent proteolytic processing. In a previous study it was shown that fusion of GFP to the C-terminus of HybC prevented endoproteolytic cleavage [27]. Our data show that improper folding, as suggested for the GFP fusion, cannot be the cause of impaired cleavage. The results of our study suggest that either an altered structure of the C-terminal peptide or, more likely, impaired recognition by its specific protease (HybD) due to addition of five amino acids might be the reason for ineffective proteolysis. Future studies will address this problem. Restoration of the natural C-terminus of HybC allowed recovery of Hyd-2 enzyme activity, indicating that the N-terminal His-tag was not the cause of the impaired processing or activity. Notably, addition of the same pentapeptide sequence to the HyaB large subunit of Hyd-1 also prevented endoproteolytic cleavage, while addition of the pentapeptide to HycE, the large subunit of Hyd-3, did not affect cleavage or maturation to an active enzyme (M. Braussemann, C. Pinske, B. Soboh and R.G. Sawers, unpublished data). This suggests that differences might exist between the mechanisms and requirements for cleavage of the large subunits of the hydrogen-oxidising enzymes compared with that of the hydrogen-evolving Hyd-3 enzyme.

Surprisingly, when isolated under strictly anaerobic conditions His_6-HybC was brown in colour and spectroscopic and metal analyses revealed sub-stoichiometric levels of a FeS cluster. Based on UV–Vis spectroscopy there appeared to be a mixture of [4Fe–4S] and [2Fe–2S] clusters in the protein. This would be consistent with a labile cluster and indeed we were unable to identify a FeS cluster when His-HybC was isolated aerobically. This is reminiscent of the oxygen-sensitive [4Fe–4S] cluster in the FNR transcription factor [25,28,29].

In a recent study in which the large subunit HyhL from *Thermococcus kodakarensis* was heterologously overproduced in *E. coli,* the authors observed that the isolated protein had both a brown colour and sub-stoichiometric amounts of iron [30], but no detailed

evidence for the binding site was reported. Our own findings have revealed that the unprocessed large subunit of Hyd-1 also has features of a FeS cluster (M. Braussemann, B. Soboh and R.G. Sawers, unpublished data), which suggests that FeS binding *in vivo* might be a general mechanism if processing is blocked or impeded. The immature hydrogenase large subunit acquires a labile FeS cluster, which is coordinated by the same cysteines used to coordinate the $NiFe(CN)_2CO$ cofactor (depicted schematically in Fig. 3). These findings suggest that if metabolic or regulatory restrictions limit $NiFe(CN)_2CO$ cofactor biosynthesis or insertion, then transient insertion of a FeS cluster might stabilize the preformed hydrogenase large subunit, maintaining a folding state appropriate for subsequent insertion of the completed cofactor. Alternatively, the FeS cluster might be an obligatory intermediate on the pathway of maturation, which becomes trapped if late steps in maturation fail (see Fig. 3). This result might also explain the recent observation [24] that the unprocessed forms of native HyaB and HybC are stable in a *hypF* mutant, yet the processed forms of the large subunit are degraded in mutant backgrounds in which the small subunits cannot be matured or in which FeS cluster biosynthesis is impaired.

Acknowledgments

We would like to thank Martin Herzberg for help performing the ICP-MS analyses. This work was supported by the DFG (Grant SA 494/3-1) and the region of Saxony-Anhalt.

References

[1] P.M. Vignais, B. Billoud, J. Meyer, Classification and phylogeny of hydrogenases, FEMS Microbiol. Rev. 25 (2001) 455–501.
[2] A. Volbeda, M.H. Charon, C. Piras, E.C. Hatchikian, M. Frey, J.C. Fontecilla-Camps, Crystal structure of the nickel––iron hydrogenase from *Desulfovibrio gigas*, Nature 373 (1995) 580–587.
[3] L. Forzi, R.G. Sawers, Maturation of [NiFe]-hydrogenases in *Escherichia coli*, Biometals 20 (2007) 565–578.
[4] A. Böck, P. King, M. Blokesch, M. Posewitz, Maturation of hydrogenases, Adv. Microb. Physiol. 51 (2006) 1–71.
[5] A. Magalon, M. Blokesch, E. Zehelein, A. Böck, Fidelity of metal insertion into hydrogenases, FEBS Lett. 499 (2001) 73–76.
[6] M.R. Leach, D.B. Zamble, Metallocenter assembly of the hydrogenase enzymes, Curr. Opin. Chem. Biol. 11 (2007) 159–165.
[7] R. Rossmann, M. Sauter, F. Lottspeich, A. Böck, Maturation of the large subunit (HYCE) of *Escherichia coli* hydrogenase 3 requires nickel incorporation followed by C-terminal processing at Arg537, Eur. J. Biochem. 220 (1994) 377–384.
[8] A. Magalon, A. Böck, Dissection of the maturation reactions of the [NiFe]-hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation, FEBS Lett. 473 (2000) 254–258.
[9] M.J. Casadaban, Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu, J. Mol. Biol. 104 (1976) 541–555.
[10] M. Redwood, I. Mikheenko, F. Sargent, L. Macaskie, Dissecting the roles of *Escherichia coli* hydrogenases in biohydrogen production, FEMS Microbiol. Lett. 278 (2007) 48–55.
[11] A. Paschos, A. Bauer, A. Zimmermann, E. Zehelein, A. Böck, HypF, a carbamoyl phosphate-converting enzyme involved in [NiFe]-hydrogenase maturation, J. Biol. Chem. 277 (2002) 49945–49951.
[12] C. Pinske, R.G. Sawers, The A-type carrier protein ErpA is essential for formation of an active formate–nitrate respiratory pathway in *Escherichia coli* K-12, J. Bacteriol. 194 (2012) 346–353.
[13] C. Pinske, S. Krüger, B. Soboh, C. Ihling, M. Kuhns, M. Braussemann, M. Jaroschinsky, C. Sauer, F. Sargent, A. Sinz, R.G. Sawers, Efficient electron transfer from hydrogen to benzyl viologen by the [NiFe]-hydrogenases of *Escherichia coli* is dependent on the coexpression of the Iron–sulfur cluster-containing small subunit, Arch. Microbiol. 193 (2011) 893–903.
[14] M. Kitagawa, T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, H. Mori, Complete set of ORF clones of *Escherichia coli* ASKA library (*A* complete set of *E. coli K*-12 ORF *a*rchive): unique resources for biological research, DNA Res. 12 (2005) 291–299.
[15] Y. Begg, J. Whyte, B. Haddock, The identification of mutants of *Escherichia coli* deficient in formate dehydrogenase and nitrate reductase activities using dye indicator plates, FEMS Microbiol. Lett. 2 (1977) 47–50.
[16] B. Soboh, C. Pinske, M. Kuhns, M. Waclawek, C. Ihling, K. Trchounian, A. Trchounian, A. Sinz, R.G. Sawers, The respiratory molybdo-selenoprotein formate dehydrogenases of *Escherichia coli* have hydrogen: benzyl viologen oxidoreductase activity, BMC Microbiol. 11 (2011) 173.
[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[18] O. Lowry, N. Rosebrough, A. Farr, R. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[19] D.J.A. Netz, C.M. Stith, M. Stümpfig, G. Köpf, D. Vogel, H.M. Genau, J.L. Stodola, R. Lill, P.M.J. Burgers, A.J. Pierik, Eukaryotic DNA polymerases require an Iron–sulfur cluster for the formation of active complexes, Nat. Chem. Biol. 8 (2012) 125–132.
[20] W.W. Fish, Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples, Meth. Enzymol. 158 (1988) 357–364.
[21] J.D. Cline, Spectrophotometric determination of hydrogen sulfide in natural waters, Limnol. Oceanogr. 14 (1969) 454–458.
[22] M. Paul, S. McSheehy, Elementspezies bestimmen mit ICP-MS, Nachr. Chem. 55 (2007) 766–769.
[23] N.K. Menon, C.Y. Chatelus, M. Dervartanian, J.C. Wendt, K.T. Shanmugam, H.D. Peck Jr., A.E. Przybyla, Cloning, sequencing, and mutational analysis of the *hyb* operon encoding *Escherichia coli* hydrogenase 2, J. Bacteriol. 176 (1994) 4416–4423.
[24] C. Pinske, R.G. Sawers, Delivery of Iron–sulfur clusters to the hydrogen-oxidizing [NiFe]-hydrogenase in *Escherichia coli* requires the A-type carrier proteins ErpA and IscA, PLoS One 7 (2012) e31755.
[25] B. Py, P.L. Moreau, F. Barras, Fe–S cluster, fragile sentinels of the cell, Curr. Opin. Microbiol. 14 (2011) 218–223.
[26] E. Theodoratou, A. Paschos, S. Mintz-Weber, A. Böck, Analysis of the cleavage specificity of the endopeptidase involved in the maturation of the large subunit of hydroenase 3 from *Escherichia coli*, Arch. Microbiol. 173 (2000) 110–116.
[27] M. Zhang, N. Pradel, M.A. Mandrand-Berthelot, Z. Yu, L.F. Wu, Effect of alteration of the C-terminal extension on the maturation and folding of the large subunit of the *Escherichia coli* hydrogenase-2, Biochemie 85 (2003) 575–579.
[28] A.J. Jervis, J.C. Crack, G. White, P.J. Atymiuk, M.R. Cheesman, A.J. Thomson, N.E. LeBrun, J. Green, The O_2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe–4S] to [2Fe–2S] conversion, Proc. Natl. Acad. Sci. USA 106 (2009) 4659–4664.
[29] E.L. Mettert, F.W. Outen, B. Wanta, P.J. Kiley, The impact of O_2 on the Fe–S cluster biogenesis requirements of *Escherichia coli* FNR, J. Biol. Chem. 384 (2008) 798–811.
[30] D. Sasaki, S. Watanabe, T. Kanai, H. Atomi, T. Imanaka, K. Miki, Characterization and *in vitro* interaction study of a [NiFe] hydrogenase large subunit from the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1, Biochem. Biophys. Res. Commun. 417 (2012) 192–196.