The [FeFe]-hydrogenase maturase HydF from *Clostridium acetobutylicum* contains a CO and CN⁻ ligated iron cofactor

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A R T I C L E  I N F O

Article history:
Received 16 November 2009
Revised 9 December 2009
Accepted 10 December 2009
Available online 14 December 2009

Edited by Stuart Ferguson

Keywords:
[FeFe] Hydrogenase
H-Cluster
Maturation
Fourier-transform infrared spectroscopy
Electron paramagnetic resonance
spectroscopy
Clostridium acetobutylicum

A B S T R A C T

Biosynthesis of the [FeFe] hydrogenases active site (H-cluster) requires three maturation factors whose respective roles are not understood yet. The clostridial maturation enzymes (CaHydE, CaHydF and CaHydG) were homologously overexpressed in their native host *Clostridium acetobutylicum*. CaHydF was able to activate *Chlamydomonas reinhardtii* [FeFe] hydrogenase apoprotein (CrHydA1) almost 100% compared to the native specific hydrogen evolution activity. Based on electron paramagnetic resonance spectroscopy and Fourier-transform infrared spectroscopy data the existence of a [4Fe4S] cluster and a CO and CN⁻ ligand coordinated di-iron cluster is suggested. This study contains the first experimental evidence that the bi-nuclear part of the H-cluster is assembled in HydF.

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1. Introduction

[FeFe] hydrogenases are [FeS] proteins that catalyze the reversible reduction of protons to molecular hydrogen. They have been found in hydrogen evolving anaerobic bacteria and unicellular eukaryotes [1,2]. The active site of [FeFe] hydrogenases is commonly known as H-cluster. This unique prosthetic group consists of a ferredoxin-type [4Fe4S] cluster, connected via a single bridging dithiolate ligand [3]. Based on a pulse electron paramagnetic resonance spectroscopy (EPR) study, the central atom of the dithiolate bridge has been recently identified as nitrogen [4].

The green alga *Chlamydomonas reinhardtii* possesses a small [FeFe] hydrogenase (CrHydA1) of 48 kDa [5]. In contrast to bacterial-type [FeFe] hydrogenases, CrHydA1 exhibits no additional [FeS] clusters besides the active site [6]. Recent spectroscopic studies of CrHydA1 [7–9] confirmed that the general structure of the active center is similar to other known [FeFe] hydrogenases. Three highly conserved proteins HydE, HydF and HydG, which are common in all organisms containing [FeFe] hydrogenases, are required for the assembly of the intact H-cluster [10]. Recently, the X-ray crystallographic structure of recombinant, reconstituted HydE from *Thermotoga maritima* (TmHydE) was solved. The structure is very similar to that of biotin synthase from *Escherichia coli*; in addition to the [4Fe4S] cluster of the radical-reaction center a second [2Fe2S] cluster is present depending on the reconstitution state [11]. HydG is proposed to be involved in the synthesis of the dithiolate ligand. Studies of Pilet et al. demonstrated its ability to cleave the substrate tyrosine into p-cresol in a radical-S-adenosyl methionine (SAM) reaction, further reaction steps of the second proposed reaction product dehydroglycine could result in a dithiolate bridge with a bridging nitrogen atom [12]. HydF contains a Guanosinetriphosphate (GTP)-binding motif, shows GTPase activity [13] and was hypothesized to act as a scaffold protein in the

**Abbreviations:** EPR, electron paramagnetic resonance spectroscopy; FTIR, Fourier-transform infrared spectroscopy; HYSCORE, Hyperfine sublevel correlation spectroscopy; CrHydA1, [FeFe] hydrogenase protein of *C. reinhardtii*; CaHydE, maturation factor HydE of *C. acetobutylicum*; CaHydF, maturation factor HydF of *C. acetobutylicum*; CaHydG, maturation factor HydG of *C. acetobutylicum*; TmHydE, maturation factor HydE of *Thermotoga maritima*; TmHydF, maturation factor HydF of *Thermotoga maritima*; [2Fe2S] moiety of the H-cluster; GTP, Guanosinetriphosphate; GDP, Guanosindiphosphate; SAM, S-adenosyl methionine; AdoH, S'-Desoxadenosine

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H-cluster maturation process [14]. The steps of H-cluster biosynthesis are largely unknown. To achieve deeper insight into the maturation process, [FeFe] hydrogenase apoprotein synthesized in the absence of maturation factors can be activated by in vitro maturation. Previous studies showed evidence that all three maturation factors have to be expressed in concert to achieve maturation activity [15]. The hydrogenase apoprotein needs to contain a [4Fe4S] cluster to get activated by HydF [16]. However, there is almost nothing known about the structure of the cofactor in HydF. In this study, a very efficient in vitro maturation system is presented, utilizing homologously overexpressed Clostridium acetobutylicum maturation factors (CaHydE, CaHydF and CaHydG) and CaHydA1 apoprotein (CrHydA1apo) expressed in E. coli. Hydrogen evolution rates close to the evolution rates of native CaHydA1 were obtained. Spectroscopic analyses by EPR and Fourier-transform infrared spectroscopy (FTIR) techniques provide information about the inorganic cofactors within the maturation HydF.

2. Materials and methods

2.1. Cloning of maturation factors

The genes hydro (hydF, hydF and hydF) were PCR-amplified from purified C. acetobutylicum ATCC 824 genomic DNA with primers containing adequate restriction sites. For cloning into pTrHydA1Opt-C-tag plasmid [18] the CrHydA1Opt was first excised with BamHI and EcoRV restriction endonucleases. The maturease-encoded genes were then cloned into the vector with primers using the same sites. The following primers, containing a BamHI restriction site in the forward primer and a EcoRV restriction site in the reverse primer, were used: 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTCTTTT-3′ for hydE, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydF, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG, 5′-GGATCCATGGGTAAGATATTAATAAAGGGCCTTTGAGGTATATAAACCATGAGATCTCCTTCTTTTT-3′ for hydG. The resulting plasmids pT hysterEca, pHydFe2Ca, and pHydFe3Ca were used to transform C. acetobutylicum ATCC824 [19].

2.2. Strains, media and growth conditions

Anaerobic overexpression of the maturation factors CaHydE, CaHydF and CaHydG in recombinant C. acetobutylicum ATCC 824 strains were performed as described earlier [18,19]. Every single overexpressed maturease was synthesized with a background of three maturation enzymes in one organism [15]. The hydrogenase apoprotein needs to contain a [FeS] cluster to get activated by HydF [16]. However, there is almost nothing known about the structure of the cofactor in HydF. In this study, a very efficient in vitro maturation system is presented, utilizing homologously overexpressed Clostridium acetobutylicum maturation factors (CaHydE, CaHydF and CaHydG) and CaHydA1 apoprotein (CrHydA1apo) expressed in E. coli. Hydrogen evolution rates close to the evolution rates of native CaHydA1 were obtained. Spectroscopic analyses by EPR and Fourier-transform infrared spectroscopy (FTIR) techniques provide information about the inorganic cofactors within the maturation HydF.

2.3. Purification and characterization of Streptococcus thermophilus tagged proteins

All purification steps were performed under strictly anaerobic conditions with oxygen-free buffers, containing 2 mM sodium dithionite (dithionite). The purification was performed as described in the appropriate protocol (IBA GmbH, Gottingen, Germany). The elution step was performed with 2.5 mM desthiobiotin in 100 mM Tris–HCl buffer pH 8, each fraction was provided with 3% glycerol, sealed and frozen at –80 °C. 10% SDS–polyacrylamide gel electrophoresis (PAGE) was used to analyze the presence and purity of the proteins. Protein concentrations were determined using Quick Start Bradford Protein Assay (Bio-Rad Laboratories GmbH, Muenchen, Germany). GTPase activity and radical–SAM activity were measured by HPLC analyses as described by Brazzolotto et al. [13] and Zhao et al. [20] using a NucleoL 100–5 C18 ec EC 250/4.6 column (Machery Nagel, Düren, Germany). SAM and GTP cleavage was determined at 37 °C after different incubation times between 0 h and 6 h.

For spectroscopic measurements proteins were concentrated to achieve 100 μM in 30 μl with Vivasin 500 centrifugal filter units (Satorius, Gottingen, Germany). For analyses of the reduced proteins, 30 mM dithionite were added to ensure complete reduction of the [FeS] clusters.

2.4. Maturation assay

CrHydA1apo was incubated with CaHydE, CaHydF and CaHydG in their as-isolated form, provided in the elution buffer. The assay had a final volume of 400 μl and was prepared in a 1.5 ml vial to a final volume of 400 μl with 100 mM potassium phosphate buffer pH 6.8 with 0.25 mM GTP (Acros Organics, Geel, Belgium) and 2.5 mM MgCl2 (JT Baker, Deventer, Netherlands) and incubated at 37 °C in an anaerobic chamber for 1 h. To determine the hydrogen evolution activity, the complete 400 μl assay was added to a hydrogenase activity assay [5].

2.5. Spectroscopic characterization

Q-band EPR spectra of CrHydA1apo, and all clostridial maturation enzymes were measured by using the 2-pulse electron spin echo (ESE) detected EPR technique. The ESE intensity was detected after two microwave (MW) pulses (π/2 and π) as a function of the external magnetic field. The delay between the MW pulses was fixed to τ = 500 ns. The length of the π/2 MW pulse was set to 40 ns and the π pulse was set to 68 ns. If not mentioned, the shot repetition time was set to 1 ms. All measurements were performed on a Bruker ELEXYS E580 Q-band spectrometer with a SuperQ–FT microwave bridge, working at 34.11 GHz. Cryogenic temperatures (10–20 K) were obtained by an Oxford CF935 flow cryostat.

FTIR measurements were performed on a Bruker IFS 66 v/s FTIR spectrometer equipped with a Bruker MCT (mercury–cadmium–telluride) detector. The sample chamber was under a constant flow of N2 purge gas to expel CO2 gas and water vapour. Low temperatures were achieved by an Oxford OptistatCF continuous helium flow cryostat. The interferograms were accumulated in the double-sided, forward–backward mode with 2000 scans. All measurements were performed with a resolution of 3 cm−1. The obtained interferograms were automatically processed by the Opus software utilizing a 32-points phase correction and a Blackman–Harris 3-term apodization window. Baseline correction was done using a cubic spline data interpolation procedure applied to manually selected points of the experimental spectra. Data processing was facilitated by home-written routines in the MATLAB™ programming environment.

3. Results and discussion

3.1. Maturation capability of homologously expressed HydF

It was shown that the facultative anaerobe bacterium C. aceto- butylicum is useful for the overexpression of correctly assembled [FeS] proteins [19]. To achieve the maturation factors (HydE, HydF and HydG) with maturation capability, it is essential to co-synthesize all three maturation enzymes in one organism [15]. C. acetobutylicum which naturally provides all three maturation factors can be purified...
with a yield of approximately 2 mg protein per liter cell culture. Further characterizations of the as-isolated proteins by HPLC analyses showed radical-SAM activity of CaHydE (0.008 AdoH min$^{-1}$) and GTase activity of CaHydF (0.017 GDP min$^{-1}$), which were in the same range as the $Tm$HydE and $Tm$HydF activities as reported previously [13,21]. The main elution fractions are light brown colored, which indicates the presence of iron in all proteins.

For analyzing the influence of several maturation factors in an in vitro maturation assay, different combinations of 0.7 ug anaerobically purified CaHydA$_{apo}$, which was heterologously expressed in E. coli BL21(DE3) with a yield of 0.8 mg per liter, with 8.4 ug of each maturation enzyme were tested. CaHydA$_{apo}$ and the three mature proteins exhibited no background activity of hydrogen evolution when tested individually. The combination of CaHydE and/or CaHydG with CaHydA$_{apo}$ also did not result in H$_2$-production.

However, by combining CaHydA$_{apo}$ with CaHydF a hydrogen evolution rate of 486 umol H$_2$ min$^{-1}$ mg$^{-1}$ was achieved. To evaluate the dependence of the molar CaHydE: CaHydA$_{apo}$ ratio and the hydrogen production, several protein ratios were tested. Maximum CaHydA$_{apo}$ activity of 98% (726 umol H$_2$ min$^{-1}$ mg protein$^{-1}$) compared to the native enzyme CaHydA was achieved by incubating 0.017 uM CaHydA$_{apo}$ with 0.466 uM CaHydF (see Fig. 1). The experiments show that the hydrogen production rate of CaHydA$_{apo}$ increases nearly linearly up to almost full native hydrogen evolution activity at rising CaHydF:CaHydA$_{apo}$ ratios.

Interestingly, an about 80-fold excess of the HydF protein is needed to yield an H$_2$ evolution rate of only 50 umol H$_2$ min$^{-1}$ mg$^{-1}$ (about 15% of the native activity) if HydF is heterologously expressed with a background of the other heterologously introduced maturation factors in E. coli [14,15,22]. Apparently, the overexpression of the maturation factors in their native host, with background expression of the intrinsic maturess, results in a much more capable CaHydF enzyme. To give evidence for the assumed cluster transfer from HydF to HydA apoprotein [14,16] in the activation process, we have examined proteins of the maturation process on presence of inorganic cofactors.

### 3.2. Spectroscopic analyses of inactive hydrogenase and active maturases

The EPR spectrum of the dithionite treated CrHydA$_{apo}$ Feveals a strong rhombic signal (Fig. 2IA). The principal g-values of the spectrum are very typical for a ferredoxin like (all-Cys ligated) [4Fe4S]$^{1+}$ cluster [23]. The relaxation properties and the temperature dependence are also typical for [4Fe4S]$^{1+}$ clusters [23]. Based on our EPR measurements we can confirm that the CrHydA$_{apo}$ already contains the cubane part of the H-cluster as it was suggested recently by Mulder et al. based on EPR and Mössbauer measurements on CrHydA$_{apo}$ [16].

To understand the structure and functional properties of the maturation proteins, it was important to perform spectroscopic analyses with homologously expressed maturation enzymes with full maturation capability. So far, only EPR studies were initiated with the HydE, HydF and HydG enzymes from T. maritima (TmHydE, TmHydF and TmHydG). The T. maritima maturases were heterologously expressed in E. coli with no background expression of the two other maturation factors, which means that they have no maturation capability. The metal cofactors were reconstituted with the cysteine desulfurase IscS protein from E. coli [13,21]. To evaluate whether [FeS] clusters of active clostridial maturation factors differ from those with no maturation capability, we performed EPR and FTIR measurements of C. acetobutylicum maturators expressed in concert with all maturases and with no need of subsequent reconstitution.

The EPR spectra of CaHydE and CaHydG reduced with dithionite are quite similar to typical spectra of [4Fe4S]$^{1+}$ clusters (Fig. 2IBC). The spectra are essentially axial with principal g-values characteristic for this class of FeS clusters ($g_{CaHydE} = 2.036, 1.932, 1.908; g_{CaHydG} = 2.035, 1.940, 1.904$). The CaHydE sample without dithionite shows a dramatic decrease of the [4Fe4S]$^{1+}$ EPR signal. This
indicates that the [4Fe4S] cluster in CaHydE is mainly in its oxidized EPR silent form when no reductant is present. We did not observe appearance of any additional signals in the spectra for this sample. Therefore, based on the obtained data we suggest that both CaHydE and CaHydG contain a single [4Fe4S] cluster in their structures. From the X-ray structure of TmHydE a [4Fe4S] and a [2Fe2S] cluster have been suggested [11]. However, the presence of the additional [2Fe2S] cluster is largely dependent on the reconstitution and soaking conditions. We did not observe any EPR signals of CaHydE that could be related to a reduced [2Fe2S] cluster. It has to be noted that elimination of the [2Fe2S] cluster by mutagenesis of TmHydE did not affect its maturation capacity [11]. Therefore, it is possible that this cluster does not play a role in the maturation process and might not be conserved in different HydE proteins.

The EPR spectra of CaHydF showed some diversity for the sample with and without dithionite (Fig. 2II). The EPR spectrum of the sample with dithionite is rather simple and resembles that of a [4Fe4S] cluster. It is possible that this cluster does not play a role in the maturation process and might not be conserved in different HydE proteins.

The EPR spectra of CaHydF showed some diversity for the sample with and without dithionite (Fig. 2II). The EPR spectrum of the sample with dithionite is rather simple and resembles that of a [4Fe4S] cluster (Fig. 2II), similar to the signal observed in TmHydF [13] (g_CaHydF = 2.049, 1.902, 1.872). In the CaHydF primary structure, three cysteine residues (Cys304, Cys353 and Cys356) together with a histidine provide the only possible motif for the ligation of an [FeS] cluster. We performed pulse EPR measurements of 14N signals to probe His ligation on the [4Fe4S] signal using hyperfine sublevel correlation spectroscopy (HYSCORE). The measured X-band HYSCORE spectrum (not shown) is identical to the one measured on TmHydF [13], indicating a histidine ligation of the [4Fe4S] cluster.

An additional and rather different EPR signal (g_CaHydF (red) = 2.045, 2.007, 1.906) was detected in the CaHydF samples that had been purified without dithionite (“oxidized form”), while the [4Fe4S] signal was found to be considerably smaller (Fig. 2II). For convenience, the spectra in Fig. 2IIA are normalized to the [4Fe4S] signals. Notably, the high-field component of the spectrum has a complex structure (i.e., an extra “step” at g = 1.906). This might indicate the presence of several overlapping signals.

We found that the longitudinal relaxation for the additional (“oxidized”) signal is about 10 times slower than for the [4Fe4S] signal. At 20 K the spectrum shows predominantly the “oxidized” signal while the [4Fe4S] signal is strongly suppressed due to fast longitudinal relaxation (Fig. 2IIIB). As it has been observed for various FeS clusters by EPR the longitudinal relaxation rates becomes slower with decreasing number of Fe atoms in the FeS cluster [24]. Although the relaxation rates can depend strongly on the actual structure and surrounding of a FeS cluster, we suggest that this additional signal might be attributed to a cluster which contains three or less irons.

Based on EPR data we can conclude that the Fe-cluster is EPR silent in the reduced state and EPR active in the oxidized state, which is rather similar to the redox behaviour of the EPR signals of the H-cluster [7]. Thus it is tempting to suggest low-valence states of the irons in the Fe cluster (e.g., [Fe(I)Fe(I)]red and [Fe(I)Fe(I)]ox configurations). Moreover, the extracted g-values for the “oxidized” EPR spectrum of CaHydF are rather different from those of the oxidized H-cluster. The electronic structure of the EPR active states of the H-cluster is governed by a strong exchange interaction between the [2Fe2S] and the [4Fe4S] subcluster resulting in principal g-values above g_L = 2.0023. The fact that the principal g-values for the “oxidized” CaHydF differs dramatically from what has been observed for the oxidized H-cluster most probably indicates that in CaHydF the [2Fe2S] cluster and the [4Fe4S] cluster are not bound like the H-cluster.

Since a part of the assembly of the bi-nuclear subcluster of the H-cluster should involve incorporation of CN− and CO ligands, we decided to further characterize the CaHydF protein by means of IR spectroscopy. We concentrated on the frequency range between 2100 cm⁻¹ and 1800 cm⁻¹ in the FTIR spectra which is predominantly governed by signals of C–O/C–N stretching vibrations [25]. It has been shown that the H-cluster and for various related biomimetic (bi-nuclear) model complexes that CN bands normally lie in the range between 2110 cm⁻¹ and 2025 cm⁻¹, while CO bands occur at the lower frequencies between 1850 cm⁻¹ and 1850 cm⁻¹ [9,25–27].

The dithionite reduced sample showed a FTIR spectrum, consisting of four bands in the region of the terminal CO and two at the CN region (see Fig. 3A). It is noteworthy that the position of the IR bands is similar to the one observed in Fe(I)–Fe(I) model complexes containing CN− ligands and no bridging CO [27]. When dithionite was removed from the sample the FTIR spectrum changed (Fig. 3B). Seven additional bands could be identified in the CO region, while the CN bands did not change significantly. Most of the additional bands shifted upwards in frequency, which is typical for oxidation of the central iron atom [9,26]. Since the number of CO bands in this sample is larger than what has been observed for the dithionite reduced sample (which is anticipated to contain a single reduced state of the additional Fe-cluster), we propose the existence of more than one additional species. Future electrochemical studies could clarify the assignment of these bands. Nevertheless, as can be seen from Fig. 3B, a clear band at 1811 cm⁻¹ has been observed which is typical for a bridging CO ligation, indicating the presence of a Fe–CO–Fe moiety. Therefore, these results suggest a bi-nuclear nature of the additional Fe-cofactor in CaHydF.

3.3. Summary

Several hypotheses have been formulated for the role of HydF in the maturation process, although no direct experimental evidence has been obtained so far [14,16,28].

The fact, that CaHydF is the only responsible maturation factor for in vitro maturation of CrHydAapo, supports the results of McGlynn et al. and encourages the hypothesis that HydF is acting as the key player for the assembly of the bi-nuclear site in HydA [14]. Furthermore, we showed that homologously expressed...
CnHydF is much more active than heterologously expressed CnHydF and that it is possible to activate CrHydA1 apo up to full native hydrogen evolution activity.

Two different Fe-containing cofactors have been detected in CnHydF. One of them can be identified as a [4Fe-4S] cluster. Considering the spectroscopic observations presented here we propose that (1) the observed additional Fe-cluster is a bi-nuclear cluster; (2) in the oxidized form the cluster is EPR active, while in the reduced form it is EPR silent; (3) iron ions in the additional Fe-cluster are coordinated by CN- and CO ligands (most likely one CN- and two CO ligands per iron); (4) in at least one of the oxidized forms the cluster contains also a bridging CO ligand. Since the properties of this additional cluster are very similar to those of the bi-nuclear subcluster of the H-cluster, it is anticipated that it can be used for the activation of the hydrogenase apoprotein.

Acknowledgements

This work was supported by the European Union/Energy Network (SOLAR-H2 contract 212508), the BMBF (H2-design cells) and the Max-Planck-Gesellschaft. We are also very grateful to L. Currell (MPI Mülheim/Ruhr) for his technical assistance.

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