Title

Isolation and characterisation of a new [FeFe]-hydrogenase from *Clostridium perfringens*

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Running title (max 50 characters)

The [FeFe]-hydrogenase of *Clostridium perfringens.*

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Abstract (50-250 words)

This paper reports the first characterisation of an [FeFe]-hydrogenase from a *Clostridium perfringens* strain previously isolated in our laboratory from a pilot-scale bio-hydrogen plant, that efficiently produces H_2 from waste biomasses. On the basis of sequence analysis, the enzyme is a monomer formed by four domains hosting various iron-sulphur centres involved in electron transfer and the catalytic centre H-cluster. After recombinant expression in *E. coli*, the purified protein catalyses H_2 evolution at high rate of 1645±16 s^{-1}. The optimal conditions for catalysis are in the pH range 6.5-8.0 and at the temperature of 50°C. EPR spectroscopy showed that the H-cluster of the oxidised enzyme displays a spectrum coherent with the H_{ox} state, while the CO inhibited enzyme has a spectrum coherent with the H_{ox}-CO state. FTIR spectroscopy showed that the purified enzyme is composed of a mixture of redox states, with a prevalence of the H_{ox}; upon reduction with H_2, vibrational modes assigned to the H_{red} state were more abundant, while binding of exogenous CO resulted in a spectrum assigned to the H_{ox}-CO state. The spectroscopic features observed are similar to those of the [FeFe]-hydrogenases class, but relevant differences were observed given the different protein environment hosting the H-cluster.

Keywords (max 6, alphabetical order, at least 3 not used in the title)

Bio-hydrogen; *Clostridium perfringens*; [FeFe]-hydrogenases; H-cluster; iron-sulphur centres; recombinant expression.

Introduction

Hydrogen is a promising energy carrier that may replace or complement fossil fuels. For this purpose, the production of this gas by renewable technologies is necessary, and, in this perspective, the exploitation of microorganisms or enzymes for high rate hydrogen synthesis from low cost substrates has been strongly supported [1-4].

In Clostridia, the very last step of H_2 production is catalysed by [FeFe]-hydrogenases, a class of redox enzymes found in several microorganisms, that display exceptionally high turnover rates [5,6].

[FeFe]-hydrogenases are characterised by the peculiar catalytic centre H-cluster, an organometallic cofactor composed by a cubane [4Fe4S] sub-cluster and a [2Fe] sub-cluster; the [4Fe4S] centre is coordinated by four conserved cysteines, while the [2Fe] centre is coordinated by a single protein cysteine and by other non protein ligands [7,8].

These enzymes have been widely investigated and their application as natural or engineered catalysts has been proposed [9-13]. Surprisingly, despite the large interest and vast availability of different natural enzymes, only few of them have been studied so far [5,14,15].

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium that is widely distributed in the environment and is also part of the normal flora of the human and animal intestine. Despite this, it is a relevant pathogen as it can cause infections and food poisoning [16,17].

Similarily to other Clostridia, its metabolism is based on anaerobic fermentation: after glycolysis, piruvate is oxidised via pyruvate-ferredoxin oxidoreductase (PFOR) resulting in the production of lactate, alcohol, acetate and butyrate together with gaseous CO_2 and H_2 [16]. *Clostridium perfringens* is often found in anaerobic digestion plants, where it is one of the responsible of H_2 generation [18-20].

In this work, the [FeFe]-hydrogenase CpHydA was studied for the first time. The gene CPF_2655, encoding for CpHydA, was cloned from the strain *Clostridium perfringens* SM09, recently isolated in our laboratory from a pilot-scale bio-hydrogen plant in Torino (Italy) which was particularly efficient in H_2 production from waste biomasses [20,21].

Previous works showed that the gene CPF_2655 is expressed during H_2 production [20] and that it is directly involved into the metabolic pathway [22].

Here, the new enzyme was recombinantly expressed in *E. coli* in the active form, purified and characterised both in its functional and spectroscopic features.

Materials and methods

Gene cloning

A DNA fragment of 2126 bp containing the entire coding sequence of CPF_2655 was amplified by PCR from the genome of *Clostridium perfringens* SM09 [20] using the following primers: ATGGCGTTGAAGAAGCAAAG and AACCGTTTTCATCCATGAGC. For this purpose, the proof-reading KOD Hot Start DNA polymerase (Merck) was used following the manufacturer's instructions.

Subsequently, the 5' of the gene was modified by PCR to insert an NdeI site and the 3' was modified to add a sequence coding for Strep-tagII and a XhoI site using the following primers: AACCATATGAATAAAATAATAATCAATGATAAGACTATCG and ATCTCGAGTTATTTTTCAAATTGAGGATGACTCCAATTTTTTTTATATTTCATGTGTAATAACTCATGAG.

The PCR fragment (1760 bp) was digested with NdeI and XhoI (Thermo Scientific) and ligated into the empty expression vector pECr1 [23] to give the new vector pECPF2655. The T4 DNA Ligase (Thermo Scientific) was used. The gene cloned into the final expression vector has been fully sequenced.

The sequence has been deposited into the GenBank database and assigned the accessions KP115260 (DNA) and AJQ21778 (protein).

A homology model of the 3D structure was built by the Swiss-Model server (http://swissmodel.expasy.org/) using CpI 3C8Y x-ray structure as template.

The sequence alignment was performed using MultAlin (http://multalin.toulouse.inra.fr/multalin/). The accessions of the other [FeFe]-hydrogenase sequences are: CaHydA (NP_346675), CpI (AAA23248), DdH large subunit (1HFE) and CrHydA1 mature form (AAL23572).

Recombinant expression and purification

Recombinant expression in *E. coli* Rosetta2(DE3) was performed as previously described [24]. Briefly, the vector pECPF2655 (harbouring CpHydA gene and CaHydE maturation gene) was co-transformed with pCaFG (harbouring CaHydF and CaHydG maturation genes) [23]. Cells were aerobically grown in Terrific Broth medium supplemented with 2 mM ammonium ferric citrate as a source of iron, 200 µg/mL carbenicillin, 50 µg/mL streptomycin and 34 µg/mL chloramphenicol. When the OD_{600} reached 0.4, the culture was supplemented with 2 mM cysteine, 25 mM sodium fumarate and 0.5% w/v glucose and the expression was induced by the addition of 1.5 mM IPTG. After the induction, cells were incubated over night under pure argon flow to maintain anaerobic conditions in a water bath at 30°C. To prevent oxygen inactivation of the active CpHydA all the

following manipulations were performed into a glove box (Plas Labs) under an anaerobic hydrogen/nitrogen atmosphere; before use, all solutions were vacuumed, equilibrated with the anaerobic atmosphere and supplemented with 2-20 mM sodium dithionite.
Purification of CpHydA was carried out under strict anaerobic conditions by affinity chromatography using Strep-Tactin Superflow high capacity cartridges (IBA) and following manufacturer's instructions. The enzyme was eluted and stored in 100 mM Tris·HCl, 150 mM NaCl pH 8.0 supplemented with 2 mM sodium dithionite and 2.5 mM desthiobiotin.
Coomassie-stained SDS-PAGE was used to determine the purity and the molecular weight of the purified enzyme. Protein concentration was assayed with Bradford assay using bovine serum albumin as standard.

Hydrogenase activity assay

Hydrogenase activity (hydrogen evolution) was tested as previously described [25]. Briefly, 10 mM dithionite-reduced methyl viologen was used as artificial electron donor at 37°C in 100 mM Tris·HCl, 150 mM NaCl pH 8.0, unless otherwise indicated. The evolution of H_2 was quantified by gas chromatography using an Agilent Technologies 7890A instrument equipped with purged packed inlet, Molesieve 5A column (30 m, ID 0.53 mm, film 25 mm) and thermal conductivity detector; argon was used as carrier gas.
For the study of the pH dependance, the following buffering agents were used: 50 mM MES·NaOH (pH range 5.2–6.5) or 50 mM ACES·NaOH (pH range 6.5–7.5) or 50 mM Tris·HCl (pH range 7.5–9.0); the total ionic strength was fixed to 50 mM with NaCl. For the study of the temperature dependance, a refrigerated water bath was used to set up the temperature in the range between 0 and 70°C. The relative activity was calculated as the ratio between the activity of each experimental point and the maximum activity. The activation energy was calculated by an Arrhenius plot.
Oxygen sensitivity was determined as previously described [26].

EPR spectroscopy

Purified CpHydA was anaerobically concentrated by ultrafiltration using Amicon Ultra 0.5 mL 30K MWCO (Millipore); the buffer was exchanged to remove traces of dithionite and 5% v/v glycerol was added. The enzyme was treated with thionine in a molar excess of 7.8 to obtain the oxidised sample; the final CpHydA concentration was 0.2 mM. The CO-treated sample was obtained by flushing the oxidised sample with CO twice for 30 seconds on ice.
X-band continuous wave (CW) EPR spectra were recorded on a Bruker EMX spectrometer equipped with a cylindrical cavity and operating at a 100 kHz field modulation. The experimental

parameters were as follows: microwave power 10 mW, modulation amplitude 0.2 mT, temperature 77K. Typical measurements were carried out on a 60 µL frozen solution in a quartz tube with internal diameter 2 mm. EPR spectra were simulated using the Easyspin tool package [27].

FTIR spectroscopy

For the characterisation of the H-cluster, purified CpHydA was anaerobically concentrated up to 1 mM. The untreated sample was measured immediately after concentration; the fully oxidised sample was obtained by addition of thionine (8 mM); the H_2-treated sample was obtained by flushing H_2 three times for 1 minute on ice; the CO-treated sample was obtained by flushing CO twice for 30 seconds on ice.

The spectra were acquired at room temperature using a Bruker Tensor 27 FT-IR spectrometer (Bruker Instruments). The sample chamber was purged with 99.9999% pure nitrogen gas.

For the characterisation of the H-cluster, a transmission cell (CaF_2 window; 50 µm pathlength) was used. Spectra were acquired with a resolution of 2 cm^{-1} accumulating 256 scans. The baseline correction was obtained using the Opus 6.0 software (Bruker Instruments) by the concave rubberband algorithm.

Results and discussion

Cloning, recombinant expression and purification

In order to produce large amount of active CpHydA, the gene CPF_2655 was cloned into the vector pECr1 [23], replacing the gene HydA1, and resulting into the new vector pECPF2655 (Fig. 1A). When co-transformed with the vector pCaFG [23], this vector is suitable for the recombinant expression in *E. coli* [23]. The two vectors allow the co-expression of CpHydA with the maturases HydE, HydF and HydG from *Clostridium acetobutylicum*, that allow the assembly of the H-cluster within the [FeFe]-hydrogenase, under strict anaerobic conditions, to avoid the inactivation of the enzyme by atmospheric oxygen [28].

CpHydA was purified by affinity chromatography with a typical yield of 1.5 mg per litre of culture. The purified protein (Fig. 1B) has the expected molecular weight of ~64.7 kDa.

Sequence analysis

CpHydA is a monomeric [FeFe]-hydrogenase phylogenetically classified in the cluster A2 with a modular structure M3 (Fig. 1C), according to the most recent literature [6].

The enzyme is composed by four domains that contain iron sulphur clusters (Fig. 1D): a [2Fe2S] plant ferredoxin-like (yellow), a [4Fe4S] coordinated by three cysteines and one histidine (red), a 2[4Fe4S] bacterial ferredoxin-like (green) and the H-domain containing the catalytic centre H-cluster (blue). The various iron sulphur centres are involved in the electron transfer from the redox partner to the H-cluster, where the reduction of protons into H_2 is catalysed.

Alignment with other known [FeFe]-hydrogenase sequences (Fig. 2) allows the identification of the key residues in CpHydA: fifteen cysteines and the histidine responsible for the binding of the four accessory iron sulphur clusters in the N-terminal and C300, C355, C497 and C501, responsible for the binding of the H-cluster in the C-terminal. The latter residues are embedded in the signature sequences L1, L2 and L3 [6,29], which are strognly conserved in all known [FeFe]-hydrogenases. Other relevant residues that can be observed are: M353, K358 and M495, that are essential for non-covalent interactions with the H-cluster [30] and E279, E282, C299 and S319 that are essential for the proton transfer [25,31].

Also, the protein sequence of CpHydA is 69% identical to CpI and 31% identical to DdH.

Functional characterisation of CpHydA

The purified CpHydA shows the typical catalytic behaviour for [FeFe]-hydrogenases and it is able to evolve H_2 from reduced methyl viologen at a rate of 1645±16 s^{-1}. The hydrogen evolution rate of

CpHydA is within the range for [FeFe]-hydrogenases [8], and it is particularly high in comparison to other recombinant enzymes such as CrHydA1, CpI and CaHydA [24,32,33].

Purified CpHydA was inactivated by oxygen with a 50% loss of activity after 2.8 minutes exposure to air. This value is within the range of 2-5 minutes determined for other [FeFe]-hydrogenases [26].

The pH dependance of the H_2 evolution rate (Fig. 3A) showed a broad peak with maximum activity between 6.5 and 8.0. In comparison to other [FeFe]-hydrogenases, this feature is very similar to that observed in CaHydA where the maximum is at pH 8.0 [25]. It is also similar to that observed in CpI where the maximum is at pH 6.3 and the activity decreases faster at higher pH, but it is much different from CpII where the maxima are at pH 5.8 and 9.1 [26]. The pH dependance is also different from CrHydA1, where the maximum is at pH 6.9 and the bell shape of the curve is much narrower [28].

The H_2 evolution activity (Fig. 3B) increases exponentially with temperature, reaches a maximum at 50°C and is mainly lost at 70°C. The temperature maximum is similar to other [FeFe]-hydrogenases, such as CpI (~50°C) [26] and CrHydA1 (60°C) [28], and it is coherent with the growth temperature of *Clostridium perfringens*, which spans from 15 to 50°C, with optimum at 45°C [34].

The calculated activation energy for H_2 evolution by CpHydA is 50.8±2.1 kJ/mol, which is similar to those calculated for CrHydA1 (55.1 kJ/mol) [35] and CpI (61 kJ/mol) [36].

The catalytic activity of CpHydA at relatively high temperatures suggests a good thermal stability of the enzyme that makes it suitable for biotechnological applications.

Spectroscopic characterisation of CpHydA

EPR and FTIR spectroscopies were used to characterise the structure, composition, geometry of the catalytic centre H-cluster in different redox states. In particular, EPR was used to investigate the electronic structure and the chemical environment, while FTIR gave information about the vibrational modes of the H-cluster ligands CO and CN.

The EPR spectra (Fig. 4) are consistent with spectra reported for other well studied [FeFe]-hydrogenases. The oxidised sample (Fig. 4A, Table 1) is characterized by a rhombic EPR spectrum with **g** tensor components $g1$=2.0892, $g2$=2.0363, $g3$=1.9954 typical for the H_{ox} state of the H-cluster [15,37-45]. The simulation analysis also indicates a minor contribution of an axial EPR pattern with **g** values $g1$ = 2.0755 and $g2$ = $g3$ = 2.0080. This spectroscopic feature has been assigned to a CO inhibited state of the H-cluster due to the release of CO molecules from damaged H-cluster [38,46,47]. Indeed upon treatment with CO the EPR spectrum of the H_{ox} state is completely converted into the typical axial spectrum of the H_{ox}-CO state (Fig. 4B).

Transmission FTIR spectra (Fig. 5) show absorption peaks that are similar to signals previously observed in other [FeFe]-hydrogenases, both in terms of the wavenumber and the relative intensity. On this basis, the peaks observed here were assigned to the H_{ox}, H_{ox}-CO and H_{red} state of [FeFe]-hydrogenases (Table 2). When the enzyme is only concentrated without further treatment (untreated), it shows a number of peaks that are due to a mixture of different redox states, with a prevalence of the H_{ox} state. The fully oxidised enzyme was obtained by thionine treatment and shows only peaks of the H_{ox} state. Treatment with H_2 results in the enrichment of peaks that can be assigned to the H_{red} state, while treatment with CO results in a very homogeneous spectrum with peak shifts that are in good agreement with previous studies on other [FeFe]-hydrogenases in the H_{ox}-CO state.

Despite the expected similarities between CpHydA and the other [FeFe]-hydrogenases, some relevant differences could be observed. For example, reduction with H_2 do not cause the appearance of signals assignable to the H_{sred} state, which was recently characterised in CrHydA1 [44,45,49]; this might be due to the effect of the accessory iron sulphur centres (the so called F-clusters) that are present in CpHydA and absent in CrHydA1. Consistently, this intermediate has never been described before in CpI. Moreover, the CN signals in the CO-treated sample showed an unusual behaviour: they are very close and appear as a single large peak that was fitted with two peaks with maximum at 2091.1 and 2088.3 cm^{-1}.

In conclusion, in this work the [FeFe]-hydrogenase CpHydA from *Clostridium perfringens* SM09 was characterised for the first time. The enzyme has sequence similarity with other enzymes of the class. The recombinantly expressed purified enzyme is properly folded and can catalyse H_2 evolution at high rates (1645±16 s^{-1}). The catalytic centre H-cluster is correctly inserted and coordinated and it can be readily oxidised with thionine to obtain the H_{ox} state, which can be converted into the H_{ox}-CO state by binding the inhibitor CO, as determined by EPR spectroscopy. Also FTIR confirmed that the H-cluster was properly bound and the typical H_{ox}, H_{ox}-CO and H_{red} signals could be observed.

The high turnover rates for hydrogen evolution and the thermal stability and optimum activity at 50°C of this newly characterised [FeFe]-hydrogenase make it an excellent catalyst for biotechnological devices and sustainable processes for clean energy production.

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Tables

Table 1. Spin-Hamiltonian parameters of the H_{ox} and H_{ox}-CO states of H-cluster of CpHydA extracted by the computer simulations of the CW EPR spectra reported in Figure 4 in comparison to selected data on other [FeFe]-hydrogenases. *nd* = not determined.

Enzyme	H_{ox}			H_{ox}-CO			Ref.
	g_1	g_2	g_3	g_1	g_2	g_3	
CpHydA	2.0892 ±0.0005	2.0363 ±0.0005	1.9954 ±0.0005	2.0755 ±0.0005	2.008 ±0.005	2.008 ±0.005	This work
CrHydA1	2.102	2.040	1.998	2.052	2.007	2.007	[42]
CaHydA	*nd*	*nd*	*nd*	2.075	2.009	2.009	[41]
CpI	2.097	2.039	1.999	2.072	2.006	2.006	[37]
DdH	2.10	2.04	2.00	2.06	2.00	2.00	[39]
	2.100	2.040	1.997	2.065	2.007	2.001	[43,47]

Table 2. FTIR signals: summary of the peaks (wavenumber in cm^{-1}) observed in CpHydA in comparison to selected works on other [FeFe]-hydrogenases in the H_{ox}, H_{ox}-CO and H_{red} states. *nd* = not determined.

Enzyme	H_{ox}			H_{ox}-CO				H_{red}		Ref.
	CNs	COs	μ-CO	CNs	CO_{exo}	COs	μ-CO	CNs	COs	
CpHydA	2087, 2080	1968, 1944	1800	2091, 2088	2013	1971, 1967	1806	2066, 2039	1897	This work
CpI	2086, 2072	1971, 1948	1802	2095, 2077	2017	1974, 1971	1810	*nd*	*nd*	[48]
CrHydA1	2088, 2072	1964, 1940	1800	2092, 2084	2013	1970, 1964	1810	2083, 2070	1935, 1891, 1793	[49]
DdH	2093, 2079	1965, 1940	1802	2096, 2088	2016	1971, 1963	1810	2079, 2041	1965, 1916, 1894	[46]

Figure legends.

Figure 1. CpHydA cloning, purification and model structure. A) Map of the expression vector pECPF2655. B) Coomassie-stained SDS-PAGE of the purified CpHydA; molecular weights are in kDa. C) Scheme of the modular domains, where yellow cross = [2Fe2S] plant ferredoxin-like; red diamond = [4Fe4S] coordinated by three cysteines and one histidine; green oval = 2[4Fe4S] bacterial ferredoxin-like; blue rectangle = H-domain. D) Homology model of CpHydA structure.

Figure 2. Sequence alignment of CpHydA with other selected [FeFe]-hydrogenases. CaHydA = *Clostridium acetobutylicum* hydrogenase A; CpI = *Clostridium pasteurianum* hydrogenase I; DdH = *Desulfovibrio desulfuricans* hydrogenase; CrHydA1 = *Chlamydomonas reinhardtii* hydrogenase A1. Grey shaded residues are fully conserved; black shaded residues are cysteines or histidines that coordinate the iron sulfur centres. Squared regions are the conserved signature motifs L1, L2 and L3 that include the H-cluster coordinating cysteines.

Figure 3. Hydrogen evolution characterisation. A) pH dependance; the maximum activity was at pH 8 and was 1645±16 s^{-1}. B) Temperature dependance; the maximum activity was at 50°C and was 1941±22 s^{-1}. The activity was assayed by gas chromatography using reduced methyl viologen as artificial electron donor.

Figure 4. Experimental (solid lines) and computer simulations (dotted lines) X-band CW EPR spectra of CpHydA frozen solutions in the A) H_{ox} and B) H_{ox}-CO states of the H-cluster. The spin Hamiltonian parameters extracted from the computer simulations are reported in Table 1. Asterisks in spectrum A indicate the features of the H_{ox}-CO state present as an impurity.

Figure 5. A) Transmission FTIR spectra of CpHydA after different treatments: untreated, thionine oxidised, H_2-reduced and CO-treated. The H_2-reduced spectrum has been magnified by a factor of 2. B) Summary of the FTIR signals assigned to the H_{ox}, H_{ox}-CO and H_{red} states.