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

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Abstract

This paper reports the first characterization of an [FeFe]-hydrogenase from a Clostridium perfringens strain previously isolated in our laboratory from a pilot-scale bio-hydrogen plant that efficiently produces H2 from waste biomasses. On the basis of sequence analysis, the enzyme is a monomer formed by four domains hosting various iron–sulfur centres involved in electron transfer and the catalytic center H-cluster. After recombinant expression in Escherichia coli, the purified protein catalyzes H2 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 oxidized enzyme displays a spectrum coherent with the Hox state, whereas the CO-inhibited enzyme has a spectrum coherent with the Hox-CO state. FTIR spectroscopy showed that the purified enzyme is composed of a mixture of redox states, with a prevalence of the Hox state upon reduction with H2, vibrational modes assigned to the Hred state were more abundant, whereas binding of exogenous CO resulted in a spectrum assigned to the Hox-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: bio-hydrogen, Clostridium perfringens, [FeFe]-hydrogenases, H-cluster, iron–sulfur centers, recombinant expression

1. 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 H2 production is catalyzed by [FeFe]-hydrogenases, a class of redox enzymes found in several microorganisms, that display exceptionally high turnover rates [5, 6].

[FeFe]-hydrogenases are characterized by the peculiar catalytic center H-cluster, an organometallic cofactor composed by a cubane [4Fe4S] sub-cluster and a [2Fe] sub-cluster; the [4Fe4S] center is coordinated by four conserved cysteines, whereas the [2Fe] center 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].

Similarly to other clostridia, its metabolism is based on anaerobic fermentation: after glycolysis, pyruvate is oxidized via pyruvate-ferredoxin oxidoreductase (PFOR) resulting in the production of lactate, alcohol, acetate, and butyrate together with gaseous CO2 and H2 [16]. C. perfringens is often found...
in anaerobic digestion plants, where it is one of the factors responsible for \( \text{H}_2 \) 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 \textit{C. perfringens} SM09, recently isolated in our laboratory from a pilot-scale bio-hydrogen plant in Torino (Italy), which was particularly efficient in \( \text{H}_2 \) production from waste biomasses [20, 21].

Previous works showed that the gene CPF\_2655 is expressed during \( \text{H}_2 \) production [20] and that it is directly involved into the metabolic pathway [22].

Here, the new enzyme was recombinantly expressed in \textit{Escherichia coli} in the active form, purified and characterized in both its functional and spectroscopic features.

## 2. Materials and Methods

### 2.1. Gene cloning

A DNA fragment of 2,126 bp containing the entire coding sequence of CPF\_2655 was amplified by PCR from the genome of \textit{C. perfringens} SM09 [20] using the following primers: ATGGCGTTGAAGAAGCAAGA and AACCGTTTTCATCCATGAGC. For this purpose, the proofreading KOD Hot Start DNA polymerase (Merck, Vimodrone, Italy) was used following the manufacturer's instructions.

Subsequently, the 5’ of the gene was modified by PCR to insert an \textit{NdeI} site and the 3’ was modified to add a sequence coding for Strept-tagII and a \textit{XhoI} site using the following primers: AACCATATGAAATAAATAATAATCAATGATAAGACTATCG and ATCTCGAGTTATTTTTCAAATTGAGGATGACTCCAATTTTTTATATATTCATGTAATTAACTCATGAG.

The PCR fragment (1,760 bp) was digested with \textit{NdeI} and \textit{XhoI} (Thermo Scientific, Rodano, Italy) 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).

### 2.2. Recombinant expression and purification

Recombinant expression in \textit{E. coli} Rosetta2(DE3) was performed as previously described [24]. Briefly, the vector pECPF2655 (harboring CpHydA gene and CaHydE maturation gene) was co-transformed with pCaFG (harboring 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 \( \mu \)g/mL carbenicillin, 50 \( \mu \)g/mL streptomycin, and 34 \( \mu \)g/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 overnight 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 inside a glove box (Plas Labs, Lansing, MI) 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, Goettingen, Germany) 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.
FIG. 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. Gray 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.

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.

2.3. 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 H2 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 dependence, 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 dependence, 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].

2.4. EPR spectroscopy
Purified CpHydA was anaerobically concentrated by ultrafiltration using Amicon Ultra 0.5 mL 30K MWCO (Millipore, Vimodrone, Italy); 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 oxidized sample; the final CpHydA concentration was 0.2 mM. The CO-treated sample was obtained by flushing the oxidized sample with CO twice for 30 Sec 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, and temperature 77 K. 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].

2.5. FTIR spectroscopy
For the characterization of the H-cluster, purified CpHydA was anaerobically concentrated up to 1 mM. The untreated
3. Results and Discussion

3.1. 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 liter of culture. The purified protein (Fig. 1B) has the expected molecular weight of ~64.7 kDa.

3.2. 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].

Sample was measured immediately after concentration; the fully oxidized sample was obtained by addition of thionine (8 mM); the H₂-treated sample was obtained by flushing H₂ three times for 1 Min on ice; the CO-treated sample was obtained by flushing CO twice for 30 Sec 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 characterization of the H-cluster, a transmission cell (CaF₂ 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, Milano, Italy) by the concave rubberband algorithm.

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**FIG. 3** Hydrogen evolution characterization. (A) pH dependence; the maximum activity was at pH 8 and was 1645 ± 16 s⁻¹. (B) Temperature dependence; 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.

**FIG. 4** Experimental (solid lines) and computer simulations (dotted lines) X-band CW EPR spectra of CpHydA frozen solutions in the (A) Hox and (B) Hox-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 Hox-CO state present as an impurity.
The enzyme is composed of four domains that contain iron sulfur clusters (Fig. 1D): a [2Fe2S] plant ferredoxin-like (yellow), a [4Fe4S] coordinated by three cysteines and one histidine (red), a [4Fe4S] bacterial ferredoxin-like (green), and the H-domain containing the catalytic center H-cluster (blue). The various iron sulfur centers are involved in the electron transfer from the redox partner to the H-cluster, where the reduction of protons into H₂ is catalyzed.

Alignment with other known [FeFe]-hydrogenase sequences (Fig. 2) allows the identification of the key residues in CpHydA: 15 cysteines and the histidine responsible for the binding of the four accessory iron sulfur 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 strongly conserved in all known [FeFe]-hydrogenases. Other relevant residues that can be observed are: M353, K358, and M495, that are essential for noncovalent 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 Cpl and 31% identical to DdH.

3.3. Functional characterization of CpHydA
The purified CpHydA shows the typical catalytic behavior for [FeFe]-hydrogenases and it is able to evolve H₂ 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 with other recombinant enzymes such as CrHydA1, Cpl, and CaHydA [24, 32, 33].

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

The pH dependence of the H₂ evolution rate (Fig. 3A) showed a broad peak with maximum activity between 6.5...
and 8.0. In comparison with 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 Cpl where the maximum is at pH 6.3 and the activity decreases faster at higher pH, but it is much different from Cpl II where the maxima are at pH 5.8 and 9.1 [26]. The pH dependence 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 H₂ 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 Cpl (50 °C) [26] and CrHydA1 (60 °C) [28], and it is coherent with the growth temperature of C. perfringens, which spans from 15 to 50 °C, with optimum at 45 °C [34].

The calculated activation energy for H₂ evolution by CpHydA is 50.8 ± 2.1 kJ/mol, which is similar to those calculated for CrHydA1 (55.1 kJ/mol) [35] and Cpl (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.

### 3.4. Spectroscopic characterization of CpHydA

EPR and FTIR spectroscopies were used to characterize the structure, composition, and geometry of the catalytic center H-cluster in different redox states. In particular, EPR was used to investigate the electronic structure and the chemical environment, whereas 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 oxidized sample (Fig. 4A and 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 Hox 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 Hox state is completely converted into the typical axial spectrum of the Hox-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 Hox, Hox-CO, and redox 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 Hox state. The fully oxidized enzyme was obtained by thionine treatment and shows only peaks of the Hox state. Treatment with H₂ results in the enrichment of peaks that can be assigned to the redox state, whereas treatment with CO results in a very homogeneous spectrum with peak shifts that are in agreement with previous studies on other [FeFe]-hydrogenases in the Hox-CO state.

Despite the expected similarities between CpHydA and the other [FeFe]-hydrogenases, some relevant differences could be observed. For example, reduction with H₂ do not cause the appearance of signals assignable to the redox state, which was recently characterized in CrHydA1 [44, 45, 49]; this might be due to the effect of the accessory iron sulfur centers (the so-called F-clusters) that are present in CpHydA and absent in CrHydA1. Consistently, this intermediate has never been described before in Cpl. Moreover, the CN signals in the CO-treated sample showed an unusual behavior: 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.

### 4. Conclusions

In this work, the [FeFe]-hydrogenase CpHydA from C. perfringens SM09 was characterized 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 catalyze H₂ evolution at high rates (1645 ± 16 s⁻¹). The catalytic center H-cluster is correctly inserted and coordinated and it can be readily oxidized with thionine to obtain the Hox state, which can be converted into the Hox-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 characterized [FeFe]-hydrogenase make it an excellent catalyst for biotechnological devices and sustainable processes for clean energy production.

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