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Oxygen Stability in the New [FeFe]-Hydrogenase from Clostridium beijerinckii SM10 (CbA5H)

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**Supporting Information**

**Experimental details.**

**Cloning**

The entire gene region for CbA5H was amplified from the genomic DNA of *Clostridium beijerinckii* SM10 by PCR, using the following primers: 5'-GACTGGCGATGCATAAAATTG and 5'-ATTGAATCCGATTTTCCCTCA. PCR was performed using the high proof-reading polymerase “KOD Hot Start DNA polymerase” (Merck) following manufacturer’s instructions. Subsequently, the 5’ and 3’ were modified by PCR with the NcoI and EcoRV restriction sites and the fragment was cloned into a vector derived from pECr1 (King 2006). This vector inserted at the C-terminus of the enzyme a spacer sequence (DIWSVGVKLFGGGSGGGG) (von Abendroth 2008) and the StrepTagII sequence (WSHPQFEK). The spacer was essential to obtain high purification yield.

**Recombinant expression**

CbA5H was expressed in *E. coli* using the maturases HydE, HydF and HydG from *Clostridium acetobutylicum*. The plasmids pECbA5H (harboring the genes hydA5H and hydE) and pCaFG (harboring the genes hydF and hydG) were co-transformed in *E. coli* Rosetta2(DE3). Cultures were aerobically grown in Terrific Broth medium supplemented with 2 mM ferric ammonium citrate, 100 μg/mL ampicillin, 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, 0.5% w/v glucose and induced with 1.5 mM IPTG. The expression was performed overnight under argon sparging. The optimal post-induction temperature (Morra 2015) for CbA5H was 20°C. Cell harvest was performed under strict anaerobic conditions in a glove box (Plas Labs) under a 5% hydrogen 95% nitrogen atmosphere.

**Affinity purification**

The purification was carried out at room temperature by affinity chromatography, using Strep-Tactin Superflow high capacity cartridges (IBA) following the manufacturer's instructions. The enzyme was finally eluted in 100 mM TrisHCl, 150 mM NaCl pH 8. Only in the case of anaerobic purification, all manipulations were performed under strict anaerobic conditions in a glove box (Belle Technology) under a pure nitrogen atmosphere; all solutions were supplemented with 2–10 mM sodium dithionite and equilibrated with the glove box atmosphere before use. Protein concentration was assayed with the Bradford assay using bovine serum albumin as standard. The typical yield was 1.2-1.8 mg pure protein/L culture.

**FTIR spectroscopy**

The enzyme was concentrated up to 0.3-1 mM by ultrafiltration using Amicon Ultra 0.5 mL 30K MWCO (Millipore) in 100 mM TrisHCl, 150 mM NaCl pH 8; 2 mM sodium dithionite was present in anaerobically purified samples. The various samples were obtained as follows. The “purified” sample was acquired just after concentration without any other treatment. The CO bound sample was obtained by sparging the enzyme with carbon
monoxide twice for 30 s on ice. The thionine oxidized sample was obtained by the addition of 8-fold molar ratio of thionine (0.3 mM CbA5H, 2.4 mM thionine). The DCIP oxidized sample was obtained by the addition of 24-fold molar ratio of 2,6-dichlorophenolindophenol (0.6 mM CbA5H, 14.4 mM DCIP). Inactivation with air was obtained simply opening the sample vial to the laboratory atmosphere for 10 minutes on ice; the sample volume was 12 μL. Before reactivation, oxygen was not intentionally removed by any specific treatment. Activation with dithionite was obtained by the addition of 10-fold molar ratio of sodium dithionite for 2 minutes (0.9 mM CbA5H, 9 mM dithionite). Activation with hydrogen was obtained by sealing the sample in a glass vial and sparging with H₂ for 10 minutes, then the purge was stopped to avoid excessive sample evaporation and the sample was incubated for further 40 minutes under H₂. CO and H₂ treatments were performed by sparging (< 0.1 bar) the headspace of the sample vial (2 mL), without bubbling the concentrated enzyme solution (12 μL). The spectra were acquired at room temperature using a Bruker Tensor 27 FT-IR spectrometer (Bruker Instruments). A transmission cell equipped with CaF₂ window and 50 μm pathlength (Specac) was used; the sample chamber was purged with pure nitrogen gas. 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.

Activity assay

H₂ evolution activity was assayed by gas chromatography at 37°C. The reaction mixture contained 10 mM methyl viologen, 20 mM sodium dithionite, 100 mM TrisHCl, 150 mM NaCl pH8 and was saturated with Ar. H₂ production was detected after 20 minutes with an Agilent Technologies 7890A instrument equipped with purged packed inlet, Moliesive 5A column (30 m, ID 0.53 mm, film 25 mm) and thermal conductivity detector; argon was used as carrier gas. H₂ uptake activity was determined spectrophotometrically at 37°C. The reaction mixture contained 10 mM methyl viologen, 100 mM TrisHCl, 150 mM NaCl pH8 and was saturated with H₂. Methyl viologen reduction was detected at 604 nm and an extinction coefficient of 13,600 M⁻¹ cm⁻¹ was adopted. The values presented are the average of 3 replicates and error bars in figure 4 represent the standard deviation.

In the case of the assays coupled with the FTIR experiments, the same samples used for spectroscopy were immediately diluted 500-fold in anaerobic buffer and promptly assayed. The protein concentration was determined for each sample in order to compensate for dilution (e.g. addition of thionine or DCIP solution) or concentration (e.g. air or H₂ treatment).

References

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Von Abendroth, G.; Stripp, S.; Silakov, A.; Croux, C.; Soucaille, P.; Girbal, L.; Happe, T. *Int. J. Hydrogen Energy* 2008, 33, 6076.
Figure S1. Sequence alignment of the H-domain of CbA5H 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. Squared regions are the conserved signature motifs L1, L2, and L3 that include the H-cluster coordinating cysteines (black shaded).
Figure S2. The same spectra as in figure 2 of the main text, including all the wavenumbers.
Figure S3. The same spectra as in figure 3 of the main text, including all the wavenumbers.
Figure S4. O₂ exposure experiment (FTIR spectra and activity assays) with Clostridium acetobutylicum hydrogenase (CaHydA/CaI) in conditions identical to those reported in Figure 2, 3 and 4 of the main text.