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Oxygen-resistant [FeFe]hydrogenases: new biocatalysis tools for clean energy and cascade reactions

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The use of enzymes to generate hydrogen, instead of using rare metal catalysts, is an exciting area of study in modern biochemistry and biotechnology, as well as biocatalysis driven by sustainable hydrogen. Thus far, the oxygen sensitivity of the fastest hydrogen-producing/exploiting enzymes, [FeFe]hydrogenases, has hindered their practical application, thereby restricting innovations mainly to their [NiFe]-based, albeit slower, counterparts. Recent exploration of the biodiversity of clostridial hydrogen-producing enzymes has yielded the isolation of representatives from a relatively understudied group. These enzymes possess an inherent defense mechanism against oxygen-induced damage. This discovery unveils fresh opportunities for applications such as electrode interfacing, biofuel cells, immobilization, and entrapment for enhanced stability in practical uses. Furthermore, it suggests potential combinations with cascade reactions for CO2 conversion or cofactor regeneration, like NADPH, facilitating product separation in biotechnological processes. This work provides an overview of this new class of biocatalysts, incorporating unpublished protein engineering strategies to further investigate the dynamic mechanism of oxygen protection and to address crucial details remaining elusive such as still unidentified switching hot-spots and their effects. Variants with improved kcat as well as chimeric versions with promising features to attain gain-of-function variants and applications in various biotechnological processes are also presented.

Introduction

One of the greatest challenges to current society is the design of cheap catalytic systems for efficient production and oxidation of carbon neutral energy carriers such as molecular hydrogen. In this context a very promising perspective is the application of hydrogenases, which is an irreversible inhibitor that destroys the active site of the enzyme. Conversely, in the absence of oxygen, it is able to return to an inactive (Hinact) protected form in aerobic environment.

Clostridium beijerinckii is a Gram positive anaerobic bacterium, isolated from various sources and previously known for its capacity to produce H2 and other industrially-relevant biomolecules with high yields. The analysis of its genome highlighted the presence of six genes encoding for [FeFe]hydrogenases, one of which codes for CbA5H (CbHydA1, NCBI accession number: KX147468, Cbei_1773). CbA5H is a cytoplasmic soluble enzyme composed of 644 amino acids, with a molecular weight of 72 kDa and a modular structure typical of the M2c category. It belongs to the A5 subgroup, hence the name CbA5H. From the N-terminal to the C-terminal, this enzyme harbours a soluble-ligand-binding β-grasp motif (SLBB), a domain hosting two [4Fe-4S] clusters or bFd (homologous to bacterial Ferredoxins), and the H-domain typical of all [FeFe]hydrogenases.

This enzyme is the first of its kind due to its resilience towards O2: it can withstand the oxidative action of oxygen by switching to an inactive (Hinact) protected form in aerobic environment. Conversely, in the absence of oxygen, it is able to return to an oxidised and fully active state (Hox). Even though it was not the first [FeFe]hydrogenase observed in which this particular Hinact state is formed and identified by a specific FTIR signature (it was the first one in which the transition from the Hox to the Hinact state was fully reversible and could be repeated several times without any activity loss or any damage observed to the H-cluster). Indeed, in DdH, once the Hinact form goes back to the Hox state, the former cannot be obtained again, and therefore the enzyme becomes O2-sensitive after the first exposure to...
The capacity to overcome O2 attack is in fact possible thanks to the special role of cysteine 367, which coordinates with Fe avoiding the binding of O2 (acting as a “safety cap”). Cys367 is normally responsible for transferring protons to Fed, via the amine group of the azadithiolate ligand that connects the distal and proximal iron atoms. This residue is highly conserved among all [FeFe]hydrogenases, but it is normally located too far away from Fed to interact with it. The X-ray structure of CbA5H in the protected form, resulting from air exposure, shows some differences in comparison to anaerobically crystallised Cpf, indeed CbA5H’s TSC-loop (T365-S366-C367) has a different conformation from the corresponding TSC-loop of Cpf. This loop appears to be more flexible than in standard [FeFe]hydrogenases, enough to allow the sulfur atom of C367 to interact with Fed, lowering their distance from 5.9 Å (which is the reported value in Cpf) to 3.1 Å. However, both the cysteine residue and the TSC-loop are strictly conserved among [FeFe]hydrogenases. Therefore, there needs to be another factor that contributes to the flexibility of the loop. In CbA5H, three residues have been identified, that probably play a role in making the TSC-loop more flexible, which are Leu364, Ala561 and Pro386. These residues do not significantly change the backbone structure from the one reported of Cpf but have smaller and more flexible lateral chains which enable the mobility of the TSC-loop. A further increase in enzyme flexibility was also recently engineered.

The selected residues were identified within the inner (4 Å) and outer (8 Å) sphere of interaction of the diiron subcluster of the H-cluster with the protein moiety. Figure 1 reports the corresponding regions (Figure 1A: 4 Å, Figure 1B: 8 Å) mapped on the reference structure of the Hox state of Cpf. The highlighted residues were also mapped onto the sequence alignment (Figure 1C) of the main studied enzymes of the [FeFe]hydrogenase class, with the same colour code (4 Å sphere positions are highlighted in green, 8 Å sphere in red). Many conserved residues are present, especially in the 4 Å sphere, but few fine differences can be spotted. Residue M393 of CbA5H is typically a glutamine in other well-characterized and oxygen-sensitive hydrogenases and it is located in a region adjacent to the bridging CO portion of the H-cluster (check Figure 2B for closer view). In the 8 Å coordination sphere, position 419 of CbA5H is also harbouring a chemically different amino acid if compared to most of the other enzymes of the group, since an alanine is present, whereas serine or threonine are the consensus conserved residues in oxygen-sensitive enzymes (Figure 1B). This implies the lack of possible H-bonding in CbA5H, while both serine and threonine in other more “standard” [FeFe]hydrogenases can grant this stabilising interaction. The longer distance to the H-cluster (Figure 2B) is not hindering a possible effect if a long-range network of H-bonding interactions is involved.

In order to assess the fine-tuning role of the protein scaffold on the oxygen resistance and check if it is possible to implement the biotechnological exploitation of this robust enzyme, or even to confer oxygen resistance to other enzymes of the class, multiple amino acids substitutions were tested.

It is not clear though if the sequence features, which allow TSC-loop flexibility and are strictly required for this purpose (as demonstrated in loss-of-functions mutants), are also sufficient to grant the resiliency to oxygen of CbA5H. The details of the peculiar and immediate switch to Hinxact and back to Hox allowed by this flexibility are still elusive, as well as the role of the modular structure and the unique SLBB domain present at the N-terminus of the protein. The crystal structure suggests the presence in the SLBB domain of a 4Fe4S cluster bound to three cysteines and one histidine, but other ligands and/or redox partners might be interacting with this part of the protein.

In this work, a protein engineering approach has been used to investigate further the domains or residues involved in oxygen resistance of this peculiar [FeFe]-hydrogenase, in the perspective of exploiting this feature for biotechnological applications. Mutated variants and chimeras obtained by shuffling domains with other hydrogenases are presented which add further information on the mechanism and lay the groundwork for robust hydrogen-driven catalysis. This can be achieved in combination with non-physiological partners or in chimeric multidomain proteins, as inspired by modular “Lego approach” for protein engineering.
crystal structure of Cpi as a reference for Hox structure, highlighted in green. B: 8 Å sphere mapped on the crystal structure of Cpi. C: 4 Å and 8 Å sphere residues mapped on the multiple alignment of CbASH from C. beijerinckii, CaHydA from C. acetobutylicum, Cpl from C. pasteurianum, DdH from D. vulgaris Hildenborough, CrHydA1 from C. reinhardtii, CpiHydA from C. perfringens. The same colour-code of A and B was used (green highlight: 4 Å sphere, red highlight: 8 Å). The alignment was performed using Cobalt.

For these reasons, both positions 393 and 419 were selected as mutagenesis targets to investigate a possible fine-tuning role in the peculiar behaviour of CbASH. Results of the mutations were screened by a colorimetric on-plate method which can directly measure, with a semi-quantitative approach, the activity of the recombinant enzyme variants co-expressed in E. coli cells with the suitable maturases, as described in the Experimental section. The test can be performed before and after air exposure of the plate, granting information also on the capacity of the expressed enzymes to access the protected state typical of sensitive enzymes to a CbASH-like structure, it could be advantageous. Also, it can be worth further investigation to elucidate the reason of such effect in CbASH.

A second set of mutations was performed on positions 424 and 425 of CbASH, where the alignment suggests a strong difference in terms of polarity when comparing CbASH to oxygen sensitive enzymes, except for DdH, in which a similarity can be highlighted. In fact, CbASH harbours hydrophilic isoleucine and alanine (IA) while normally more polar residues are present (TR, TS, ND), with DdH containing instead similarly hydrophilic residues VA (Figure 1C). These two amino acids are located in the vicinity of the 4Fe4S cubane subcluster of the H-cluster (Figure 3B) and changes in the protein environment has been demonstrated to affect potential and therefore properties of FeS clusters.37-40 The results of mutagenesis of positions 424-425 are reported in Figure 3A.

Figure 3: Mutations in the cubane subcluster vicinity, H-domain. A: results of the on-plate activity screening before and after air exposure on mutants M393Q, A419S of CbASH and Q195M, S221A of CrHydA1. B: detail of the corresponding sites on the crystal structure of Cpi as representative of the Hox state.

<table>
<thead>
<tr>
<th>Protein</th>
<th>H₂ production activity kcat (s⁻¹) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbASH NS</td>
<td>2404±424</td>
</tr>
<tr>
<td>CbASH TS</td>
<td>5887±691</td>
</tr>
<tr>
<td>CbASH NA</td>
<td>2221±361</td>
</tr>
<tr>
<td>CbASH A419S</td>
<td>6757±693</td>
</tr>
<tr>
<td>CbASH C236A</td>
<td>9214±400</td>
</tr>
<tr>
<td>CbASH WT batch 1</td>
<td>5449±473</td>
</tr>
<tr>
<td>CbASH WT batch 2</td>
<td>4199±544</td>
</tr>
</tbody>
</table>

Table 1: H₂ production activity (evolution) tested on WT CbASH and on mutated variants. The test was performed in two independent
vials, following the assay protocol described in the Experimental section. The headspace of each vial was sampled twice with an airtight syringe to evaluate the technical variability of the GC measurement. The mean is calculated on the two independent vials values.

The $k_{\text{cat}}$ is expressed as TOF (turnover frequency) already normalised for the protein concentration. Results are reported in Table 1, which also includes the activity of the purified variant A419S already discussed as for on-plate screening results, and of mutant C236A. This last was engineered to test the role of atypical cysteines present in CbASH in the vicinity of the consensus sequences (Figure 4C) required to coordinate cubane $\text{Fe}$ of CbASH (not visible in the crystal structure$^{13}$, to be possibly oriented towards cubane 1 (FSA) of this domain (Figure 4B), suggesting a possible alternative site to the typical coordinating cysteines of the cluster or a cluster with unusual structure, which could be important for the peculiar features of CbASH.$^{41}$ The tested replacement with an alanine yielded the results reported in Figure 4A: on-plate screening shows that the single replacement C236A is not affecting oxygen resistance of CbASH and the variant has apparently a wild type-like behaviour, although a further investigation on the purified variant C236A suggests a subtle tuning effect.

Figure 4: Mutation in the bFd domain. A: results of the on-plate activity measurement before and after air exposure on mutant of position 236 of CbASH B: detail of the corresponding position of Cys236 and cubane 1 (FSA) on CbASH structure obtained by homology modelling on the Hox Cpl structure (CbASH bFd domain could not be resolved in the crystal). C: alignment of CbASH from C. beijerinckii, CaHydA from C. acetobutylicum, Cpl from C. pasteurianum, DdH from D. vulgaris Hildenborough, CrHydA1 from C. reinhardtii, CpHydA from C. perfringens with consensus sequences for cubane 1 (FSA) highlighted in pink and cubane 2 (FSB) highlighted in pale blue. The three extra cysteines of CbASH (Cys236 is indicated) are highlighted in orange. DdH and CrHydA1 were inserted in the alignment even if their cluster arrangement is expected to be different. The alignment was performed using Cobalt.

In fact, by analysing the activity data obtained for the selected mutants, it is interesting to observe that the catalytic constant $k_{\text{cat}}$ of the wild type protein (values for two independent batches are reported, highlighting a relatively high variability) has values comparable only to that of the CbASH T5 mutant; the CbASH NS and CbASH NA mutants have a lower turnover than the wild type, while the point mutations CbASH A419S and CbASH C236A have a much higher turnover frequency. The data obtained therefore suggest that the mutations have generated subtle but significant changes in the protein environment. Although the mutations did not affect the mechanism of oxygen resistance, they could still have an important role in regulating the catalytic activity of the enzyme.

A further interesting single position, located in the proximity of the cubane of the H-cluster (identified within the 8 Å sphere represented in Figure 1B and 1C) is serine 370 of CbASH. In oxygen sensitive [FeFe]hydrogenases the corresponding residue is usually a glycine or an alanine, except for CpHydA from C. perfringens, a strain normally present and isolated from the same environments in which C. beijerinckii is found,$^{6}$ which could therefore represent an intermediate variant. Serine 370 is located in CbASH in a position crucial for possible hydrogen-bonding in the vicinity of the cubane and the comparison of the crystal structure of CbASH in the air exposed form to Cpl in the Hox structure (Figure 5A) shows that there is a shift in the backbone highlighting a possible role as switch between the two states. Also, recently a symmetrical mutation on Cpl, where the corresponding position is numbered 302 (Figure 5B), highlighted that the replacement of the canonical glycine with a serine can enhance the stability to oxygen exposure of Cpl, possibly by a combined effect of the local blocking of $O_2$ diffusion and the two newly formed H-bonds which trigger conformational changes onto S357 and might influence the flexibility of the protein, modifying the $O_2$ diffusion channels.$^{42}$

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Figure 5: Mutation of Serine 370. A: Comparison of the crystal structure of CbASH with Serine 370 in the air exposed form to the corresponding Glycine 302 of Cpl in the Hox structure. B: corresponding alignment of the same region in CbASH from C. beijerinckii, CaHydA from C. acetobutylicum, Cpl from C. pasteurianum, DdH from D. vulgaris Hildenborough, CrHydA1 from C. reinhardtii, CpHydA from C. perfringens. The colour-code is the same indicated in Figure 1. The alignment was performed using Cobalt. C: Gas chromatography signals of sampled headspace after 30' of incubation at 37°C of lyastes expressing positive control WT CbASH, with $H_2$ peak at 1.5 minutes. D: Gas chromatography signals of sampled headspace after 30' of incubation at 37°C of lyastes (obtained as for WT) expressing mutant S370A of CbASH, with $H_2$ peak at 1.5 minutes.
Preliminary tests were performed on a CbA5H S370A mutant, engineered and expressed in small scale. If compared to the wild type protein obtained with the same experimental set-up, the activity measured as H₂ evolution by GC assay (Figures 5C and 5D) is lowered to approximately 22% of the wild type, but further characterisation is needed to confirm an effect on the Hinact formation kinetics.

**Domain shuffling**

As a second strategy, different modular arrangement of the domains involved in electron transfer (ET) and possible key regulators in catalysis were engineered in truncated forms of CbA5H and in chimeras designed by combination with other oxygen sensitive [FeFe]hydrogenases such as CrHydA1 and CaHydA, producing variants in which the catalytic H-domain and the ET domains were shuffled between oxygen resistant and oxygen sensitive enzymes (Figure 6).

Figure 6: Strategy of domain swapping. In the figure the domains contained in CbA5H, CaHydA, CbA5H-Trunc and CbA5H-ΔSLBB proteins are schematically represented. In order to produce the CaHydA/CbA5H chimeras the accessory domains of CaHydA were transferred in the CbA5H's truncated forms, following the diagram shown in the figure. Since the ΔSLBB mutant has an internal bFd domain, only the pFd and HCCC domains were transferred. The CbA5H-CrHydA Chimeras was produced by linking the accessory domains of CbA5H to CrHydA which only contains the H-domain.

SLBB bFd H-domain
CbA5H

SLBB bFd H-domain
CbA5H-ΔSLBB

pFd HCCC bFd H-domain
CaHydA

pFd HCCC bFd H-domain
Chimera CaHydA/CbA5H-ΔSLBB

pFd HCCC bFd H-domain
Chimera CaHydA/CbA5H-Trunc

pFd HCCC H-domain
CrHydA1

pFd HCCC H-domain
Chimera CaA5H/CrHydA1

Results of the effect of deleting domains is reported in Figure 7A. Truncated forms of CbA5H (either removing SLBB alone or both SLBB and bFd) show no activity in the on-plate assay although both forms can be detected as expressed (Figure 7B). Previously, truncated active versions were produced for [FeFe]hydrogenase MHydA from *Megaplastera elsdenii* and for CaHydA from *C. acetobutylicum*. Nonetheless, in the latter case a dramatic reduction in catalytic activity was reported, therefore it is not surprising that the truncated forms of CbA5H do not show activity in the semi-quantitative on-plate assay.

Figure 7: Truncated variants of CbA5H. A: results of the on-plate activity screening before and after air exposure on truncated form of CbA5H lacking SLBB only (CbA5H-ΔSLBB) or both SLBB and bFd domains (CbA5H-trunc). B: Western blot analysis of lysates expressing the two truncated forms, showing the bands at 55 kDa (CbA5H-ΔSLBB) and at 40 kDa (CbA5H-trunc) in the induced cell lysates only.

This lack of activity of the truncated forms hindered further investigation on the fine role of the atypical SLBB domain on the CbA5H oxygen resistance features. The other chimeras proposed in Figure 6 were alternative strategies put in place to discriminate whether the SLBB domain, alone or in combination with the CbA5H bFd domain, is necessary for CbA5H to achieve the Hinact protected state and to elucidate the recovery of activity upon air exposure, or if, conversely, SLBB can by itself grant protection to damage in oxygen sensitive [FeFe]hydrogenases.

The transfer of *Clostridium acetobutylicum* CaHydA accessory domains into the plasmids harboring the Trunc and ΔSLBB truncated sequences of CbA5H was successfully achieved, as confirmed in sequencing data, attesting the correct acquisition of the inserts in both samples. Figure 8 reports preliminary results obtained for Chimeras CaHydA/CbA5H-ΔSLBB and CaHydA/CbA5H-TRUNC using cell lysates prepared in aerobic conditions and then tested, after reductive reactivation, in the GC H₂ evolution assay. Compared to the negative control (Figure 8A), in which non induced cells were used, assays reported H₂ production activity for the CaHydA/CbA5H-ΔSLBB expressing lysates (Figure 8C), similarly to the positive control cells expressing CbA5H (Figure 8B), although at lower levels, while in lysates which should express CaHydA/CbA5H-TRUNC chimera no H₂ production could be detected (Figure 8D).
**Figure 8:** Gas chromatography signals of sampled headspace after 24h of incubation at 37°C of air exposed lysates from small-scale expression of **A:** not induced cells as negative control, showing only the oxygen and nitrogen signals due to small air contamination in the inlet syringe, **B:** cells expressing WT CaBASH as positive control, with the hydrogen production signal at 1.5 min, **C:** cells expressing Chimera CaHydA/CbA5H-ΔSLBB, with the hydrogen production signal at 1.5 min, **D:** cells expressing Chimera CaHydA/CbA5H-TRUNC, with the no signal at 1.5 min and only oxygen and nitrogen signals as negative control.

The results obtained allowed to highlight that the oxygen resistance is not severely affected by the absence of the SLBB domain, which could nonetheless have a role in allosteric regulation, since a significantly reduced activity was observed in the CaHydA/CbA5H-ΔSLBB chimera when compared to that of the wild type CaBASH. A dramatic impairment is instead caused by replacing the whole N-terminal part (including the bFd domain) of CaHydA with the corresponding N-terminal of CaHydA, although bFd is in principle a domain with higher similarity among the two [FeFe]hydrogenases than SLBB to the two domains (pFd and HCCC) transferred to replace the SLBB in the active CaHydA/CbA5H-ΔSLBB chimera. It is to be noted that the bFd domain of CaBASH, as discussed in the site specific mutagenesis part of this section, contains some peculiarity as for non-canonical sequences, i.e. the extra cysteines (like C236) in the cubane 1 and 2 (FSA and FSF) consensus regions. If not taken alone (given the high activity of the C236A mutant) these single changes could cumulatively make a difference in the whole structure and catalytic activity of CaBASH in combination with other unique features like the presence of SLBB.

As expected from previous data, granting to the H-domain of CaBASH a main role in driving the O₂ resistance properties, it was confirmed (Figure 9A) that CaBASH accessory domains (SLBB+bFd) are not sufficient to determine oxygen stability in the oxygen-sensitive CrHydA1. It is anyway very interesting to observe that the wild type (WT) CaBASH/CrHydA1 chimera is fully active in the on-plate assay if kept in anaerobic environment. The CaBASH/CrHydA1 chimera was also used as a platform for mutagenesis to evaluate the combined effects of the two engineering strategies (site specific mutations and domain shuffling). Mutations were introduced on the previously identified positions (Figure 1B): M393 and A419 of CaBASH, in the proximity of the 2Fe subcluster, replacing the corresponding Q195 and S221 of CrHydA1 (Chimera MA) and I424 and A425 of CaBASH, in the proximity of the cubane subcluster, replacing T226 and R227 of CrHydA1. The two sets of mutations were also combined to generate the chimera MAIA (Figure 9A and 9B).

**Figure 9:** Chimeras CaBASH/CrHydA1 WT and mutants. **A:** results of the on-plate activity screening before and after air exposure on WT CaBASH/CrHydA1 Chimera and on mutated forms. **B:** Domain arrangements of the chimera and graphical scheme of position of the mutations introduced.

All mutated chimeras based on the CaBASH/CrHydA1 architecture were fully active in the on-plate screening in anaerobic conditions, but none gained any detectable resistance to air exposure in the on-plate assay. Minor improvement might be evaluated with a deeper analysis, but we expect further mutations to be necessary to attain a gain-of-function variant. The chimera CaBASH/CrHydA1 and its MAIA variant represent good platforms to introduce further changes, since they provide a “CbA5H-like” environment as for accessory ET domains and key positions, maintaining a good level of expression/activity and showing a high plasticity/tolerance to introduced mutations. In fact, it is to be noted that the mutation of R227 into A in the chimera did not affect the activity, while the symmetrical replacement in CaBASH (A425R in the TR variant, Figure 3A) was the only observed to completely abolish catalysis even in anaerobiosis. Also, CrHydA1 is an extremely well-studied model among [FeFe]hydrogenases, thus granting a “gold standard” comparison for data obtained on CaBASH/CrHydA1 chimeras.

**Experimental**

**Materials**

Culture media for bacterial growth and chemicals were analytical grade, purchased from Merck (Darmstadt, Germany) and Carlo Erba Reagents (Milan, Italy). Desthiobiotin, StrepTag resin and HABA were purchased from IBA Lifesciences GmbH (Göttingen, Germany).

**Mutagenesis and domain shuffling**

Mutants were obtained by following an in-house adapted protocol based on the QuikChange strategy. Materials used were those of the QuikChange Lightning Kit (Agilent). The concentrations employed were those of the manufacturer’s protocol except for plasmid and primers which were doubled. Mutagenic primers were designed according to Agilent’s guidelines, following the criteria of melting temperature ≥ 55 °C and equal performances between forward and reverse primer, and purchased from Eurofins Genomics. DNA sequencings were outsourced to Eurofins Genomics, Germany, where they were carried out with the Sanger method. Samples were prepared according to the company’s instructions (for plasmid DNA: 15 μL at 50-100 ng/μL) and shipped.

**On plate colorimetric screening**

The screening procedure was previously developed in our group for site saturation mutagenesis. Briefly, E. coli BL21 (DE3) competent cells were co-transformed with the desired hydrogenase plasmid and the maturation plasmid pFG, plated on LBA supplemented with antibiotics (Ampicillin 150 μg/ml and Streptomycin 50 μg/ml) and incubated at 37°C for 16-18 hours. After, 6 cm diameter plates were prepared containing 8 ml of...
M63 Agar medium (12 g/l KH₂PO₄, 28 g/l K₂HPO₄, 8 g/l (NH₄)₂SO₄, 5 g/l Casamino acids, 120 mg/l anhydrous MgSO₄, 1.5% w/v bacteriological grade agar, pH 7.3, 0.5 mg/l FeSO₄ x 7H₂O), supplemented with 206.5 µl of a mix solution containing 490 µl Glucose 50% w/v, 490 µl Ferric Citrate 10 mM, 245 µl methyl viologen, 73.5 µl IPTG 1M, 98 µl Ampicillin 75 mg/ml and 49 µl Streptomycin 50 mg/ml.

In each plate squares were drawn and appropriate E. coli BL21 (DE3) transformed colonies were transferred onto plates in triplicate (typically positive control, negative control, and samples to test for each plate); CbASH-expressing colonies were used as positive control for oxygen resistance, while CbHydA1 WT-expressing colonies were used as negative control.

Plates were then placed in a sealed glass jar, with gas inlet and outlet, and flushed with pure argon at 30 °C for 5 hours in order to remove the oxygen from the inside. Subsequently, the jar was incubated at 30 °C for further 20-21 hours to let the hydrogenases be expressed.

After this time, the enzymatic activity (hydrogen uptake) was detected by flushing H₂ for 60 minutes, during which the colourless [MV]²⁻ was reduced to [MV]⁺⁺ yielding a blue spot around the colonies which harbour active hydrogenases. Oxygen stability was then determined by flushing H₂ for another 60 minutes after having exposed the plates to the air for 20 minutes to let the MV regress to colourless again. Colonies that redeveloped the blue spot were those whose hydrogenase activity was not compromised by air oxidation.

**Protein expression and purification**

CbASH, mutants and chimeric variants were expressed as reported. Briefly, plasmid pCbASH, containing the CbASH gene  or the mutated genes and the maturase gene hydE from *Clostridium acetobutylicum* (containing the plasmid pCaFG, with the maturase genes hydF and hyd G from *C. acetobutylicum*). Plates with streptomycin and ampicillin were used to grow the cells. For the expression a single colony was used to inoculate 20 ml culture to inoculate 1L culture in 2L total volume flasks. Each flask contained: potassium phosphate solution (2.2 g KH₂PO₄, 9.4 g K₂HPO₄), 2 mM ferric ammonium citrate, 100 µg/ml ampicillin, 50 µg/ml streptomycin. The cultures were incubated aerobically at 37°C, 200 rpm util OD₆₀₀ = 0.4-0.6. When culture reached the desired OD, it was supplemented with 0.5% glucose, 25 mM sodium fumarate, 2 mM cysteine and protein expression was induced with 1.5 mM IPTG.

The cultures were transferred in 1L bottles and kept under anaerobic conditions flushing argon for 24h. The optimal post-induction temperature was 20°C. Cells were harvested under anaerobic conditions and stored at -20°C in in airlight glass bottles.

Cell lysis was performed in Lysis Buffer (TrisHCl 100 mM, NaCl 150 mM, , Glyceral 5% v/v, Triton X-100 1% v/v, 1/4 tablet of COMPLETE protease inhibitor, Lysozyme 1 mg/ml, DNAse I 1U/ml from Thermo Fisher, pH 8) as previously described, typically in glove box if not otherwise indicated.

Each purification was performed in anaerobic conditions, using Strep-Tactin Superflow high-capacity resin (IBA), following the manufacturer’s protocol, the protein was eluted with desthiobiotin in Buffer W (100mM TrisHCl, 150 mM NaCl, pH 8.0), checked for purity via SDS-PAGE and finally the enzyme was concentrated and stored with Buffer W for further tests. Protein concentration was evaluated via the Bradford assay with bovine serum albumin as a standard. Typical yields were between 1 and 1.5 mg of pure protein/l culture.

**Western blotting**

All the hydrogenases and chimeras carried a Strep-tag II (WSHPQFEK) against which the Strep-Tactin®-horseradish peroxidase conjugate (IBA Life Sciences) can be used for detection. Proteins separated by SDS-PAGE were electro-transferred onto a PVDF Transfer Membrane 0.45 µm (Thermo Scientific) with a Semiphor semi-dry transfer unit (Hoefer). The PVDF membrane was activated by dipping it in pure methanol for 5 minutes and after washing with deionized water the membrane was put in Towbin buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol, 0.1% w/v SDS, pH 8.3) for 5 minutes. The SDS-PAGE gel was prepared by soaking it in the Towbin buffer for 15 minutes. Inside the electro-transfer unit, a 6-layer system was assembled as described by the protocol: starting from the anode (+), 3 sheets of blotting paper (8.5 x 6 cm) imbedd in Towbin buffer, the PVDF membrane, the SDS-PAGE gel and 3 more blotting paper sheets imbedd in Towbin buffer were stacked on top of each other to finish the “sandwich”. The proteins were transferred during 60 minutes at 76 mA and at room temperature. After the run the membrane was incubated with 20 ml PBS-blocking buffer (PBS buffer with 3% BSA and 0.05% w/v Tween 20), overnight at 4°C under gentle agitation. Subsequently the membrane was washed three times with 20 ml PBS-Tween buffer (PBS buffer with 0.1% w/v Tween 20) for 5 minutes each. After the last washing step, the membrane was incubated with gentle shaking for 1 hour with 10 ml PBS-Tween buffer and 2.5 µl Strep-Tactin® horseradish peroxidase conjugate (1:4000). After this step the membrane was washed two times with PBS-Tween buffer for 1 minute each and then two times with PBS-buffer for 1 minute each. From this point forth, IBA’s “Chromogenic detection with horse radish peroxidase (HRP)” protocol was followed, except for the substitution of 3 % w/v 4-chloro-1-naphtol in methanol with 0.5 mg/ml 3,3’-diaminobenzidine (DAB) in PBS buffer (4 mM KH₂PO₄, 16 mM Na₂HPO₃, 115 mM NaCl, pH 7.4) as chromogenic reagent.

**HC₇ evolution activity assay**

H₂ evolution activity was evaluated by gas chromatography (GC) at 37°C. The assay was conducted so as to recreate the ideal conditions for proteins re-activation. 20 ml glass vials were filled with 5 ml of Buffer W (pH 8) containing 1% v/v of Methyl Viologen 1M, closed with rubber stoppers, sealed and saturated with Argon (upside down) for 10 minutes. Subsequently 500 µl of 1M Sodium dithionate were added with a syringe and saturated with Argon for other 10 minutes.

Finally, few µl of pure protein (adjusted depending on the concentration) were added and vials were incubated at 37°C. H₂ production was detected in time by analysing the headspace
with 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.

Conclusions

The peculiarity that generates considerable interest in the protein CbA5H is its ability to induce a spontaneous and reversible highly stable state Hinact that can be converted into the active form simply through reductive treatment with dithionite or H₂. A second intriguing feature is the presence of an SLBB domain, reported to be an adapter module, both in ancient procaryotic organisms and in eukaryotes, for protein-protein and protein-ligand interaction in many redox-driven catalytic complexes.²³,⁵⁷–⁵⁹

Mutagenesis was here applied to gain insight into the dynamic mechanism of oxygen protection and to localise still unidentified switching hot-spots and their effects. Two selected mutants in the ZFe-subcluster proximity did not determine oxygen stability change in CbA5H or CrHydA1 but highlighted a key position for CbA5H catalytic activity (M393) and granted a mutant (A419S) with increased kcat for H₂ production. Changing the polarity environment of the cubane subcluster of CbA5H to increase hydrophilicity in positions 424–425 has variable results, ranging from no effect to a complete inactivation and in two cases lowering the kcat of the evolution reaction. On a recently identified position in which oxygen sensitivity can be decreased in Cpl,⁴² our data suggest that the mechanism of oxygen resistance in CbA5H is not affected by the absence of Serine 370, as the mutant has shown activity after exposure to oxygen resistance in CbA5H is not affected by the absence of a role in the kinetic of switching and in general on catalysis cannot be excluded and the comparison with the data obtained on the symmetrical position in Cpl suggest further experiments, for instance the replacement of S370 with glycine and a more detailed study by FTIR and electrochemistry to precisely dissect the replacement effects.

The production of chimeras by domain swapping clarified that SLBB is not strictly required for CbA5H to attain the O₂ protected state. A robust and active hybrid chimeras, with the SLBB and bFd domains of CbA5H at the N-terminal and the H-domain of CrHydA1 at the C-terminal was built and further modified to drive the H-domain environment to a higher similarity to CrHydA1. This will represent an excellent starting platform for protein engineering with the challenging aim of designing and producing a gain-of-function variant with O₂ protection.

The availability of WT CbA5H and the design of derived chimeras, with alternative modular arrangements, open new perspectives of exploitation for applicative purposes. Robust devices, in which electrode interfacing of the enzyme, immobilization and entrapment for bio fuel-cells attain enhanced stability, can be proposed and be relevant for real application.

Presently we are envisaging combination of CbASH with other biocatalysts, either in soluble form or via “Molecular-Lego approach”²⁵,²⁸–³⁰,²⁶,²⁷ for cascade reactions, focusing on H₂-driven CO₂ conversion and on cofactors-regeneration (NADPH/NADH) with simplified product separation and improved sustainability.

Author Contributions

Conceptualization: FV, SM, GC, GG, SJS; Data curation: SM, LB, SD, AR, GC. Funding acquisition: FV, SJS, GG. Investigation: SM, LB, SD, AR. Methodology: SM, FV, LB, SD, GC, SJS, Project administration: FV, SM, GG. Resources: FV, GG, SJS, Validation: SM, GC, FV, GG. Visualization: SM, LB, SD, AR. Writing – original draft: FV, SM, LB, SD, GC. Writing – review & editing: all authors

Conflicts of interest

There are no conflicts to declare.

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