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Increasing the O₂ resistance of the [FeFe]-hydrogenase CbA5H through enhanced protein flexibility

Andreas Rutz¹, Chandan K. Das², Andrea Fasano³, Jan Jaenecke^{1†}, Shanika Yadav⁴, Ulf-Peter Apfel^{4,5}, Vera Engelbrecht¹, Vincent Fourmond³, Christophe Léger³, Lars V. Schäfer², Thomas Happe^{1*}

¹Photobiotechnology, Department of Plant Biochemistry, Ruhr-Universität Bochum, 44801 Bochum, Germany.

²Theoretical Chemistry, Ruhr-Universität Bochum, 44801 Bochum, Germany.

³Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Aix-Marseille Université, Institut de Microbiologie de la Méditerranée, 13009 Marseille, France.

⁴Inorganic Chemistry I, Department of Chemistry and Biochemistry, Ruhr-Universität Bochum 44801 Bochum, Germany.

⁵Fraunhofer UMSICHT, 46047 Oberhausen, Germany.

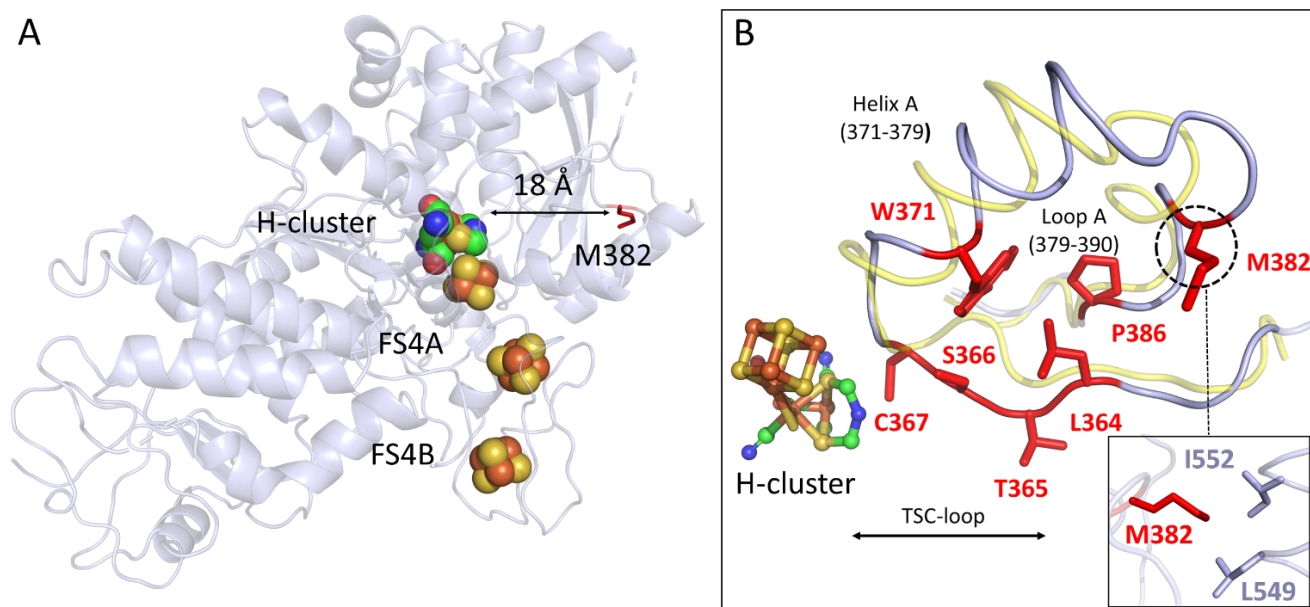
Metalloenzymes, [FeFe]-hydrogenase, spectroscopy, protein film voltammetry, molecular dynamics

ABSTRACT: The high turnover rates of [FeFe]-hydrogenases under mild conditions and at low overpotentials provide a natural blueprint for the design of hydrogen catalysts. However, the unique active site (H-cluster) degrades upon contact with oxygen. The [FeFe]-hydrogenase from *Clostridium beijerinckii* (CbA5H) is characterized by the flexibility of its protein structure, which allows a conserved cysteine to coordinate to the active site under oxidative conditions. Thereby, intrinsic cofactor degradation induced by dioxygen is minimized. However, the protection from O₂ is only partial, and the activity of the enzyme decreases upon each exposure to O₂. By using site directed mutagenesis in combination with electrochemistry, ATR-FTIR spectroscopy and molecular dynamics simulations, we show that the kinetics of the conversion between the oxygen protected inactive state (cysteine-bound) and the oxygen-sensitive active state can be accelerated by replacing a surface residue that is very distant from the active site. This sole exchange of a methionine for a glutamate residue leads to an increased resistance of the hydrogenase to dioxygen. With our study we aim to understand how local modifications of the protein structure can have a crucial impact on protein dynamics and how they can control the reactivity of inorganic active sites through outer sphere effects.

1. Introduction

[FeFe]-hydrogenases catalyze the reversible oxidation and production of hydrogen at high rates.^{1,2} These redox enzymes use a unique active center, the so-called “H-cluster”, which consists of two sub-clusters. The cubane subcluster, [4Fe]_H, is a standard [4Fe-4S]-cluster ligated by four cysteine ligands to the protein backbone. One of the cysteine ligands also bridges the [4Fe]_H cluster and the binuclear FeS cluster, [2Fe]_H, which is additionally coordinated by two CN⁻ and three CO ligands.^{3,4}

35 Both iron atoms, Fe_p and Fe_d (the subscripts p and d refer to “proximal” and “distal” with respect to
36 the cubane subcluster), are connected via an azadithiolate bridge (ADT), which plays an important
37 role during the catalytic hydrogen turnover.³⁻⁶ The vacant site at Fe_d is involved in hydrogen oxida-
38 tion/evolution and is prone to the binding of various inhibitors (O₂, CO, SH).⁷⁻¹⁴ In contrast to in-
39 dustrial platinum-based hydrogen production catalysts, [FeFe]-hydrogenases rely on earth abun-
40 dant metals such as iron and can be considered as biological blueprints for the design of synthetic
41 catalysts.¹⁵⁻¹⁸ However, their catalytic performance is limited by a high O₂-sensitivity. The binding
42 of oxygen to the vacant coordination site is coupled to further protonation and reduction steps that
43 eventually lead to the formation of reactive oxygen species (ROS), which subsequently results in an
44 irreversible destruction of the active site.^{7,8,19-22} Note that in standard hydrogenases, the destruc-
45 tion of the enzyme by O₂ is mainly irreversible, but a partial reactivation, resulting from the reduc-
46 tion of O₂ at the active site, can be detected after short exposure to O₂.^{7,23} Computational and exper-
47 imental studies suggested that O₂-binding leads either to the decomposition of the [2Fe]_H subsite,
48 retaining an intact [4Fe]_H²¹ or ROS (O₂⁻, OOH⁻) diffusion initiates the decomposition at the
49 [4Fe]_H.^{13,24} However, an *in crystallo* study could demonstrate that both pathways can coexist and
50 are highly dependent on the electron supply.²² The enzyme CbA5H from the bacterium *Clostridium*
51 *beijerinckii* is so far the only characterized enzyme, which tolerates prolonged exposure to O₂, by
52 reversibly switching between the active H_{ox} state to the inactive but O₂-protected H_{inact} state.^{25,26}
53 The H_{inact} state was originally identified in DdH from *Desulfovibrio desulfuricans* and provided an
54 irreversible protection against O₂, albeit only limited to the first reductive reactivation.^{14,27,28} Stud-
55 ies by Rodríguez-Maciá et al., could provide evidence that this effect is attributed to the binding of
56 exogenous sulfide under oxidizing conditions.⁹ Sulfide treatment to other [FeFe]-hydrogenases
57 could reproduce this inactivation, enabling effective protection against O₂ prior to the first reactiva-
58 tion.^{10,29} In contrast to that, CbA5H possesses an intrinsic protection mechanism towards O₂, in-
59 dependent of exogenous sulfide, which is governed by the coordination of an adjacent cysteine res-
60 idue (C367). Upon oxidizing conditions, C367 can coordinate to Fe_d and thus prevent the binding
61 of O₂ and the subsequent destruction of the active site.^{25,26,30} It has been shown that various resi-
62 dues are crucial for the movement of the cysteine residue and, based on spectroscopic and kinetic
63 data, we proposed a detailed model for the conformational change of the “TSC” peptide loop that
64 relocates the conserved cysteine close enough to Fe_d to allow coordination.³⁰



65

66 **Figure 1.** Structural overview of the [FeFe]-hydrogenase CbA5H^{WT}. A) X-ray structure of CbA5H^{air}
67 (PDB: 6TTL) shown as cartoon. H-cluster and accessory FeS clusters are depicted as spheres. Resi-
68 duce M382 is represented as stick in red. B) Structural alignment of CpI (PDB: 4XDC, yellow) and
69 CbA5H^{air} (light blue). H-cluster and residues that are expected to influence the loop flexibility are
70 depicted as sticks and highlighted in red. Inset shows residues that are in close vicinity to M382 as
71 sticks.

72 A structural comparison of CbA5H and the standard [FeFe]-hydrogenase CpI from *Clostridium pas-*
73 *teurianum* unveiled a complex interplay of different residues which prevents the coordination of
74 the cysteine residue in conventional [FeFe]-hydrogenases. Substituting these amino acids with an-
75 analogues of CpI resulted in slower conformational changes and more O₂-sensitive CbA5H variants. In
76 the WT enzyme, the reductive reactivation is limited to a few cycles of O₂-exposure and reductive
77 reactivation: a fraction of the enzyme is irreversibly degraded in each reversible conversion from
78 active (A) to inactive (I) form, as observed in independent investigations of CbA5H.²⁶ In this work,
79 we show that methionine 382, an amino acid positioned more than 18 Å away from the active site
80 (see Figure 1A) is an important structural determinant of the O₂ resistance of CbA5H. Due to the
81 inherent properties of methionine residues, M382 anchors the protein framework and stabilizes a
82 particular configuration of the peptide chain. A substitution of this residue results in structural
83 changes within the protein, which increase the flexibility of a loop (termed “Loop A”) adjacent to
84 the TSC-loop. By combining site-directed mutagenesis, IR spectroscopy, molecular dynamics simu-
85 lations and electrochemical kinetic studies, we show that the residue at position 382 remotely con-
86 trols the active site chemistry of CbA5H and that its substitution towards and glutamic acid accel-
87 erates the conformational changes and renders the enzyme more resistant to oxygen.

88 **2. Experimental Section**

89 **Site directed mutagenesis.** QuikChange PCR was used to generate hydrogenase genes with differ-
90 ent codons according to the protocol described earlier.³¹ Expression plasmids were amplified by
91 using mismatch primers (Table SI). Products were treated with endonucleases DpnI and trans-
92 formed into *E. coli* strain DH5 α by using heatshock method. All DNA constructs were subsequently
93 verified via sequencing.

94 **Expression and purification:** *E. coli* strain BL21 (DE3) Δ iscR was transformed with expression
95 plasmid pET21b containing the codon optimized CBA5H gene (NCBI GenBank: KX147468.1) and
96 was used to express apo CbA5H (lacking the [2Fe_H] subcluster).³² Expression and purification was
97 conducted under strictly anaerobic conditions as described previously.³³ Affinity chromatography
98 was performed used Strep Tacting High-capacity resin (IBA GmbH). Protein concentration was de-
99 termined by Bradford assay³⁴ and purity was verified by SDS-PAGE (polyacrylamide gel electro-
100 phoresis).³⁵ Proteins were stored at -80 °C in 100 mM Tris-HCl buffer pH 8 with 2 mM sodium di-
101 thionite (NaDT).

102 **In vitro maturation:** Apo proteins were incubated on ice for 1 hour with a 10-fold molar excess of
103 the artificially synthesized [2Fe]_H cofactor mimic ([2Fe₂[μ -(SCH₂)₂NH](CN)₂(CO)₄]²⁻)³⁶ in order to
104 reconstitute active protein as described earlier.³⁷ Holo proteins and excess [2Fe]_H were separated
105 by size exclusion chromatography (NAP 5 column, GE healthcare).

106 **Enzyme activity assay (H₂ production) with multiple inactivation cycles:** H₂ production activity
107 was assayed with 10 mM methyl viologen (MV) as electron mediator, 200 mM NaDT as electron
108 donor and 400 ng holo-enzyme in 100 mM potassium phosphate buffer (K₂HPO₄/KH₂PO₄) at pH 6.8.
109 The reaction mixture was degassed with 100 % argon and incubated for 20 min at 37 °C in a sealed
110 8 ml glass vessel. The amount of H₂ was determined by gas chromatography. The inactivation was
111 performed by incubating the enzyme solution for 15 min under an atmosphere of air, on ice, making
112 sure the solution is aerated. After the first inactivation and prior to every further inactivation with
113 air, the solution was treated for 20 min with 100 % H₂ in order to ensure full activation. All protein
114 solutions were prepared in the absence of NaDT. After each inactivation, aliquots were taken for
115 subsequent standard *in vitro* assays as described before. Each measurement was performed in tri-
116 ple determination with three technical replicates.

117 **Protein film electrochemistry.** Cyclic voltammetry and the amperometry experiments employed
118 to elucidate the kinetics of the conformational change leading to H_{inact}-formation were carried out

119 in a JACOMEX glovebox filled with N₂ in a thermostated (T = 5 °C) electrochemical cell with two
120 compartments. The main compartment contained the rotating working electrode (pyrolytic graph-
121 ite edge, 3 mm diameter, typical rotation rate 3000 rpm, mounted on a Pine MSR rotator) and the
122 counter electrode (a platinum wire) as well as the inlet of H₂. The main compartment was filled with
123 a mixed buffered solution (consisting of 5 mM of each MES, HEPES, sodium acetate, TAPS, and CHES
124 and 0.1 M NaCl). The main compartment was connected via a Luggin capillary to a second compart-
125 ment, containing the calomel reference electrode in 0.1 M NaCl. All potentials are quoted with re-
126 spect to the standard hydrogen electrode, calculated using $E_{\text{SHE}} = E_{\text{calomel}} + 0.241 \text{ V}$. To prepare the
127 protein films, the pyrolytic graphite “edge” (PGE) rotating disc working electrode was polished with
128 an aqueous alumina slurry (Buehler, 1 μm), rinsed, then painted with 0.5 μl of an enzyme solution
129 (~ 5 mg/mL in 100 mM Tris- HCl, pH 8 with 2 mM NaDT) and let dry for 2 min. The measured
130 catalytic currents were between 1- and 10 μA, so that mass transport towards the rotating electrode
131 was not limiting.³⁸ (CVs and chronoamperometric experiments were recorded with the program
132 GPES and analyzed with the open source program QSoas.³⁹ Chronoamperometric experiments were
133 started and ended at a potential 30 mV below the H⁺/H₂ equilibrium potential (pH 7: -0.459 V vs
134 SHE) to ensure complete activation of the enzyme film. The intermediate steps at oxidative poten-
135 tial were of 100 mV amplitude. Before analyzing the changes in H₂-oxidation current, we subtracted
136 the background capacitive current recorded in a control experiment without enzyme.

137 **ATR-FTIR spectroscopy.** Attenuated total reflectance fourier transform spectroscopy was per-
138 formed on a Bruker Tensor II spectrometer (Bruker Optik, Germany), equipped with a 9 reflection
139 ZnSe/Si crystal (Microm ATR Vision, Czitek). All measurements were conducted under anoxic con-
140 ditions (1.5 % H₂, 98.5 % N₂) at 25 °C. The FTIR spectra were recorded from 4000 – 1000 cm⁻¹ with
141 a resolution of 2 cm⁻¹. The protein film was dried with a H₂ gas stream for around 10 minutes and
142 re-humidified with an aerosol by purging a buffer containing compartment.

143 **Molecular Dynamics Simulations.** All molecular dynamics (MD) simulations were performed with
144 GROMACS version 2021.4.⁴⁰ The structure of the H-domain was taken from the previously reported
145 crystal structure of WT CbA5H captured in the H_{inact} state (PDB ID 6TTL).³⁰ The M382E variant
146 structure was generated by replacing the M382 side-chain in 6TTL. The CHARMM36 force field was
147 employed for the protein together with the CHARMM-specific TIP3P water model. The force field
148 parameters for the H-cluster and other Fe-S clusters were taken from the work of Chang et al. and
149 McCullagh et al.^{41,42} The H_{ox} state was simulated, with the C367 side-chain protonated (-SH). The
150 protein structures were solvated in dodecahedron-shaped periodic simulation boxes filled with

151 13206 water molecules and sodium ions to neutralize the systems. Prior to the MD simulations, the
152 systems were energy-minimized, followed by stepwise equilibration for 2.7 ns, during which har-
153 monic position restraints were applied to different sets of atoms. In the first NVT equilibration
154 phase (0.2 ns), position restraints with force constants of 1000 kJ/mol/nm² were applied to all non-
155 hydrogen atoms of the protein and the Fe-S clusters. Subsequently, the second equilibration phase
156 was continued for another 2.5 ns in the NpT ensemble. During the initial 0.5 ns of this second equi-
157 libration phase, the position restraints were still applied to all non-hydrogen atoms of the protein
158 and the Fe-S clusters. During the final 2.0 ns, the position restraints were only applied to all non-
159 hydrogen atoms of the protein backbone. The MD simulations were conducted withing periodic
160 boundary conditions in the NpT ensemble at constant temperature of 300 K and 1 bar pressure, by
161 coupling to the velocity rescaling thermostat of Bussi and coworkers and weak coupling barostat,
162 respectively.^{43,44} The Coulomb and Lennard-Jones interactions were described with a buffered Ver-
163 let pair list with a 1.2 nm cutoff, with the forces smoothly switched to zero between 1.0 nm and 1.2
164 nm.⁴⁵ Long-range electrostatic interactions were treated with the particle mesh Ewald (PME)
165 method with 0.12 nm grid spacing.⁴⁶ The LINCS and SETTLE constraint algorithms were employed
166 to constrain all protein bonds involving H-atoms and internal degrees of freedom of water mole-
167 cules, respectively, allowing to integrate the equations of motion with 2 fs time steps.^{47,48} Finally,
168 starting with the equilibrated systems, two 1500 ns production MD simulations for each of the two
169 different systems (WT enzyme, M382E) were carried out in the NpT ensemble, initiated with dif-
170 ferent random seeds to generate the initial atomic velocities from a Maxwell distribution at 300 K.
171 Because the starting structures of the H_{ox} states were built from the H_{inact} crystal structure, the ini-
172 tial 200 ns of each simulation were considered to be equilibration and discarded in the analyses.

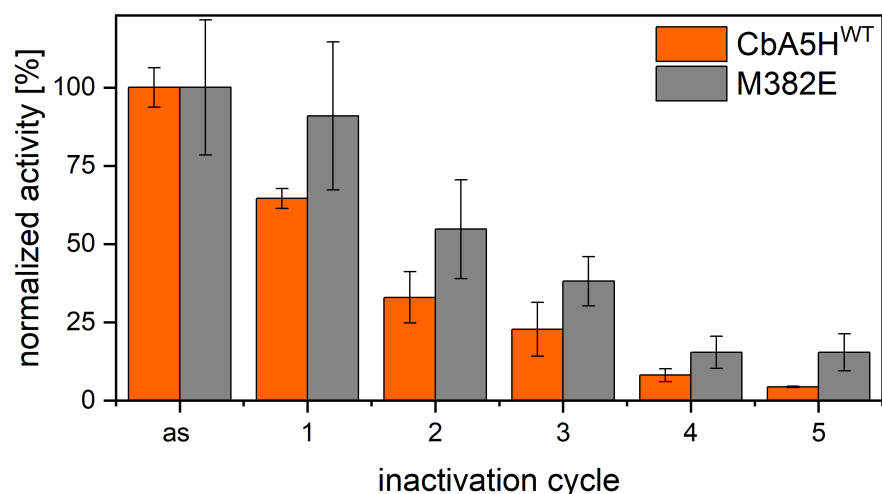
173 **3. Results and Discussion**

174 We focus on the position of methionine 382, which is located in a bridging position between Helix A
175 and Loop A, close to the TSC-loop (Figure 1B). The crystal structure of CbA5H suggests that M382
176 forms hydrophobic contacts with residues L549 and I552 (Figure 1B, inset), which might coordi-
177 nate the peptide chain. Methionine residues often stabilize protein structures by forming hydro-
178 phobic interactions with nearby residues.^{49,50} The highly flexible side chain with the unique prop-
179 erties of the thioether sulfur render this amino acid as highly versatile.^{49,51-53} It was proposed that
180 methionines form tighter van der Waals contacts than other hydrophobic side chains, which pro-
181 vides a natural anchor point in the protein environment.⁵⁴ The replacement of a methionine can
182 abolish such interactions and therefore may increase the flexibility of peptide chains.⁴⁹ We aimed

183 to alter the protein structure at this position by replacing M382 and studying the influence of the
184 mutation on the conformational dynamics and on the (in)activation mechanism.

185 **Enhanced O₂ stability of the M382E variant**

186 We replaced M382 with glutamic acid, isoleucine, and alanine. The M382A variant did not exhibit
187 measurable activity, while the isoleucine exchange maintained high catalytic performance. The ex-
188 change of methionine to glutamic acid (M382E) resulted in an H₂-production activity of about
189 50-60 % compared to the CbA5H^{WT} protein. We investigated the stability of the variant M382E after
190 repeated cycles of exposure to air and H₂ using methyl viologen based H₂-production assays.



191

192 **Figure 2.** H₂ production activities after aerobic inactivation of CbA5H^{WT} and M382E. Normalized
193 activity measurements of three independent measurements after cycles of air exposure (15 min at
194 4°C). After every inactivation step the protein solution was reactivated under 100 % H₂ atmosphere
195 for 20 min. Individual data sets are summarized in Figure S3 and Supplementary table S2.

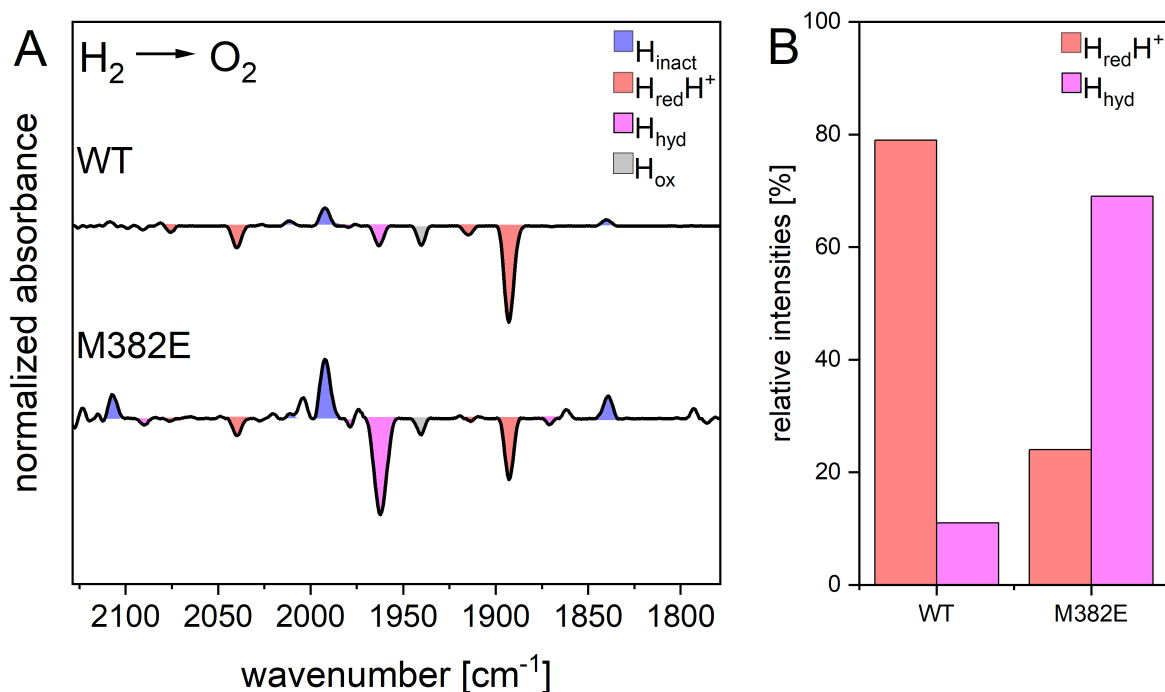
196 The results in Figure 2 show a decrease in activity after each exposure to air for the WT and the
197 variant. The observed decay of CbA5H^{WT} activity is in agreement with earlier reports (~40 % after
198 the 1st exposure, ~75 % after the 2nd exposure²⁶). However, the relative decrease in activity is
199 smaller for the M382E variant and indicates an improved protection, which might be associated
200 with the easier or faster coordination of the C367 side chain to the distal Fe.

201

202 **Influence of the M382E substitution on the catalytic cycle**

203 We examined the protein variants using FTIR spectroscopy to assess the extent of incorporation of
204 the [2Fe]_H subsite into the protein and to monitor catalytic relevant redox states. We observed a
205 reduced H-cluster occupancy in the M382E variant which is consistent with the reduced activity

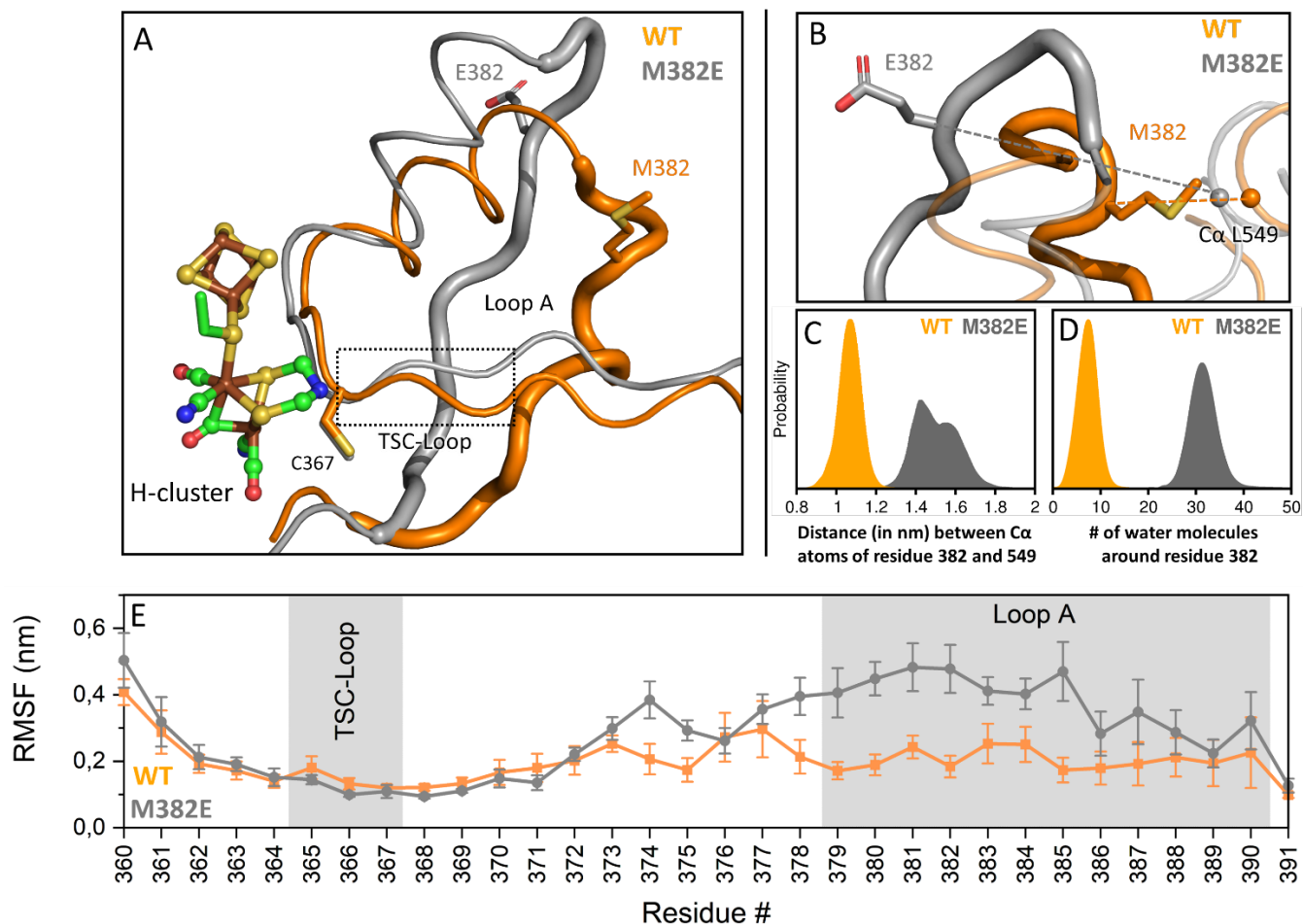
206 (see SI Figure S4). The difference spectrum in Figure 3 A shows that the treatment of the WT en-
207 zyme with a hydrogen saturated aerosol led to the accumulation of the bands at 1893 cm⁻¹, 1916 cm⁻¹,
208 1,2040 cm⁻¹ and 2075 cm⁻¹, which are assigned to the H_{red}H⁺ state with minor contributions of H_{hyd}
209 and H_{ox}.



210

211 **Figure 3.** IR-frequencies of CbA5H^{WT} and M382E. A) ATR-FTIR difference spectra of CbA5H^{WT} and
212 M382E (200-500 μM, 0.1 M potassium phosphate buffer, pH 7) after 100 % H₂ gassing and after
213 treatment with air. Blue (H_{inact}), red (H_{red}H⁺), purple (H_{hyd}), grey (H_{ox}). B) Relative band intensities
214 of WT protein and variant in respect to the relative absorbance spectra.

215 The H_{red}H⁺ state features a reduced [2Fe]_H and a proton bound to the ADT bridge.^{55,56} A one-electron
216 reduction leads to the formation of the H_{sred}H⁺ state which enables the subsequent formation of the
217 H_{hyd} state, featuring a terminal hydride species bound to Fe_d.⁵⁷ In contrast to that we could observe
218 that the spectrum of the M382E variant exhibited a significant contribution of the H_{hyd} state (2091
219 cm⁻¹, 2083 cm⁻¹, 1979 cm⁻¹, 1963 cm⁻¹ and 1855 cm⁻¹) after prolonged exposure to hydrogen (Figure
220 3).



221

222 **Figure 4.** MD simulations of CbA5H^{WT} and M382E. A) Cartoon representation of CbA5H showing
 223 WT protein in orange and M382E variant in grey. H-cluster and residues C367, M382 (CbA5H^{WT})
 224 and E382 (M382E variant) are shown as sticks. Loop A is shown in thick and the TSC-loop is indi-
 225 cated by a box. B) Structural overlay of representative snapshots from the MD simulations of WT
 226 and M382E variant. Distances between C α atoms of residues 382 and 549 are indicated by dashed
 227 lines. C) Histogram of the distance between C α atoms of residues 382 and 549 in CbA5H WT and
 228 M382E variant. D) Number of water molecules around residue 382 in CbA5H WT and variant. A
 229 water molecule were counted if its O-atom was within 0.5 nm of any atom of residue 382. E) RMSF
 230 plot depicting the fluctuations of the residues in WT and M382E. The TSC-loop and Loop A are high-
 231 lighted with grey boxes.

232 Standard [FeFe]-hydrogenases accumulate this intermediate usually in proton transfer deficient
 233 variants or under acidic conditions in a combination with an atmosphere of H₂.⁵⁷ The accumulation
 234 of H_{hyd} in the M382E variant might suggest a conformational change of the native proton transfer
 235 pathway. This notion is supported by our MD simulations (see below), which show that in the
 236 M382E variant, residue S387, which is a key residue in the native proton transfer pathway (PTP),
 237 is dislocated and no longer participates in an H-bond network with E341 and E344, which would
 238 be required for efficient proton transfer along this pathway (Figure S5). We speculate that the en-
 239 hanced spatial freedom influences the protonation dynamics of the H-cluster, hence providing a

240 reasonable explanation for the H_{hyd} accumulation in M382E. Previous studies could report a similar
241 observation for proton transfer deficient CpI variants, which exhibited an increased population of
242 the hydride bound state.^{57,58} Additionally, it could be shown that an impaired protonation of O_2^-
243 species due an interrupted PTP prevent the formation of mobile ROS and reduces the degradation
244 of the H-cluster.¹⁹ The structural deviations in the PTP of the variant M382E and the resulting pres-
245 ence of a terminal bound hydride might support the resistance towards oxygen. The subsequent
246 exposure of the enzymes to air led to the formation of the O_2 protected H_{inact} state (2107 cm^{-1} ,
247 2080 cm^{-1} , 2011 cm^{-1} , 1991 cm^{-1} and 1840 cm^{-1}).

248

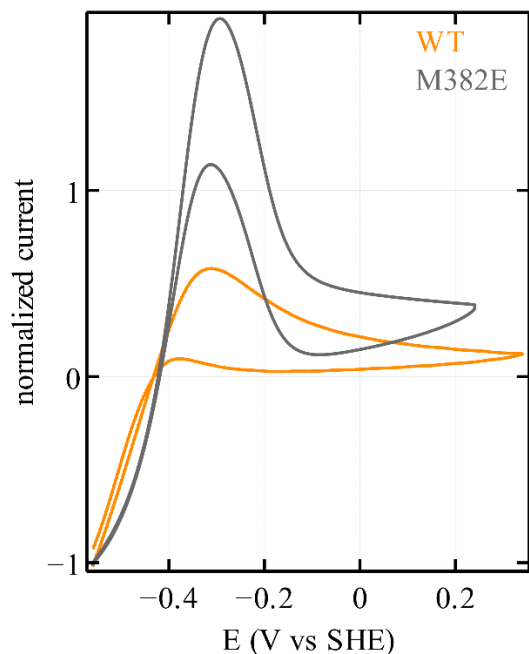
249 **The M382E substitution changes the conformational dynamics of the protein**

250 To understand how the mutation at site 382 modulates the conformational ensembles of CbA5H, we car-
251 ried out all-atom MD simulations of the WT enzyme and the M382E variant. The simulations were started
252 from the H_{ox} state, which corresponds to the A_1 state (see below). The MD simulations were based on
253 the X-ray crystal structure of CbA5H from *C. beijerinckii* (PDB code 6TTL). The atomic coordinates
254 of the M382E variant were generated from the crystal structure by replacing the M382 side chain.
255 Figure 4A-C show that in the M382E variant Loop A undergoes structural changes, which also affect
256 the vicinity of the TSC-loop. This conformational change is triggered by solvation forces, which dis-
257 place the charged E382 side chain from the hydrophobic environment in which M382 is located in
258 the WT protein, such that E382 becomes fully hydrated by moving away from the helix bearing the
259 hydrophobic contact-forming residue L549 (Figure 4B-D). This conformational change goes along
260 with a pronounced increase of the structural flexibility in this region (see RMSF profile showing the
261 fluctuations of the residues in Figure 4E). Besides residue S387 of the PTP, this part of the peptide
262 chain bears a residue that is relevant for the positioning of C367 near Fe_d (P386) and may influence
263 the kinetics of the cysteine coordination to Fe_d . These observations are in line with our previous
264 results, which suggested that at least three distinct positions (L364, P386, and A561) are crucial for
265 the conformational change.³⁰ To further investigate the link between the structural dynamics and
266 the inactivation kinetics, we employed protein film electrochemistry.

267 **Position M382 influences anaerobic inactivation**

268 Protein film electrochemistry (PFE) was used to examine the kinetics of the conformational changes
269 that lead to reversible inactivation in $CbA5H^{\text{WT}}$ and the variant, using the method described

270 earlier.³⁰ The activity of the enzyme adsorbed onto a graphite electrode is measured as a current
271 following sweeps or steps of the applied potential, which trigger its (in)activation. All electrochem-
272 ical experiments were performed at 5°C to minimize film loss. Figure 5 compares the cyclic voltam-
273 mograms (CVs) of the WT protein and the M382E variant (see Figure S7 for the M382I variant and
274 Figure S8 for the effect of varying the scan rate).



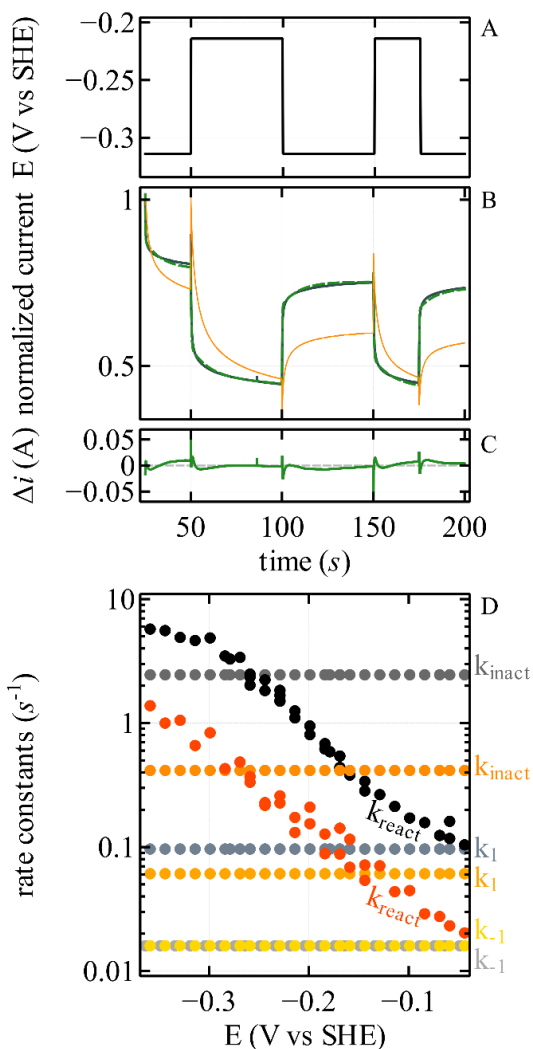
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276 **Figure 5.** Cyclic voltammograms showing the anaerobic (in)activation of CbA5H (orange) and the
277 M382E variant (gray) under conditions of direct electron transfer with a rotating disc pyrolytic
278 graphite edge electrode (pH 7, 5°C, 1 bar H₂, scan rate 20mV/s, electrode rotation rate 3000 rpm).
279 See SI Figure S7 for the comparison between the data obtained with CbA5H and M382I variant. Note
280 that in protein film voltammetry the magnitude of the signal cannot be interpreted because it de-
281 pends on the unknown enzyme coverage. Only the shape of the signal matters, and therefore the
282 potential chosen for the normalization of the current is arbitrary. See SI Figure S6 A for the raw
283 (non-normalized) data plotted in this figure.

284

285 In both cases, a negative current reveals H₂ production at low potential and H₂ oxidation is seen as
286 a positive current at high potential. The current response is dependent on the direction of the sweep
287 (the CVs show a very pronounced hysteresis). The H₂ oxidation current decreases at low potential
288 (positive of the H⁺/H₂ Nernst potential) due to oxidative inactivation and the enzymes reactivate
289 during the reductive sweep. These processes are slow on the voltametric time scale, hence the hys-
290 teresis. The high potential inactivation observed with CbA5H differs from that observed with

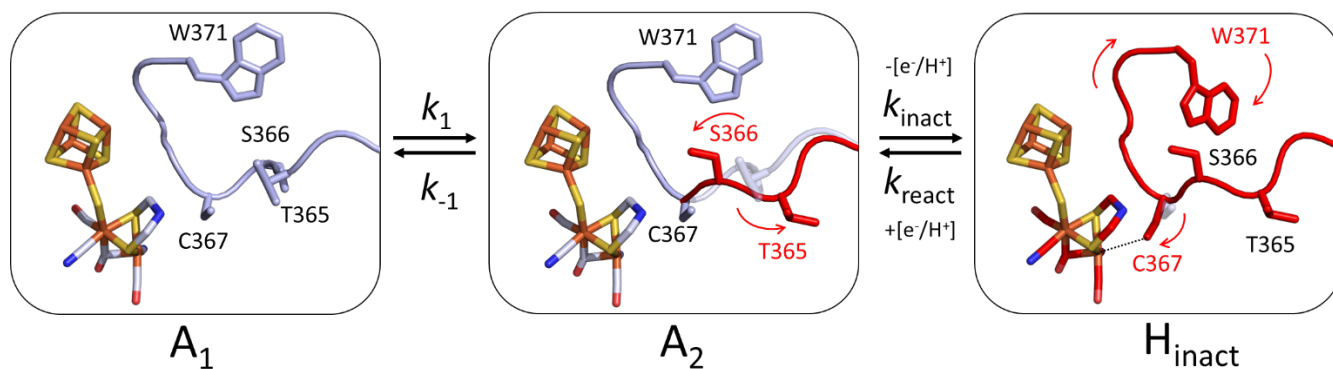
291 standard hydrogenases: it occurs at much lower potential than that of standard hydrogenases and
292 it is not due to the presence of chloride anions in the buffer (Figure S9 in reference 30 for the WT,
293 and SI Figure S9 for the M382E mutant).



294

295 **Figure 6.** Potential steps experiments used to quantify the anaerobic inactivation kinetics of the
296 CbA5H^{WT} and the M382E variant. See SI Figure S11-12 for the comparison between the data ob-
297 tained with CbA5H and the M382I variant. A) an example of potential steps applied to the electrode
298 onto which the enzymes are adsorbed (pH 7, 5°C, 1 bar H₂, 3000 rpm). B) the current responses
299 (orange: WT, gray: M382E) to the potential steps in panel A. The best fit of the model is described
300 earlier and is shown in dashed green.³⁰ C) residual of the fit. D) the four rate constants determined
301 by fitting the model to a series of chronoamperograms obtained with different potential steps (see
302 SI Figure S10). Three rate constants are potential independent and shown with dark orange, orange
303 and yellow circles (k_{inact} , k_1 and k_{-1} , respectively) for the WT protein, and in gray, slate gray and dark
304 gray (k_{inact} , k_1 and k_{-1} , respectively) for the M382E mutant. k_{-1} is ill-defined (cannot be accurately
305 determined) and was set to the same value for both enzymes. The values of k_{react} are shown as red
306 and black circles for the WT enzyme and for the mutant, respectively. The WT data are replotted
307 from ref. 30.

308 The inactivation of CbA5H WT disappears when C367 is replaced with aspartate and was therefore
309 related to the formation of the H_{inact} state.³⁰ Figure 5 shows that the (in)activation of the variant is
310 more pronounced. The kinetics of the transformation is easily assessed by interpreting potential
311 steps experiments such as those in Figure 6B, where the electrode potential is repeatedly stepped
312 up and down, and the current response is analyzed using a kinetic model. Note in this figure that
313 the change in current following the potential steps is faster for the variant than the WT.



315 **Figure 7.** AAI model for the reversible conversion between active and inactive forms of CbA5H^{WT}.
316 Structural alignment of Cpl (A₁, light blue) and CbA5H_{air} (H_{inact}: red). Three main states are identi-
317 fied³⁰, the active species A₁, a 2nd active species A₂ after conformational change of the TSC-loop
318 (central panel), and the inactive species H_{inact}, where C367 is coordinated to Fe_d. Arrows indicate
319 the rate constants k_1 , k_{-1} , k_{inact} , k_{react} .

320

321 The previously established kinetic model³⁰ considers three distinct species (A₁, A₂ and I, Figure 7),
322 and accounts for the biphasic kinetic traces that are observed in chronoamperometry experiments.
323 “A₁” and “A₂” are active forms of the enzyme, “I” is an inactive form, the “H_{inact} state”, in which cys-
324 teine C367 is coordinated to the distal iron, competing with hydrogen binding and thus preventing
325 turnover. The potential-independent rate constants k_1 and k_{-1} describe the conversion between A₁
326 and A₂. Due to the slow transition between these two forms ($1/(k_1 + k_{-1}) \approx 10$ s), we proposed that
327 this step is associated with a conformational change. The final coordination of the cysteine ligand
328 itself occurs during the transformation of A₂ to I and the bond is labile ($1/(k_{\text{inact}} + k_{\text{react}}) \approx 1$ s) due
329 to the relatively long distance of 3.1 Å. Note that the inactive state is formed upon anaerobic or
330 aerobic oxidation and provides effective protection against O₂. Hence the faster the inactivation, the
331 more resistant the enzyme. This correlation has already been observed in our previous work, although
332 the mutants examined therein were slower to inactivation and thus less O₂ resistant.³⁰ The second step

333 involves the transfer of one proton and one electron, but the analysis of the data shows that only
334 the backward rate constant k_{react} is potential dependent (see reference 30 and Figure 6). We ana-
335 lyzed a number of chronoamperometric traces such as those in Figure 6B (see SI figure S10) to
336 measure the rate constants of interconversion between the active and inactive forms of the en-
337 zymes under various conditions of electrode potential. The results are shown in Figure 6D for the
338 WT protein and the M382E variant (see S12 for the M382I variant). The results in Table 1 show that
339 the M382E mutation has no significant effect on the k_1/k_{-1} transition (note that, as mentioned in our
340 previous work, k_{-1} cannot be accurately determined and was fixed to the WT protein value in all
341 fitting procedures), but it increases the rate constants k_{inact} and k_{react} , about 5.5-fold, respectively,
342 leaving the ratio unchanged. The 5.5-fold acceleration corresponds to a 4 kJ/mol decrease in acti-
343 vation energy of step 2. Together with the increased structural flexibility observed in the MD simu-
344 lations (see above) this provides a reasonable explanation why C367 coordinates faster to Fe_d in
345 the M382E variant than in the WT. The dynamic conversion between the different states
346 ($A_{1/2} \leftrightarrow H_{\text{inact}}$) determines the efficiency of the inactivation mechanism of CbA5H. Our study illus-
347 trates how the replacement of a residue is associated with an increased local flexibility of Loop A,
348 which directly affects the TSC loop and thus the catalytic properties of the enzyme.

349 **Table 1. Values of k_1 , k_{-1} , k_{react} , k_{inact} for CbA5H^{WT} and M382E.** Measured at pH7, 5°C. All rate
350 constants are potential independent, except k_{react} which is given here at $E = -199$ mV vs SHE. The
351 value of k_{-1} is the least well-defined in the fitting procedure (as observed previously³⁰), and was
352 fixed to the WT. The WT data are taken from ref. 30.

	k_1 (s^{-1})	k_{-1} (s^{-1})	k_{inact} (s^{-1})	k_{react} (s^{-1})	$k_{\text{inact}}/$ k_{react}
CbA5H^{WT}	0.061	0.016	0.42	0.17	2.5
M382E	0.096	0.016	2.45	0.81	3.0

353

354 It is reported that local perturbations can affect protein dynamics over large distances ($> 25 \text{ \AA}$)⁵⁹
355 with implications on different biological processes.^{60,61} Loops, which are structural relevant ele-
356 ments for protein stability,^{62,63} specificity,⁶⁴ and protein-protein interactions⁶⁵, often facilitate al-
357 losteric communication⁶⁶ and catalysis.⁶⁷ Detailed studies of various enzyme provide important
358 insights into the role of protein dynamics in catalysis. In enzymes such as protein tyrosine

359 phosphatase, a wide range of conformational changes can be observed, and loop flexibility can be
360 significantly affected by the sequence composition.^{66,68,69} Similar loop dynamics were studied in
361 *E.coli* dihydrofolate reductase *EcDHFR*, showing that two dynamically coupled loops play a critical
362 role in controlling protein dynamics and function.⁵⁹ Experimental and computational work sug-
363 gested a dynamic network that includes residues that remotely control catalysis and dynamic fluc-
364 tuations of the loops.^{59,70} On the other hand, allosteric effects are rarely reported for metallopro-
365 teins. For the FeS protein family mitoNEET, conformational changes of a loop 20 Å away from the
366 metal center showed to affect the properties of the metal center.⁷¹ Our observations in the [FeFe]-
367 hydrogenase CbA5H complement several previous studies, showing that coupled networks of
368 amino acids exist in proteins and that distant effects can influence active site events.

369 **4. Conclusions**

370 Up to now, two independent H_{inact} forming protection mechanisms are known in [FeFe]-hydrogen-
371 ases, both of which rely on the protective function of the thiol groups that are coordinated to the
372 vacant site of the H-cluster. While the initially described sulfide dependent inactivation is enabled
373 due to the presence of sulfide in sulphate reducing microorganism like *D. desulfuricans*, CbA5H de-
374 veloped an intriguing inactivation mechanism on the basis of a flexible cysteine side chain that can
375 coordinate to the H-cluster. Both examples represent a convergent mechanism to resist O₂ and may
376 result as a consequence of their ecological niche. This work demonstrates how individual amino
377 acid exchanges can influence the catalytic properties of enzymes, even though they are located far
378 away from the active site. Using site directed mutagenesis we investigate how local changes in the
379 protein network can alter the dynamic interplay of the protein environment and fine tune the in-
380 trinsic O₂-tolerance of CbA5H. Our results contribute to the understanding of long-range effects and
381 their modulation of dynamical conformational changes with respect to the prominent O₂-protection
382 mechanism of CbA5H. With these findings we aim to provide new mechanistic insights into the de-
383 velopment of advanced hydrogen catalysts for biotechnological purposes.

384

385 AUTHOR INFORMATION

386 Corresponding Author

387 thomas.happe@rub.de

388

389 Notes

390 *The authors declare no competing financial interest.*

391 Present Addresses

392 †Technical University of Munich Campus Straubing for Biotechnology and Sustainability, Profes-
393 sorship for Electrobiotechnology, Uferstrasse 53, 94315 Straubing, Germany

394

395 ASSOCIATED CONTENT

396 **Supporting Information.**

397 FTIR spectra, in solution assays and detailed kinetic characterization of the protein variants with
398 protein film electrochemistry, including Figures S1–S12 and Table S1-S3

399

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408

409 ABBREVIATIONS

410 ADT, azadithiolate; FeS, iron-sulfur; ATR-FTIR, Attenuated total reflectance fourier transform infra-
411 red; IR, Infrared; NaDT, sodium dithionite; MV, methyl viologen; CVs, cyclic voltammograms;
412 $[2Fe]_H$, $[[2Fe_2[\mu-(SCH_2)_2NH](CN)_2(CO)_4]^{2-}]$; PFE, Protein film electrochemistry;

413

414

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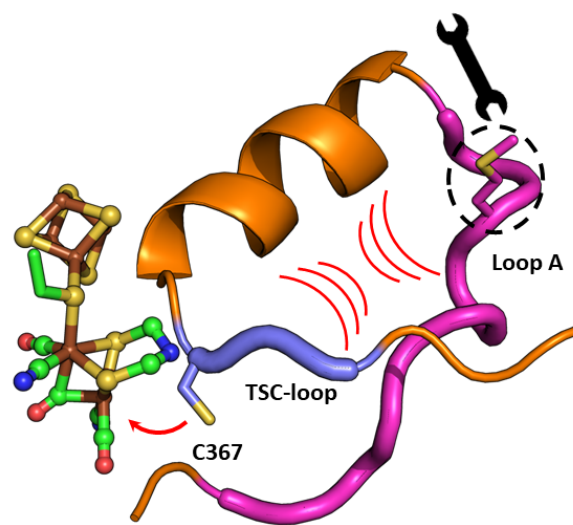
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Increasing the O₂ resistance of the [FeFe]-hydrogenase CbA5H through enhanced protein flexibility

Andreas Rutz¹, Chandan K. Das², Andrea Fasano³, Jan Jaenecke^{1†}, Shanika Yadav⁴, Ulf-Peter Apfel^{4,5}, Vera Engelbrecht¹, Vincent Fourmond³, Christophe Léger³, Lars V. Schäfer², Thomas Happe^{1*}

¹Photobiotechnology, Department of Plant Biochemistry, Ruhr-Universität Bochum, 44801 Bochum, Germany.

²Theoretical Chemistry, Ruhr-Universität Bochum, 44801 Bochum, Germany.

³Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Aix-Marseille Université, Institut de Microbiologie de la Méditerranée, 13009 Marseille, France.

⁴Inorganic Chemistry I, Department of Chemistry and Biochemistry, Ruhr-Universität Bochum 44801 Bochum, Germany.

⁵Fraunhofer UMSICHT, 46047 Oberhausen, Germany.

*Correspondence to: thomas.happe@rub.de

Supporting Information

Generation of site-directed mutagenesis variants of CbA5H

Supplementary table S1. Primers used for QuikChange PCR to generate variants of CbA5H.

M382E_for:	TGGCGATGAACTGGATGTTCCGAG
M382E_rev:	GAACATCCAGCTTATCGCCATAGTTC
M382I_for:	TGGCGATATTCTGGATGTTCCGAG
M382I_rev:	GAACATCCAGTAAATCGCCATAGTTC
M382A_for:	TGGCGATGCGCTGGATGTTCCGAGCAG
M382A_rev:	CATCCAGCGCATCGCCATAGTTCTGTTC

Biochemical characterization

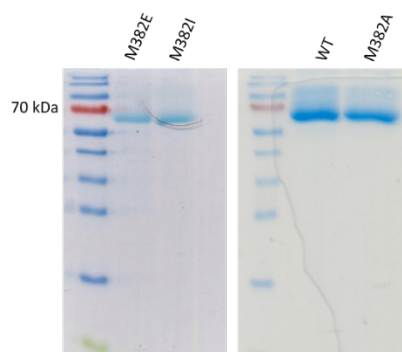


Figure S1. SDS PAGE of CbA5H WT and variants after purification. Left) Protein Marker and variants M382E and M382I. Right) Protein Marker and WT.

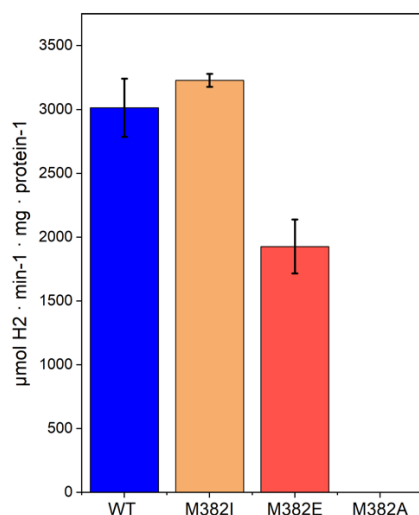


Figure S2. H₂-production activities of CbA5H and variants M382I, M382E and M382A. Activities for anaerobic proteins were determined via hydrogen production activity assay. 400 ng enzyme was mixed with 0.1 M potassium phosphate buffer (pH 6.8), 100 mM NaDT and 10 mM methyl viologen. The assay solution was incubated in a sealed glass vessel for 20 min at 37° C under constant shaking. The H₂ production rate was determined via GC-FID (gas chromatography-flame ionization detector). Determined values were calculated for WT: 3013,76 ± 227,7 $\mu\text{mol H}_2 \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, M382E: 1927,3 ± 211.2 $\mu\text{mol H}_2 \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$, M382I: 3229,3 ± 51, M382A did not show any activity. Error bars represent the standard deviations from three technical replicates.

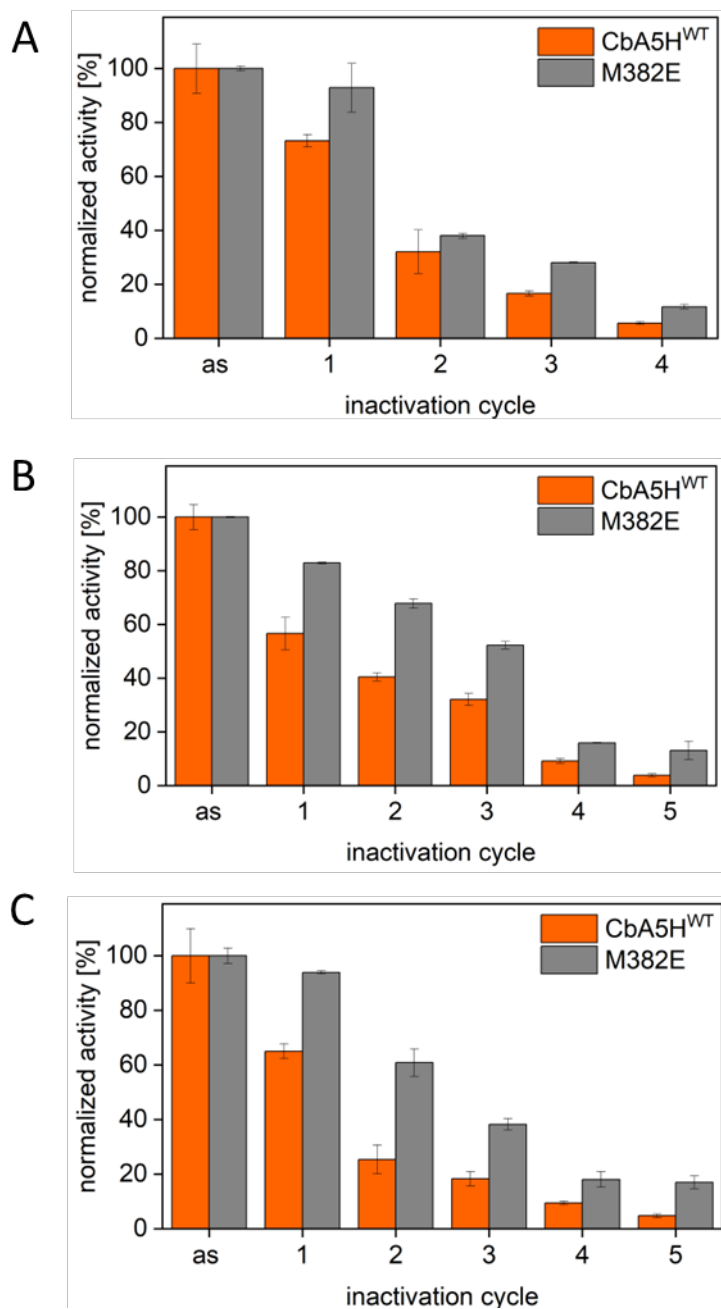


Figure S3: H₂ production activities after aerobic inactivation of CbA5H^{WT} and M382E. Normalized activity measurements after cycles of air exposure. After every inactivation step the protein solution was reactivated under 100 % H₂ atmosphere for 20 min.

Supplementary table S2: H₂ production activity measurements after aerobic inactivation and reactivation of CbA5H^{WT} variant and M382E

A	$\mu\text{mol H}_2 \times \text{min}^{-1} \times \text{mg}$				
	average	standard deviation		average	standard deviation
WT as is	3827,7 (100 %)	350,1 (9 %)	M382E as is	2394,6 (100 %)	20,7 (1 %)
WT -1	2803,3 (72 %)	87,0 (2 %)	M382E-1	2223,8 (92 %)	215,7 (9 %)
WT -2	1227,0 (32 %)	309,7 (8 %)	M382E-2	910,3 (38 %)	21,0 (1 %)
WT -3	636,8 (17 %)	36,1 (1 %)	M382E-3	671,6 (28 %)	5,7 (0,2 %)
WT-4	214,6 (6 %)	19,0 (0,5 %)	M382E-4	279,9 (12 %)	19,1 (1 %)

B	$\mu\text{mol H}_2 \times \text{min}^{-1} \times \text{mg}$				
	average	standard deviation		average	standard deviation
WT as is	4418,4 (100 %)	206,0 (5 %)	M382E as is	1700,3 (100 %)	3,1 (0,2 %)
WT -1	2501,3 (57 %)	267,5 (6 %)	M382E-1	1409,2 (83 %)	5,9 (0,3 %)
WT -2	1789,3 (41 %)	66,1 (1,5 %)	M382E-2	1153,3 (68 %)	30,0 (2 %)
WT -3	1416,9 (32 %)	99,7 (2 %)	M382E-3	887,9 (52 %)	23,7 (1 %)
WT-4	405,0 (9 %)	40,2 (1 %)	M382E-4	270,4 (16 %)	2,6 (0,2 %)
WT-5	166,5 (4 %)	26,4 (1 %)	M382E-5	222,2 (13 %)	58,9 (3 %)

C	$\mu\text{mol H}_2 \times \text{min}^{-1} \times \text{mg}$				
	average	standard deviation		average	standard deviation
WT as is	3940,0 (100 %)	393,9 (10 %)	M382E as is	2935,9 (100 %)	82,5 (3 %)
WT -1	2561,4 (65 %)	107,4 (3 %)	M382E-1	2756,5 (94 %)	17,5 (1 %)
WT -2	998,3 (25 %)	207,7 (5 %)	M382E-2	1786,9 (61 %)	147,3 (5 %)
WT -3	721,6 (18 %)	104,3 (3 %)	M382E-3	1122,3 (38 %)	62,8 (2 %)
WT-4	372,0 (9 %)	26,5 (1 %)	M382E-4	530,0 (18 %)	84,2 (3 %)
WT-5	186,3 (5 %)	25,7 (1 %)	M382E-5	499,0 (17 %)	69,5 (2 %)

ATR-FTIR spectroscopy

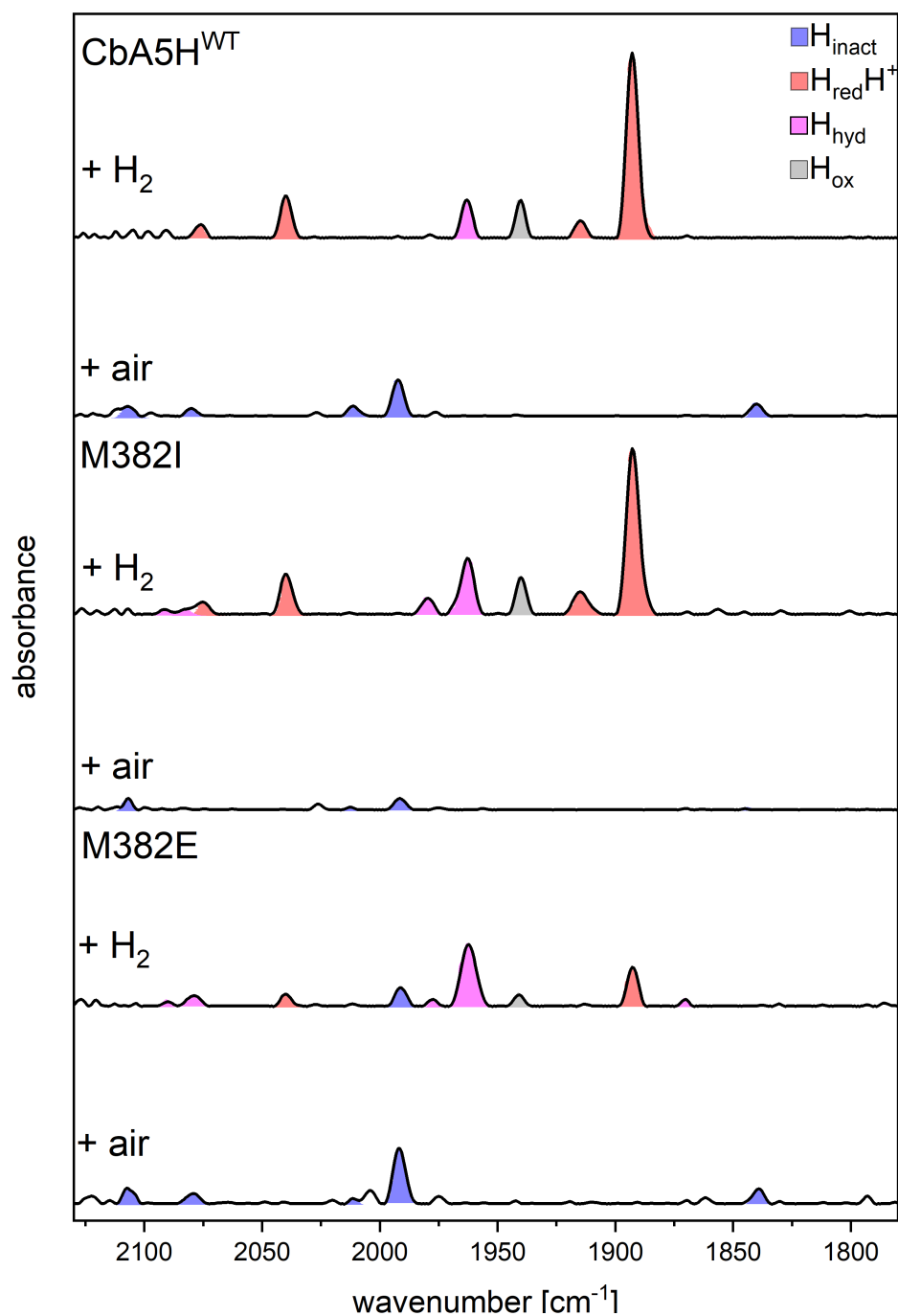


Figure S4. IR-frequencies of CbA5HWT and hydrogenase variants. A) ATR-FTIR absorbance spectra of CbA5H^{WT}, M382I, M382E (200-500 μ M, 0.1 M potassium phosphate buffer, pH 7) with 100 % H₂ gassing and after treatment with air depicting the accumulation of H_{inact}. Blue (H_{inact}), red (H_{red}H⁺), purple (H_{hyd}), grey (H_{ox}). Spectra were normalized to amide band II.

MD-simulations

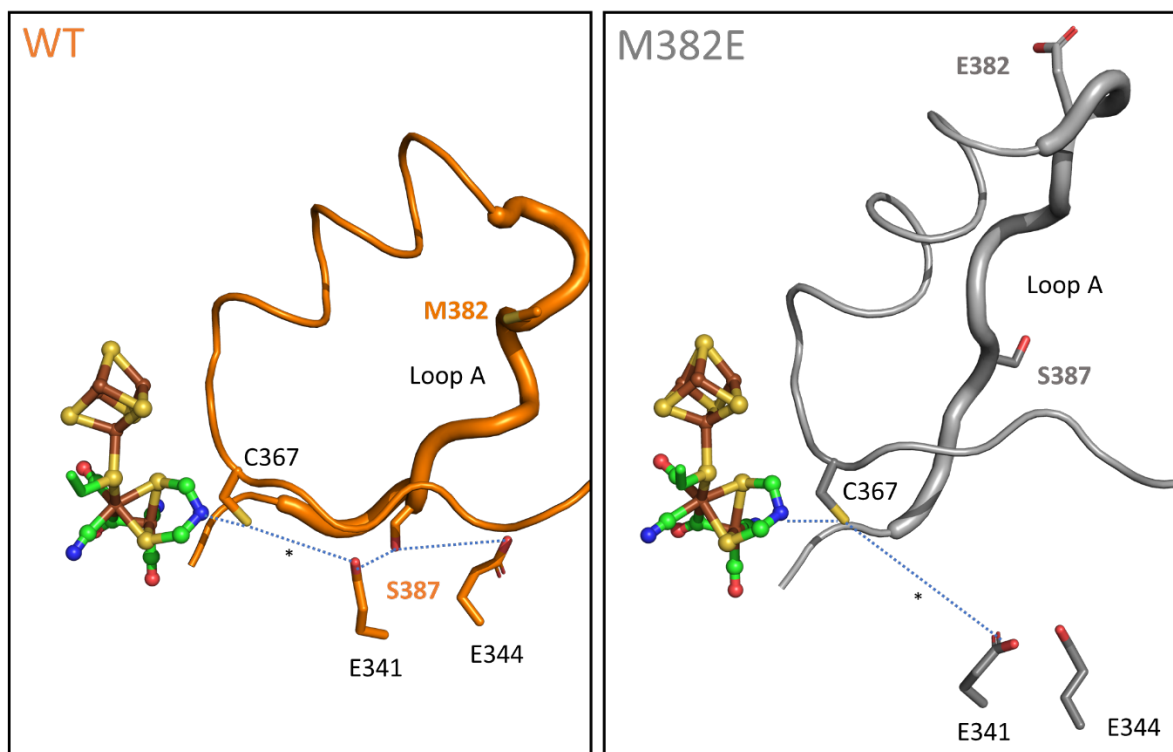


Figure S5: MD simulations of CbA5H^{WT} and M382E. Cartoon representation of A) CbA5H WT (orange) and B) variant M382E (grey). Residues of the proton transfer pathway are shown as sticks. C367 and E341 are connected through water molecules based on the structure of Cpl which is indicated by an asterisk (PDB: 4XDC). H-bond network is represented by dashed lines. Conformational change of Loop A in Variant M382E leads to a reorientation of residue S387.

Protein film electrochemistry

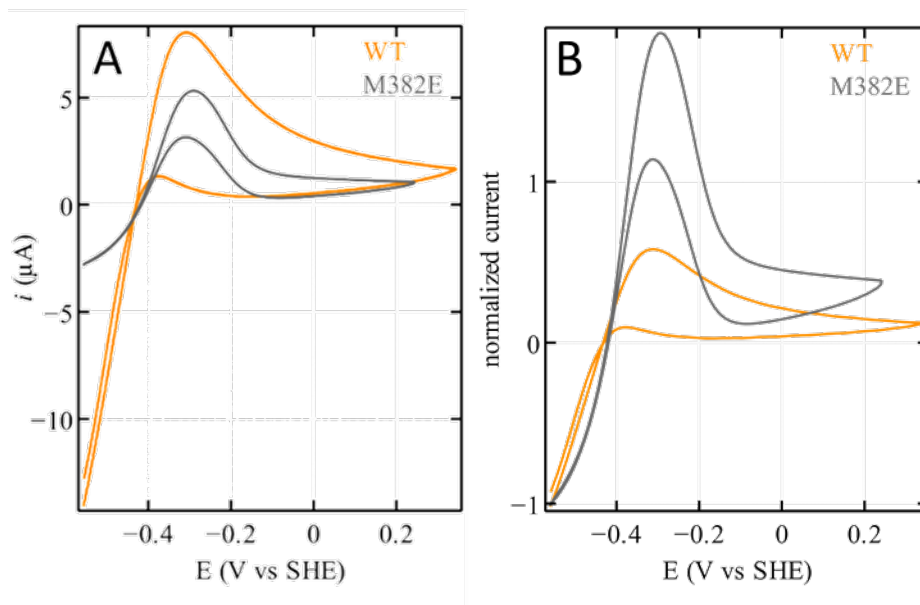


Figure S6: Cyclic voltammogram traces of CbA5H (orange) and the variant M382E (gray). A) raw B) normalized cyclic voltammograms of CbA5H WT and the variant M382E. Conditions: 5°C; pH 7; 3000 rpm; 20 mV/s

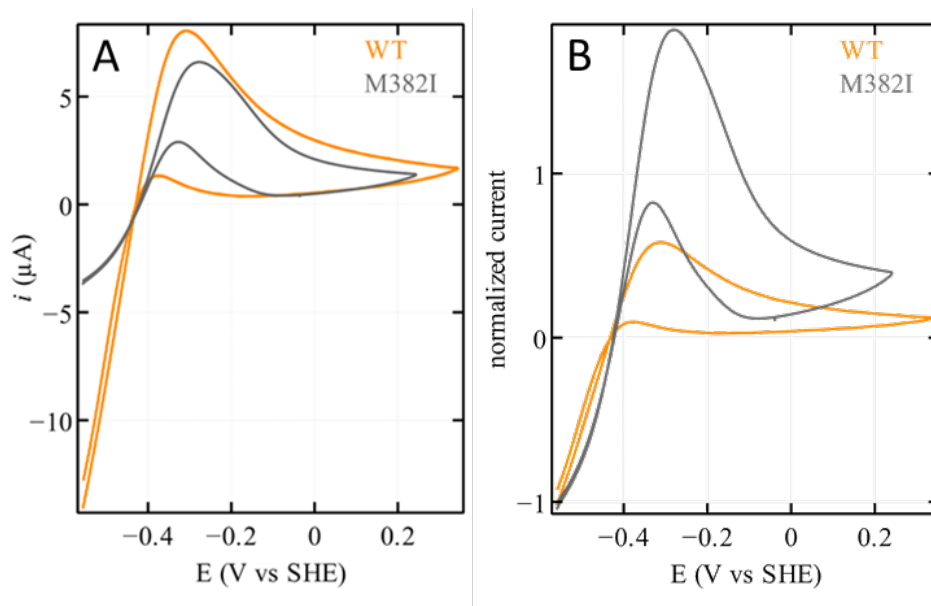


Figure S7: Cyclic voltammogram traces of CbA5H (orange) and the variant M382I (gray). A) raw B) normalized cyclic voltammograms of CbA5H WT and the variant M382I. Conditions: 5°C; pH 7; 3000 rpm; 20 mV/s

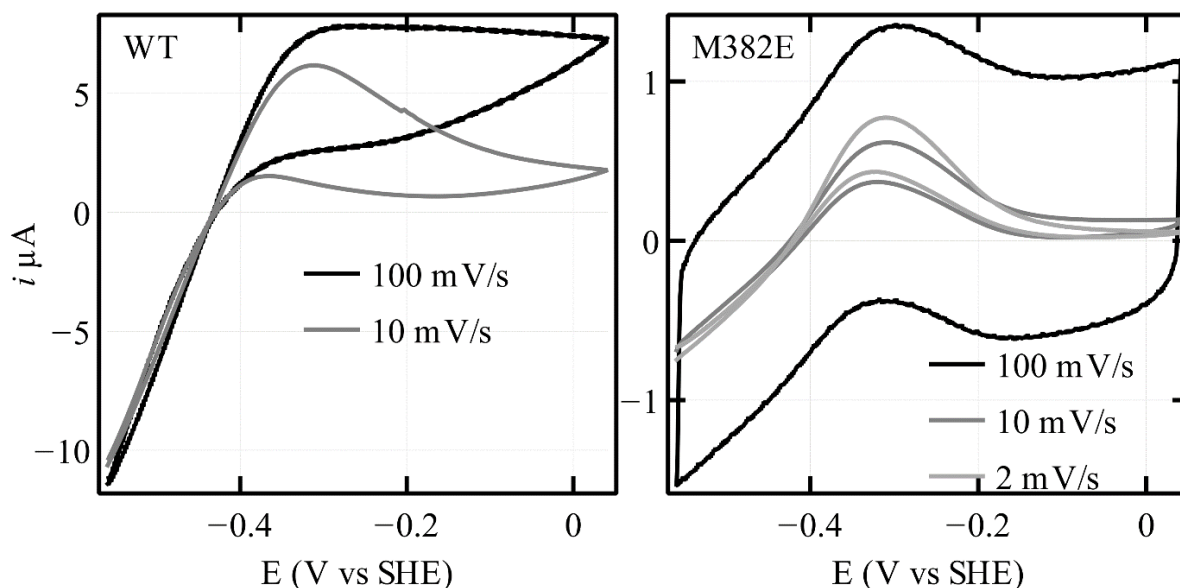


Figure S8: Cyclic voltammograms at different scan rates for CbA5H WT and the variant M382E. For the wt enzyme the dependence of oxidative inactivation on scan rate is very clear since the inactivation is slow on the time scale of the performed experiments, 100 and 10 mV/s. Sweeping the potential more slowly, a larger fraction of enzyme inactivates at high potentials and then reactivated during the reverse sweep. Increasing the scan rate leads to a smaller fraction of inactive enzyme at the end of the oxidative scan, and therefore a smaller reactivation during the backward sweep. The variant M382E inactivates and reactivates more quickly than the wt, and therefore scanning at 100 mV/s is not fast enough to significantly decrease the fraction of inactive enzyme at the end of the oxidative sweep. Scanning faster leads to a larger contribution of the capacitive current, which blurs the electrochemical response.

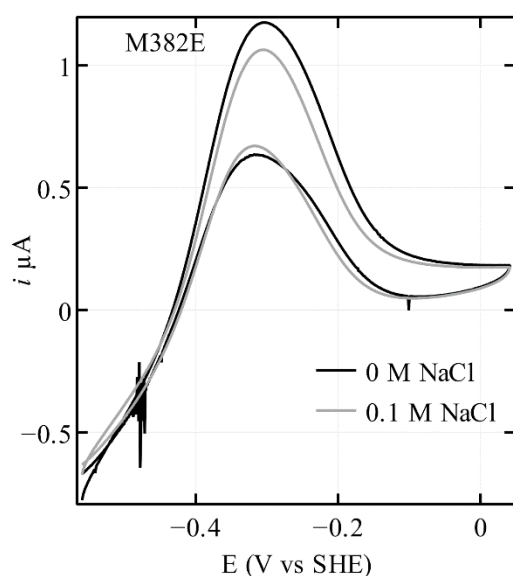


Figure S9. The anaerobic inactivation of M382E CbA5H is not due to chloride inhibition. The figure shows two consecutive scans at 20 mV/s recorded with the same film. The black trace has been recorded into a buffer containing 0.1M Na_2SO_4 as supporting electrolyte, instead of NaCl. Between the two CVs, 0.1 M NaCl was introduced in the cell (the injection at -0.5V is seen as a spike). The second scan (gray) has been recorded in the presence of 0.1M NaCl. See also fig S9 in ref (1), which demonstrates the absence of effect of chloride on the voltammetry of CbA5H WT

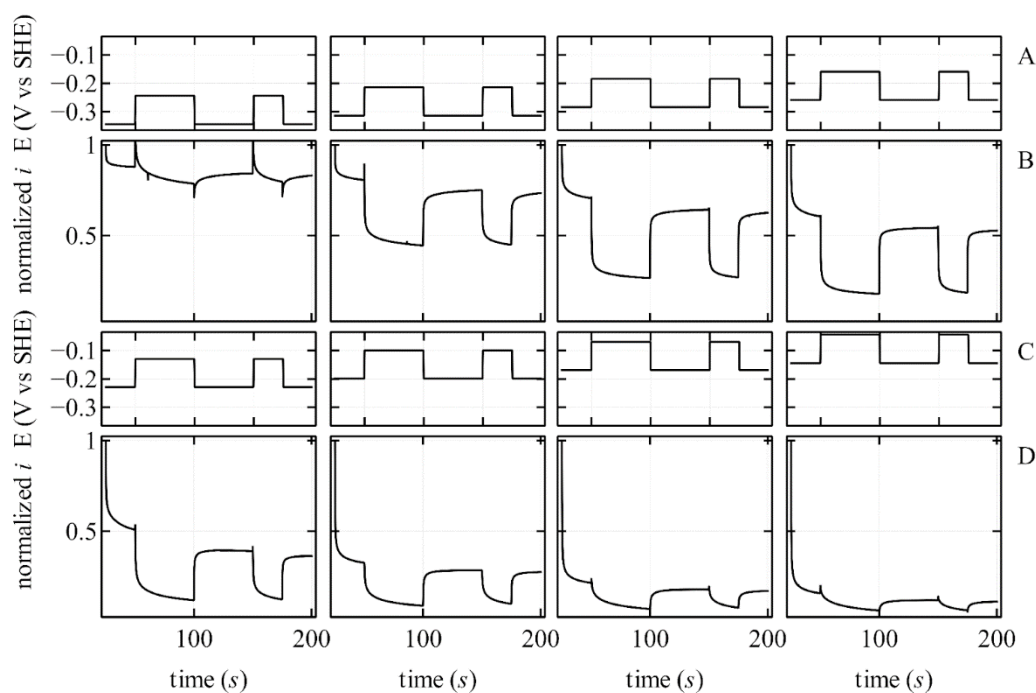


Figure S10. Chronoamperograms performed with the mutant M382E to measure the rate constants plotted in figure 6D in the main text. In these chronoamperograms (panels B and D) the potential has been stepped between 2 values always separated of 100mV (shown in panels A and C), after having completely activated the enzyme at -0.459 V vs SHE (not shown in the figure). When the potential is stepped up toward more oxidative values a fraction of enzyme inactivates at a certain rate, which depends on both the processes of inactivation and reactivation occurring at the same time at that potential, and the current therefore decreases as a function of time. Then the potential is stepped 100 mV up, triggering further inactivation. During the second step the potential goes back to the first value, triggering reactivation of the enzyme fraction that was inactivated in the previous step. Looking at the experiments from left to right the potential window (of always 100mV) shifts towards more oxidative values. The experiments at less oxidative potential (left panels in row A and B) show little inactivation followed by fast and complete reactivation. Shifting the potential window of the steps towards higher potentials lead to more pronounced inactivations, always followed by fast and complete reactivations. Shifting the potential window to even more oxidative values (row C and D, from left to right) lead to a more pronounced first inactivation, meaning that a larger fraction of enzyme is now inactive, and therefore, during the following potential steps the fraction of enzyme that inactivates/reactivates is smaller and smaller.

Fitting these 8 chronoamperograms (together with others at intermediate potentials) at the same time, with the kinetic model shown in figure 7 in the main text, allows the determination of the rate constants plotted in figure 6D.

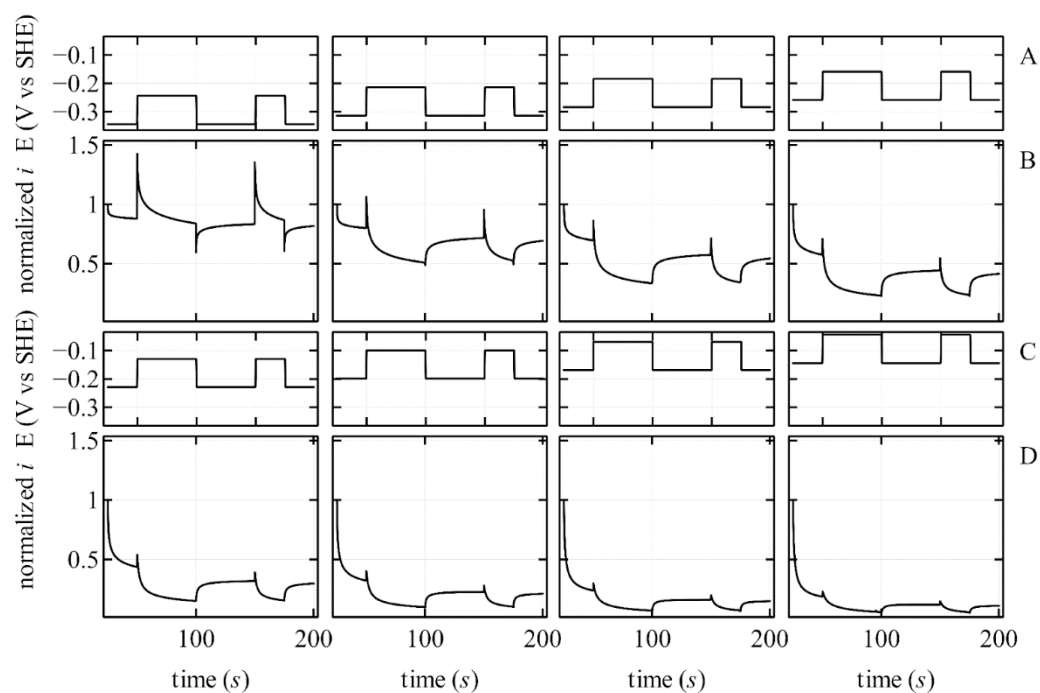


Figure S11 chronoamperograms performed with the mutant M382I, to measure the rate constants plotted in figure S12D. At every potential step, inactivation and reactivation occur more slowly than the respective experiments with the variant M382E, which is consistent with the slower rate constants plotted in figure S12D.

Supplementary Table S3. Values of k_1 , k_{-1} , k_{react} , k_{inact} for CbA5HWT and variants. Measured at pH7, 5°C. All rate constants are potential independent, except k_{react} which is given here at $E = -199$ mV. The value of k_1 is the least well-defined in the fitting procedure (as observed previously¹) and was fixed to the WT value in this analysis. The WT data are replotted from ref. (1).

	k_1 (s^{-1})	k_{-1} (s^{-1})	k_{inact} (s^{-1})	k_{react} (s^{-1})	$k_{\text{inact}}/k_{\text{react}}$
CbA5H^{WT}	0.061	0.016	0.42	0.17	2.5
M382I	0.062	0.016	0.59	0.20	3
M382E	0.096	0.016	2.45	0.81	3

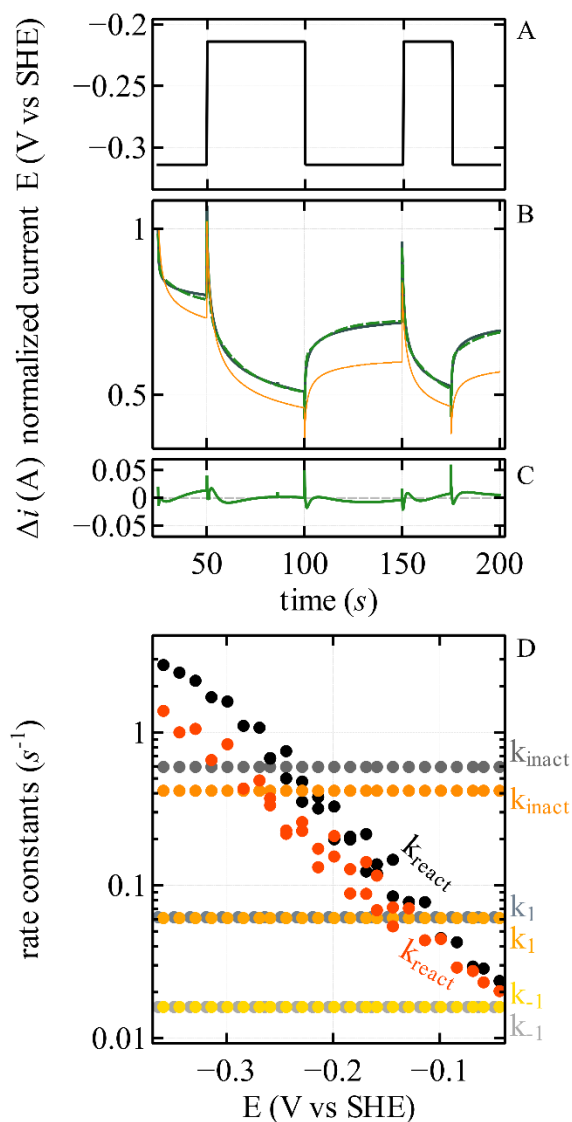


Figure S12: Potential steps experiments used to quantify the anaerobic inactivation kinetics of CbA5H and the M382I variant. Panel A: an example of potential steps applied to the RD PGE electrode onto which the enzymes are adsorbed. Panel B: the current responses (orange: WT, gray: M382I) to the potential steps in panel A. The best fit of the model is described earlier and is shown in dashed green.¹ Panel C: residual of the fit. Panel D: the four rate constants determined by fitting the model to a series of chronoamperograms obtained with different potential steps. Three rate constants are potential independent and shown with darkorange, orange and yellow circles (k_{inact} , k_1 and k_{-1} , respectively) for the WT, and in gray, slate gray and darkgray (k_{inact} , k_1 and k_{-1} , respectively) for the mutant. k_{-1} is ill-defined and set to the same value for both enzymes. The values of k_{react} are shown as red and black circles for the WT enzyme and for the mutant, respectively. Experimental conditions: T=5°C, pH 7, 3000 rpm. The WT data are replotted from ref. (1).

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