A safety cap protects hydrogenase from oxygen attack

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[FeFe]-hydrogenases are efficient H₂-catalysts, yet upon contact with dioxygen their catalytic cofactor (H-cluster) is irreversibly inactivated. Here, we combine X-ray crystallography, rational protein design, direct electrochemistry, and Fourier-transform infrared spectroscopy to describe a protein morphing mechanism that controls the reversible transition between the catalytic Hox-state and the inactive but oxygen-resistant Hₐct-state in [FeFe]-hydrogenase CbASH of Clostridium beijerinckii. The X-ray structure of air-exposed CbASH reveals that a conserved cysteine residue in the local environment of the active site (H-cluster) directly coordinates the substrate-binding site, providing a safety cap that prevents O₂-binding and consequently, cofactor degradation. This protection mechanism depends on three non-conserved amino acids situated approximately 13 Å away from the H-cluster, demonstrating that the 1st coordination sphere chemistry of the H-cluster can be remote-controlled by distant residues.
[Fe]-hydrogenases catalyze the reversible reduction of protons to dihydrogen at low over-potential and high turnover rates1-3. Their catalytic cofactor consists of a [4Fe-4S]-cluster ([4Fe4S]4+) that is covalently bound to a dirion complex ([2Fe2]H) by a bridging cysteine4. The two Fe-sites of the [2Fe2]H complex, referred to as proximal (FeP) and distal (FeD) according to their distance to the [4Fe4S]-cluster, are coordinated by three carbon monoxide (CO) and two cyanide (CN-) ligands. They exhibit strong vibrational absorption signals at discrete infrared frequencies, which respond to changes in redox and protonation states of the H-cluster5-7. The diatomic ligands are responsible for the low-spin state of the [2Fe2]H sub-cluster and stabilize it in a configuration that creates an open coordination site at FeD, allowing the binding and heterolytic splitting of dihydrogen8,9. The open coordination site at FeD is also the major target of inhibitors such as CO and dioxygen10-14. The pending bridgehead amine-group of the azadithiolate-ligand which connects FeP and FeD in the [2Fe2]H-cluster shuttle protons between FeD and a nearby located cysteine residue (C367 in CbA5H), which is part of the highly conserved proton transfer pathway15-18.

Dioxygen irreversibly damages the H-cluster of most [FeFehydrogenases10-12,14. After reaching the active center by diffusion via packing defects11,19-21, O2 binds to the open coordination site at FeD, forming a transient adduct which is further transformed by successive protonation and reduction steps21,31,22-23. To a limited extent it can be fully reduced to water, partial reduction and protonation however, lead to the production of reactive oxygen species (ROS) that cause H-cluster destruction21,11,22,26-29. To the best of our knowledge, among the [FeFe]-hydrogenases that have been characterized so far, only the recently isolated CbA5H from Clostridium beijerinckii, a gram-positive, anaerobic bacterium isolated from soil and feces, has been shown to resist long-term exposure to O2 by reversibly switching from the active oxidized ready state Hox to the O2-protected but inactive H-cluster state Hinact27. This could be demonstrated by monitoring the spectroscopic signature of the enzyme that is repeatedly oxidized in air and reduced by H2 (ref. 27 and Supplementary Figure 1). The Hinact-state was originally identified as the ‘as-isolated’ form of [FeFe]-hydrogenase DdH from Desulfovibrio desulfuricans after homologous expression and aerobic purification28-29. Hinact provides full protection against O2 prior to the first reductive activation to Hox and can be identified spectroscopically by a characteristic set of IR-signals. However, DdH becomes O2-sensitive after gaining catalytic activity10,19. For DdH and some other [FeFe]-hydrogenases, sulfide-addition under oxidizing conditions (either aerobic or anaerobic) also produces the Hinact-state; the anaerobic formation of Hinact is detected in cyclic voltammetry by the early onset of oxidative inactivation30,31. Rodriguez-Maciá et al. concluded that Hinactformation under oxidizing conditions is the consequence of sulfide-binding to FeD30, but this protected state is also formed in CbA5H27,31 and CpIII ([FeFe]-hydrogenase III from Clostridium pasteurianum)32 in the absence of exogenous sulfide. The sulfide-independent, reversible transition between Hinact and the inactive, O2-resistant Hinact-state is therefore an uncharacterized and intriguing feature of certain [FeFe]-hydrogenases, which significantly enhances their utilization potential (Fig. 1; CbA5H).

In this work, we elucidate the mechanism that protects CbA5H from dioxygen employing a multidisciplinary approach. We describe the structural rearrangement of a polypeptide-loop close to the active site that attributes a ‘safety cap’ function to the conserved cysteine at position 367. It shields the open coordination site of FeD from O2 by enabling the thiol-group to reversibly bind to FeD, thus providing an intrinsic source for the protective sulfide-ligand.

Results
Crystal structure of O2-exposed CbA5HWT. To uncover the structure of the O2-protected enzyme state, we crystallized wild-type CbA5H (CbA5HWT) under aerobic conditions (CbA5Hair) (for details on the crystal and overall structural features see Supplementary Discussion 1, Supplementary Figs. 2-5 and Supplementary Tables 1, 2). The overall structure of the H-cluster domain of CbA5Hair is similar to that of standard [FeFe]-hydrogenases like CbA5H and DdH4,33. Interestingly, it embeds the H-cluster with nearly full occupancy (>90%) (Supplementary Table 2); this stability of the active site in the air-exposed crystal contrasts with the observation that the H-cluster of standard [FeFe]-hydrogenases is destroyed under air26. Since we could not crystallize CbA5H under anaerobic conditions, we compared the structures of CbA5Hair and CbA5HR, a “standard” [FeFe]-hydrogenase. The structure of CbA5Hair shows localized structural differences with anaerobically purified CbA5H (PDB ID: 4XDC9 (Supplementary Figure 3)). The peptide-loop that spans T365, S366, and C367 in CbA5H (hereafter “TSC-loop”), corresponding to T297, S298, and C299 in CpI) is shifted from the conformation observed in the structures of CbA5H, DdH, or HydA1 (Fig. 2e and Supplementary Figure 5). The bulky side chain of the strictly conserved tryptophan 371 (W303 in CpI), adjacent to the TSC-loop, exhibits a different conformation in CbA5Hair, hinging away from the loop. The alpha helix carrying this residue is slightly shifted away from the H-cluster. Consequently, the orientation of the residue of the conserved cysteine C367 in CbA5Hair is different from that of the corresponding side chain in standard [FeFe]-hydrogenases (Fig. 2d, e and Supplementary Figs. 3 and 5). This observation is consistent with a recently published hypothesis according to which the loop that bears C367 in CbA5H may be flexible enough to allow the binding of the cysteine to FeD31. The strictly conserved cysteine residue is involved in long-range proton transfer to and from the H-cluster (Supplementary Figure 6)14,16,18. In CbA5Hair, the distance between the sulfur atom of C367 and FeD is only 3.1 Å, compared to 5.9 Å in CpI (Fig. 2e) indicating bond-formation. The length of this C367-FeD bond is longer than the average length of 2.4 Å.

Fig. 1 The unusual O2-resistance of [FeFe]-hydrogenase CbA5H. The H-cluster of CbA5H and other [FeFe]-hydrogenases is irreversibly destroyed when exposed to O2. CbA5H is a rare exception, as it reversibly converted into an inactive but O2-protected state (Hinact), even in the absence of exogenous sulfide. From the O2-protected state, CbA5H can be reactivated by reduction under anaerobic conditions. ROS: reactive O2-species resulting from O2-activation after FeP-binding. Green bar: unknown feature or mechanism, protecting the H-cluster of CbA5H from O2-attack in Hinact.
for a covalent bond between iron and sulfur, but short enough to prohibit the insertion of any molecule (including H₂ and O₂) between the sulfur atom and Fe₅. This observation suggests that the structure of CbA₅Hair is that of an inactive state and explains why the H-cluster is stable under air. That the infrared spectrum of air-exposed CbA₅HWT (Fig. 3c and Supplementary Figure 1) shows the typical signature of the H₅act state suggests that the X-ray structure of CbA₅Hair, with C₃₆₇ attached to Fe₅, is actually that of H₅act.

The crucial role of C₃₆₇ for H₅act-state formation. We compared the properties of CbA₅HWT and two site-directed variants in which C₃₆₇ is replaced with either aspartate or alanine. Below, we demonstrate that each of the three distinctive properties of CbA₅HWT is dependent on the presence of a cysteine at position 367: (1) the non-standard anaerobic oxidative inactivation which occurs at low oxidative potential, (2) the resistance to O₂ that results from this inactivation, and (3) the reversible formation of the H₅act-state upon exposure to O₂. In standard [FeFe]-hydrogenases, the replacement of the conserved cysteine in the proton transfer pathway with aspartate is the only substitution that preserves a significant level of enzyme activity. Consistent with previous results with CpI and HydA₁, we observed that replacing C₃₆₇ with alanine renders CbA₅H inactive while variant C₃₆₇D retains 20% of the H₂-production activity measured for CbA₅HWT and exhibits a shifted pH-optimum (Supplementary Figure 7).

H₅act can be accumulated anaerobically by incubating CbA₅HWT with oxidants (DCIP or thionine). Such oxidative treatment can be mimicked in protein film electrochemistry (PFE) experiments by applying high enough electrode potentials as a result of anaerobic, oxidative

Fig. 2 X-ray structure of CbA₅HWT crystallized under aerobic conditions (CbA₅HWT). a Cartoon structure of CbA₅HWT (PDB ID: 6TTL, chain A; see Supplementary Figure 2 for the homodimer). H- and F-domain are colored in yellow, the N-terminal SLBB domain (soluble ligand binding β-grasp) is presented in green. b Anomalous electron density map, depicting the positions and distances between iron-sulfur clusters, including the H-cluster (stick model), the two F-clusters (FS₄A and FS₄B) and the additional N-terminal cluster within the SLBB domain (FS₄*) located more than 20 Å away from any other cluster. c Potential cluster coordination site within the SLBB domain, consisting of 3 cysteine and one histidine ligand. d, e CbA₅HWT exhibits a characteristic structural displacement of the peptide loop containing C₃₆₇ (TSC-loop) nearby the [2Fe]₅ cluster. d The omit map (Fo-Fc) was contoured at 2σ (see Supplementary Fig. 17 for a more detailed structural comparison between the loop regions of CpI and CbA₅HWT). e Structural alignment, depicting conformational differences between CbA₅HWT (yellow) and CpI (white). Panels d and e focus on the [2Fe]₅ cluster and side chains of amino acids which influence anaerobic inactivation, O₂-resistance, and H₅act formation. f Selected parts of an amino acid sequence alignment of CbA₅HWT and CpI, including the polypeptide positions that influence TSC-loop reconfiguration and H₅act state formation in CbA₅HWT.
inactivation, as recently observed in another electrochemical investigation of CbASH\textsuperscript{31}. This inactivation at relatively low potential is reminiscent of the shift in the onset of anaerobic inactivation to lower potentials observed for DdH and HydA1 in the presence of exogenous sulfide\textsuperscript{30}. When subsequently sweeping the potential down, a faint increase in current, starting below \(-0.25\) V, reveals the beginning of enzyme reactivation, which is complete only when the potential reaches \(-0.6\) V. The C367D exchange causes a striking effect on the voltammetry (blue trace in Fig. 3a): the onset of inactivation occurs at a significantly
higher potential than for CbA5HWT, thus restoring a "standard" voltammetric signature. The latter is illustrated for CbA5HWT and standard hydrogenases and CbA5H-variant C367D on the one hand, and CbA5HWT on the other hand, therefore, result from distinct mechanisms.

To quantify and compare the O₂-sensitivity of CbA5HWT and variant C367D, we employed a PFE chronoamperometric procedure where the enzyme is exposed to O₂ under oxidizing conditions, and the level of activity is measured under reductive conditions before and after exposure to O₂ (Fig. 3b). The measured catalytic H₂-evolution currents were normalized by the initial value recorded at the end of the 1st step at low potential (−0.3 V). Upon switching the potential to 0 V, CbA5HWT instantly inactivates whereas C367D retains >95% of its H₂-oxidation activity. Injecting 50 μM O₂ into the electrochemical cell fully inhibits the C367D variant. After removal of O₂ from the system by a five-fold buffer exchange and subsequently shifting the potential back to −0.8 V, CbA5HWT returns to its original H⁺ reduction activity, unlike variant C367D, which appears to have been completely and irreversibly inactivated by O₂-exposure. These experiments demonstrate that anaerobic inactivation at relatively low potential and resistance to O₂-induced damage both depend on C367D.

As observed by IR-spectroscopy, exposure of CbA5HWT to air induces a quantitative transition from Hox to Hinact (Fig. 3c and Supplementary Figure 1)⁴⁷. In contrast to wild-type enzyme, the O₂-treated variants C367D and CbA5HWT exhibit none of the IR-vibrational signals characteristic of the H_inact state (Fig. 3c and Supplementary Figure 10). Under N₂ atmosphere, the C367D variant exhibits strong H-cluster signals which demonstrate a mixture of Hₐ and Hox-CO. O₂-treatment of C367D leads, beside a small fraction of the inert Hox-CO state (indicative of H-cluster degradation as in ref. ⁴⁵), to a fast and nearly complete loss of the H-cluster specific IR-vibrational signals; this suggests that substantial cofactor degradation occurs, as observed for standard [FeFe]-hydrogenases²⁴-²⁶ (Supplementary Figure 10 and Supplementary Discussion 2 for the corresponding IR-spectroscopy data of variant C367A).

We have therefore shown that (1) anaerobic inactivation, (2) O₂-resistance, and (3) aerobic formation of H_inact all occur due to the presence of C367D which according to our structure of CbA5H₈HWT binds to Fe₃ in the H_inact state. This saturates the coordination sphere of Fe₃ and thus prevents the binding of substrate (H₂) and inhibitor (O₂) (Fig. 2d-e), rendering the enzyme inactive but protected from O₂-induced degradation. That CbA5H is quantitatively locked in the H_inact configuration is not only obvious from the homogenous IR-spectra of O₂ treated enzyme but also from the low b-factor values observed for the H-cluster environment in CbA5H₈HWT (6TTL) including the fully shifted TSC-loop (Supplementary Fig. 16).

That both, aerobic and anaerobic oxidative conditions induce the formation of H_inact suggests that the sensing of oxidative conditions occurs via the two accessory [4Fe-4S]-clusters (FS4B and FS4A in Fig. 2b). They mediate electron transfer between external redox partners and the H-cluster and can be oxidized either by the electrode at high potential or by transferring electrons to molecular oxygen. This reaction with O₂ appears to be reversible.

Kinetics and mechanism of reversible anaerobic inactivation. We examined the kinetics and potential-dependence of anaerobic (in)activation, which can be quantitatively probed using PFE by potential-step experiments⁴⁰. Figure 4a shows a typical sequence of steps and the resulting change in current. Each initial current peak (e.g., at 50 or 100 s) results from the turnover frequency of the fully active enzyme instantly changing after the potential is stepped up or down; the subsequent slow change in current results from the accordingly slow change in the concentration of the H_inact state. The data can be interpreted by assuming various kinetic models, which we tested by fitting them to the corresponding current traces⁴¹. A model that considers just two species (corresponding to the cysteine being either unbound or bound to Fe₃) and resulting in mono-exponential changes in current after each potential step is not satisfactory (green trace in Fig. 4a): the kinetic traces are multiphasic, and any good kinetic model must therefore include the conversions between at least three species (red trace in Fig. 4a). After having analyzed the data recorded at different potentials and pH values, we concluded that the simplest good model is the following, where A means "active", and A₁ and A₂ are two distinct active species.

\[ A \underset{k_1}{\overset{k_2}{\rightleftharpoons}} A_1 \overset{k_{react}}{\underset{k_{inact}}{\rightleftharpoons}} H_{inact} \]

Fig. 4a shows the fit (red dotted line) of the above model to the chronoamperometric data, from which the values of the four rate constants can be determined at the two potentials used in the experiment. (see Supplementary Notes on the kinetic modeling of anaerobic inactivation of CbA5H and Supplementary Figure 11).

Since the C367D mutation prevents the formation of H_inact and gives back CbA5H standard catalytic properties, we assume that the structure of the active form A₁ of CbA5H is similar to that of standard hydrogenases, and that the difference between the structures of CbA5H and CbA5HWT shown in Fig. 2e illustrates the conformational change that occurs upon the formation of H_inact from the active form A₁ of the enzyme. The conversion between the active forms A₁ and A₂ occurs on the time scale of seconds (1/(k₁ + k⁻¹) = 10 s) which implies that there must be a large activation energy barrier to overcome, probably due to a conformational change. In state A₂, C367 cannot be bound to Fe₃, since A₂ is still active (about half as active as A₁,
Three distal residues determine reversible \( H_{\text{inact}} \)-formation.

The formation of \( H_{\text{inact}} \) as a result of cysteine binding to \( F_{\text{ed}} \) must therefore occur in the final step (A2 to I). That this bond is labile \((1/(k_{\text{inact}} + k_{\text{react}}) \approx 1 \)s at high potential) is consistent with the relatively long distance of 3.1 Å between \( F_{\text{ed}} \) and the S-atom of C367 observed in the structure of air-oxidized CbA5H\(^{WT}\). The reactivation from \( H_{\text{inact}} \) to A2 is the only step whose rate constant \((k_{\text{react}})\) depends on pH and the applied potential, showing that reactivation is triggered by a reduction step coupled to a protonation. At high potential, the reactivation rate constant \( k_{\text{react}} \) is lower than the inactivation rate constant \( k_{\text{inact}} \), locking down the enzyme in the \( H_{\text{inact}} \) state. The fact that \( k_{\text{inact}} \) is potential-independent points to a classical “CE” mechanism for step 2, in which one or a series of chemical step(s) (“C”) precede(s) proton-coupled oxidation (“E”). Strong coupling implies that the sites of deprotonation and oxidation are very close to one another. We therefore consider likely that this deprotonation occurs from the nitrogen atom of the azaditiolate bridge, or from the bound cysteine, to produce a thiolate ligand.

Supplementary Figure 12). The binding of the cysteine sulfur to \( F_{\text{ed}} \) must therefore occur in the final step (A2 to I). That this bond is labile \((1/(k_{\text{inact}} + k_{\text{react}}) \approx 1 \)s at high potential) is consistent with the relatively long distance of 3.1 Å between \( F_{\text{ed}} \) and the S-atom of C367 observed in the structure of air-oxidized CbA5H\(^{WT}\). The reactivation from \( H_{\text{inact}} \) to A2 is the only step whose rate constant \((k_{\text{react}})\) depends on pH and the applied potential, showing that reactivation is triggered by a reduction step coupled to a protonation. At high potential, the reactivation rate constant \( k_{\text{react}} \) is lower than the inactivation rate constant \( k_{\text{inact}} \), locking down the enzyme in the \( H_{\text{inact}} \) state. The fact that \( k_{\text{inact}} \) is potential-independent points to a classical “CE” mechanism for step 2, in which one or a series of chemical step(s) (“C”) precede(s) proton-coupled oxidation (“E”). Strong coupling implies that the sites of deprotonation and oxidation are very close to one another. We therefore consider likely that this deprotonation occurs from the nitrogen atom of the azaditiolate bridge, or from the bound cysteine, to produce a thiolate ligand.

Three distal residues determine reversible \( H_{\text{inact}} \)-formation.

The formation of \( H_{\text{inact}} \) as a result of cysteine binding to \( F_{\text{ed}} \) is a unique property of CbA5H, and yet C367 and the other residues of the shifted TSC-loop are strictly conserved (Supplementary Figure 5). Structural differences in their environment must therefore account for the loop-flexibility that enables the translocation of C367 in CbA5H\(^{WT}\). The most prominent differences between standard hydrogenases and CbA5H\(^{WT}\) near the TSC-loop concern the residues at positions 364 (leucine), 561 (alanine) and 386 (proline) (Fig. 2e, f and Supplementary Figure 5): their backbones align with those of their counterparts in standard hydrogenases, but the latter exhibit bulkier residues.

To assess whether the residues at these positions determine the possibility of a conformational change and are responsible for the unique properties of CbA5H, we produced the CbA5H-variants L364F, A561F, P386L and the double-exchange variant L364F-A561F, and characterized them using the same combination of experiments as described above. Only variant P386L shows a reduced \( H_{2} \)-evolution activity (55% compared to CbA5H\(^{WT}\), Supplementary Figure 7).

The cyclic voltammograms recorded with the four variants show reversible anaerobic inactivation, but at a significantly higher potential than observed for CbA5H\(^{WT}\) (Fig. 3d). We could fit the above kinetic model to the chronoamperometric data recorded with each of the mutants; the results in Table 1 confirm that the mutations hinder the A1 to \( H_{\text{inact}} \) conversion and inform about their individual effects on each of the two steps. The L364F and P386L substitutions decrease \( k_{\text{inact}} \) and increase \( k_{\text{react}} \) (the P386L substitution also increases \( k_{-1} \)). The A561F substitution merely reaches 34% of the activity (Table 1). The A561F substitution only significantly affects the 1st step (A\(_1\) to A\(_2\)): it decreases \( k_{1} \) and increases \( k_{-1} \) (Table 1). The double-exchange variant L364F-A561F combines the individual effects of the two single exchanges.

Figure 3e shows that each of the substitutions also decreases \( O_{2} \)-resistance. Each of the variants exhibited some residual level of activity just after the first potential step from \(-0.8 \) V to 0 V, followed by an inactivation that is slower than that of CbA5H\(^{WT}\). After buffer exchange and the final step to \(-0.8 \) V, the residual activities of all single-exchange variants are down to 76–64% and the double-exchange variant merely reaches 34% of the activity recorded prior to \( O_{2} \)-exposure, compared to 100% for CbA5H\(^{WT}\) (also in Fig. 3b). These results show that resistance to \( O_{2} \) correlates with the overall rate of formation of the inactive state.

**Table 1 Values of** \( k_{1}, k_{-1}, k_{\text{inact}}, k_{\text{react}} \) **(the latter at \(-0.196 \) V, all at \( 5^\circ \)C) for CbA5H\(^{WT}\) and variants.**

|                  | \( k_{1} \) (s\(^{-1}\)) | \( k_{-1} \) (s\(^{-1}\)) | \( k_{\text{inact}} \) (s\(^{-1}\)) | \( k_{\text{react}} \) (s\(^{-1}\)) |
|------------------|-----------------|-----------------|-----------------|-----------------|
| WT (pH 10)       | 0.095           | 0.019           | 0.65            | 0.015           |
| WT (pH 8.5)      | 0.068           | 0.018           | 0.54            | 0.069           |
| WT (pH 7)        | 0.061           | 0.016           | 0.42            | 0.17            |
| A561F (pH 7)     | 0.025           | 0.058           | 0.28            | 0.19            |
| L364F (pH 7)     | 0.055           | 0.017           | 0.14            | 1.45            |
| P386L (pH 7)     | 0.049           | 0.051           | 0.15            | 1.50            |
| A561F-L364F (pH 7) | 0.017         | 0.067           | 0.13            | 2.09            |
| Accuracy         | 8%              | 28%             | 7%              | 6%              |

Rate constants were obtained by analyzing kinetic data of anaerobic inactivation such as those shown in Fig. 4a with the “AAI” model (Supplementary Fig. 14 shows the dependence of \( k_{\text{inact}} \) on \( \phi \)). The CbA5H\(^{WT}\) reference for the kinetic parameters of the variant proteins, measured at pH7 is marked in bold. The % accuracy is our estimate of the maximal error for the determination of each rate constant.
under oxidizing conditions, which increases in the order WT < L364F ≈ P386L < A561F < L364F-A561F < C367D.

Monitoring the IR-signatures of the H-cluster confirms that the residual O₂-resistance of the variants results from the formation of a residual fraction of H₃act (Fig. 3f and Supplementary Figure 13). Prior to O₂-treatment, all variants show the signature of the Hox state, with minor contributions from Hox-CO. However, unlike the case of CbASHWT (Fig. 3c), these signals disappear upon exposure to air. The only remaining signals after O₂-treatment result from Hox-CO and a small fraction of Hinact bound cysteine. This oxidation step should favor cluster oxidation and deprotonation (probably of the α-subunit cysteine). This oxidation step finally locks down the enzyme in the Hinact-state.

The protective function of the thiolate group described here is analogous to that of exogenous sulfide demonstrated for several standard [FeFe]-hydrogenases. Sulfide, the main product of the sulphate metabolism of D. desulfuricans, could be trapped near the H-cluster and be quickly relocated under oxidative conditions to the open coordination site of Fe₂ (red) and Ser (blue) in its original position of state A₁ in Fig. 5a) and of the alpha helix that bears it (Fig. 5b, c; step 2). The substitution L364F favors state A₂ over Hinact (Table 1) because it removes the slight steric clash between L364 and the W371 in state A₂ (Fig. 5b, blue Trp). The conformational shift of W371 in step 2 should be slowed when P386 is replaced with leucine (see clashes between W371 of CbASHWT and L318 of Cpi in Fig. 5b), which is consistent with the transition from A₂ to Hinact being disfavored in variant P386L (Table 1). Step 2 brings C367 close enough to Fe₂ to allow its coordination (Fig. 5c, step 3), which should favor cluster oxidation and deprotonation (probably of the bound cysteine). This oxidation step finally locks down the enzyme in the Hinact-state.
than merely providing a cofactor cavity that stabilizes and tunes the properties of the active site, and substrate/product pathways. The intrinsic cofactor protection mechanism of ChA5H is an illustrative showcase for the level of complexity that such additional contributions may reach under corresponding evolutionary pressure.

**Methods**

**Protein preparation.** The cDNA used for heterologous overexpression of ChA5H was codon optimized for *Escherichia coli* strain K12 and synthesized with a C-terminus tag (see Supplementary Table 1). The resulting plasmid was transformed into E. coli strain BL21 (DE3) for the overproduction of the protein. After induction, the E. coli cells were harvested by centrifugation and suspended in PrePac lysis buffer (Promega). The cell pellets were disrupted by lysozyme treatment, and the resulting crude extract was treated with sonication, followed by centrifugation at 15,000 x g for 30 minutes at 4°C. The supernatant was collected and stored at −80°C until further use.

**Crystallization and structure determination.** Despite the strictly anaerobic handling of ChA5H, the protein crystallized as a ferric monomer and a ferrous monomer in separate crystals. Crystals of the ferric monomer were grown in hanging drops consisting of 2% (v/v) PEG 3350, 100 mM Tris-Cl (pH 8.5), and 0.1 M NaCl. Crystals of the ferrous monomer were grown in hanging drops consisting of 2% (v/v) PEG 3350, 100 mM Tris-Cl (pH 8.5), and 0.1 M NaCl. The crystals were then harvested and flash-frozen in liquid nitrogen before being cryoprotected in a cryoprotectant solution containing 28% PEG 400 and 10% glycerol.

**In vitro activity assays.** To perform H2-production activity assays, 400 ng enzyme was mixed with 100 mM NaDT (sodium dithionite) as sacrificial electron donor and 10 mM MV (methyl viologen) as electron mediator in 100 mM H2PO4/KH2PO4 buffer (pH 6.8). The sealed reaction vessel was kept at 37 °C in a shaking incubator (100 rpm) for 1 hour. The headspace of the reaction tube via gas chromatography (Shimadzu) was analyzed for the H2-oxidation current, we subtracted the background capacitive currents were between 1- and 10 µA, so that mass transport towards the rotating electrode was not limiting. The recorded spectra were baseline corrected via OPUS (Bruker GmbH) and then analyzed by analyzing the H2-oxidation current, we subtracted the background capacitive currents were between 1- and 10 µA, so that mass transport towards the rotating electrode was not limiting. For the chronoamperometric experiments, a potential of 0.8 V vs SHE was used as a counter electrode. The reference potential was measured using a 1 M NaCl (solute side) and a saturated calomel electrode (SCE) as the reference electrode.

**Electrochemical experiments**

**Cyclic voltammetry**

**Chromoamperometry**

**Chronoamperometry**

**Data availability**

The coordinates and structure factors of ChA5H have been deposited in protein data bank (PDB) under PDB-ID: 6T7L. All data are available in the main text or the supplementary materials. Further data supporting findings of this study are available from the corresponding authors upon reasonable request. Further publicly available datasets used or indicated in this study comprise PDB-ID: 4XDC, PDB-ID: 3LX4 and PDB-ID: 1HFE. Source data are provided with this paper.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

T.H., M.W. and C.L. conceived and supervised the experiment, which was initiated during a collaboration between C.W.G and F.V. D.J.K., N.S. and S.E. were responsible for recording cyclic voltammograms of the protein and kinetic modeling. A.R. crystallized the protein and J.D. and E.H. solved the structure. J.D., M.W. and E.H.
performed structural analysis. M.W. and J.D. designed the mutagenesis variants. L.S. and J. J. purified the protein and measured the activity. M.W., O.L., J.I. and J.D. carried out the chronoamperometry experiment. A.B. and L.S. performed the ATR-FTIR experiments. U.P.A. synthesized the [2Fe]H complex for in vitro maturation. M.W., C.L., T.H., J.D. and C.F. wrote the manuscript with input from other coauthors. All authors discussed and commented on the manuscript.

**Competing interests**
The authors declare no competing interests.

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