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Formaldehyde—A Rapid and Reversible Inhibitor of Hydrogen Production by [FeFe]-Hydrogenases

Annemarie F. Wait,[†] Caterina Brandmayr,[†] Sven T. Stripp,[‡] Christine Cavazza,[§] Juan C. Fontecilla-Camps,[§] Thomas Happe,[‡] and Fraser A. Armstrong*[,†]

[†]Inorganic Chemistry Laboratory, Department of Chemistry, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K.

[‡]Lehrstuhl fur Biochemie der Pflanzen, AG Photobiotechnologie, Ruhr-Universität, 44780 Bochum, Germany

[§]Laboratoire de Crystallographie et Crystallographie des Protéines, Institut de Biologie Structurale, J.P. Ebel, CEA, CNRS, Université Joseph Fourier, 41, rue J. Horowitz, 38027 Grenoble Cedex 1, France

Supporting Information

ABSTRACT: Dihydrogen (H_2) production by [FeFe]-hydrogenases is strongly inhibited by formaldehyde (methanal) in a reaction that is rapid, reversible, and specific to this type of hydrogenase. This discovery, using three [FeFe]-hydrogenases that are homologous about the active site but otherwise structurally distinct, was made by protein film electrochemistry, which measures the activity (as electrical current) of enzymes immobilized on an electrode; importantly, the inhibitor can be *removed* after addition. Formaldehyde causes rapid loss of proton reduction activity which is restored when the solution is exchanged. Inhibition is confirmed by conventional solution assays. The effect depends strongly on the direction of catalysis: inhibition of H_2 oxidation is much weaker than for H_2 production, and formaldehyde also protects against CO and O_2 inactivation. By contrast, inhibition of [NiFe]-hydrogenases is weak. The results strongly suggest that formaldehyde binds at, or close to, the active site of [FeFe]-hydrogenases at a site unique to this class of enzyme—highly conserved lysine and cysteine residues, the bridgehead atom of the dithiolate ligand, or the reduced Fe_d that is the focal center of catalysis.

Hydrogenases catalyze the very rapid interconversion of protons and dihydrogen (H_2) and have potential uses, direct or inspirational, in future energy technologies such as H_2 production.[1] Hydrogenases are unusual enzymes: the deeply buried active sites contain the biologically rare ligands CO and CN^- and must (ideally) act selectively on the lightest of chemical species. There are two main classes: [NiFe]-hydrogenases contain Ni and Fe atoms and are predominantly H_2 oxidizers, whereas [FeFe]-hydrogenases, more commonly regarded as better H_2 producers, contain a complex structure known as the "H-cluster" (Figure 1), in which two Fe atoms, bridged by a non-protein dithiolate ligand, are linked to a [4Fe-4S] cluster via a cysteine thiolate. During catalysis, the Fe (Fe_d) that is distal to the [4Fe-4S] cluster is thought to cycle between Fe(II) and Fe(I) in states known as H_{ox} and H_{red}, respectively.[2] The H-cluster and its immediate environment are highly conserved among [FeFe]-hydrogenases. Much information on hydrogenases stems from their inhibition by small ligands: notably, both CO and O_2 are

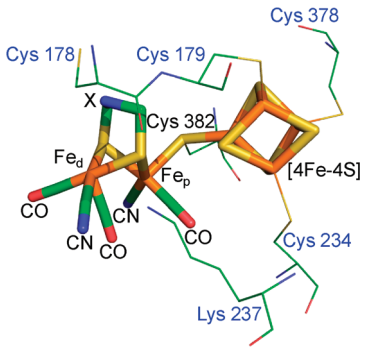

Figure 1. H-cluster of *Dd*HydAB in the reduced H_{red} state, highlighting the bridgehead atom, X (assigned as a N-atom), and conserved nucleophilic residues close to the active site (within 5 Å of component atoms) that are possible targets for formaldehyde.[7]

potent inhibitors through their ability to coordinate to low-spin d-metals by synergic σ-donor and π-acceptor (back-bonding) interactions.[3] In the case of O_2, the H-cluster is subsequently destroyed.[4,5] We now report that proton reduction by [FeFe]-hydrogenases is rapidly and *reversibly* inhibited by formaldehyde (H_2CO, methanal), an agent much more familiar as an electrophile and comparatively little reported as a ligand.[6]

Formaldehyde is a small molecule (molecular mass 30 Da) that is gaseous at room temperature. Formaldehyde dissolves in water (up to a maximum of 37% w/v, known as formalin solution) to form a diol (hydrate, K_{eqm}(hydration) $\approx$ 2000 at 25 °C[8]), which tends to form oligomers in solution. Formalin is widely used as a fixative, as it chemically modifies proteins irreversibly, although the first stages of such reactions are reversible, such as the reaction of an aldehyde with a free thiol to form a thioacetal, RS-C(OH)H_2, or with an amine to form a Schiff-base adduct, >C═N−. Formaldehyde is used to methylate lysine ($-NH_2$) residues by reducing the Schiff base with borohydride.[9]

It is difficult to demonstrate the action of formaldehyde as a *reversible* enzyme inhibitor because the aldehyde must be removed in order to check for recovery of activity. Protein film electrochemistry (PFE) solves this problem: the enzyme is immobilized onto a graphite electrode surface as an electroactive film so that the contacting solution can be exchanged, allowing

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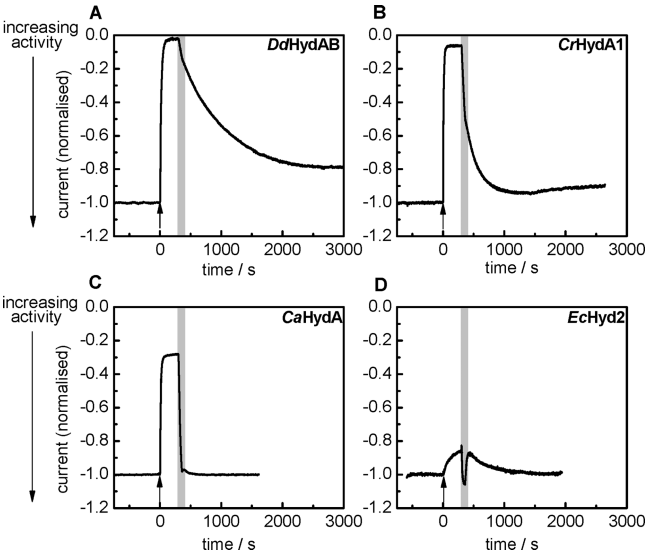

Figure 2. Inhibition of H^+ reduction by formaldehyde for various hydrogenases: (A) *Dd*HydAB [FeFe]-, (B) *Cr*HydA1 [FeFe]-, (C) *Ca*HydA [FeFe]-, and (D) *Ec*Hyd2 [NiFe]-hydrogenases. In each case, formaldehyde was injected at time = 0 s (arrow) to give a final concentration of 4.5 mM. After 300 s, the cell was rinsed with 50 mL of buffer (rinsing took ca. 100 s, gray stripe). Other conditions: electrode potential, −558 mV vs SHE; pH 6.0; 10 °C; A, B, C under 100% H_2, D under 100% N_2; electrode rotation rate, 2500 rpm.

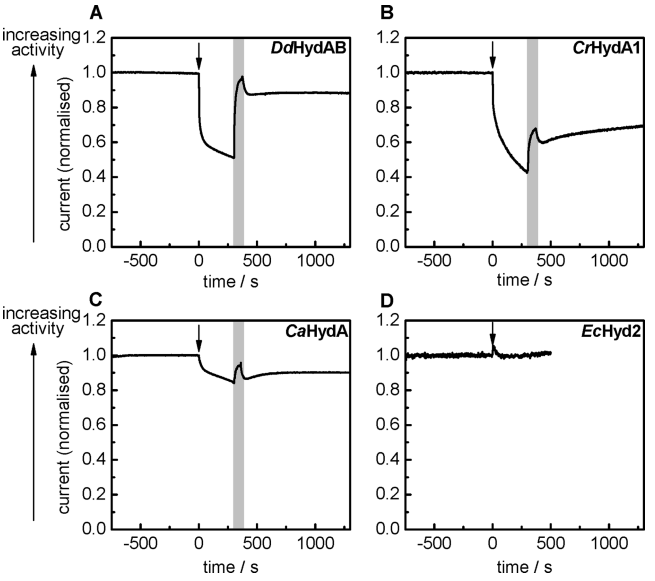

Figure 3. Inhibition of H_2 oxidation by formaldehyde for various hydrogenases: (A) *Dd*HydAB [FeFe]-, (B) *Cr*HydA1 [FeFe]-, (C) *Ca*HydA [FeFe]-, and (D) *Ec*Hyd2 [NiFe]-hydrogenases. In each case, formaldehyde was injected at time = 0 s (arrow) to give a final concentration of 4.5 mM. After 300 s, the cell was rinsed with 50 mL of buffer (ca. 100 s, gray stripe), except for panel D, where no inhibition of *Ec*Hyd2 was detected at this formaldehyde concentration. Other conditions: electrode potential, −58 mV vs SHE; pH 6.0; 10 °C; 100% H_2; electrode rotation rate, 2500 rpm.

both introduction and removal of inhibitor while the catalytic activity is recorded simultaneously as electrical current.[1,10]

The bridging dithiolate ligand in [FeFe]-hydrogenases has been definitively assigned, from HYSCORE, as a di(thiomethyl)-amine; i.e., the bridgehead atom is a N-atom.[11] In addition, the active-site region contains a highly conserved lysine close to the CN^- ligand on Fe_d (N−N distance 3.0 Å) and a cysteine that lies close to the bridgehead-N (S−N distance 3.1 Å). These residues are L237 and C178, respectively, in HydAB from *Desulfovibrio desulfuricans* (*Dd*HydAB, Figure 1).[12] Each of these amine or thiol groups is a nucleophilic center and a strong candidate for a formaldehyde target site and may be directly involved in proton transfer.[13] The reduced Fe_d in H_{red} is also a candidate, as it is implicated in the binding and redox conversion of H^+.[2]

The three [FeFe]-hydrogenases studied in this work vary significantly in quaternary structure, away from the active-site containing H-domain. Apart from the [4Fe-4S] cluster located in the H-cluster, the bacterial hydrogenases from *Desulfovibrio desulfuricans* (*Dd*HydAB) and *Clostridium acetobutylicum* (*Ca*HydA) contain additional FeS clusters ({2[4Fe-4S]} for *Dd*HydAB[12] and {3[4Fe-4S],1[2Fe-2S]} for *Ca*HydA, based on analogy with *C. pasteurianum* hydrogenase 1[14]) to relay electrons between the active site and protein surface. In contrast, the algal hydrogenase from *Chlamydomonas reinhardtii* (*Cr*HydA1) contains only the H-cluster.[13]

Pure samples of *Dd*HydAB, *Ca*HydA, and *Cr*HydA1 were prepared according to previously reported protocols.[15,16] The PFE experiments were performed as described in the Supporting Information. The temperature (10°) was chosen to minimize film loss for *Dd*HydAB which is unstable on the electrode. The all-glass cell included two inlets: one was sealed with a septum and used to inject formaldehyde solution; the other fed two tubes into the cell solution. Each of these tubes was connected to a 50 mL syringe, one empty and the other charged with buffer, equilibrated with respect to temperature and gas concentration before charging. This two-syringe assembly allowed rapid exchange of solution and thus rapid removal of formaldehyde from the cell.

Figure 2 shows electrochemical experiments in which H^+ reduction, indicated by negative current, is rapidly inhibited by formaldehyde. The electrode was poised at −558 mV, and at time = 0 s, 1 mL of 13.4 mM formaldehyde (in buffer of identical composition to that already in the electrochemical cell) was injected into the cell solution (2 mL), to give a final concentration of 4.5 mM (corresponding to ca. 2 μM anhydride). After 300 s, the cell was rinsed with 50 mL of buffer solution using the two syringes. The syringe-driven solution replacement minimized disturbance of the enzyme since it was not necessary to remove the electrode from the cell solution. The rinsing solution was saturated with H_2 or N_2 and equilibrated to the correct temperature before transferring to the syringe in order to minimize the extent of temperature change, which is the likely cause of the dips (current overshoots) frequently observed. To aid comparison, data were normalized by fitting the slow decrease in current over time ("film loss") as a single exponential. Unnormalized data for Figures 2 and 3 are shown in the Supporting Information.

For all three [FeFe]-hydrogenases, injection of formaldehyde causes a rapid loss of activity that is recovered when the formaldehyde is removed. In contrast, the [NiFe]-hydrogenase (Hyd2 from *Escherichia coli*), investigated under 100% N_2 (as H_2 is a strong inhibitor of H^+ reduction), shows a much slower and limited degree of inhibition (about 15% after 300 s). Experiments with other [NiFe]-hydrogenases showed a similarly weak response (not shown). Numerous experiments on the [FeFe]-hydrogenases under the conditions of Figure 2 established that

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inhibition of H^+ reduction is complete for *Dd*HydAB, >90% complete for *Cr*HydA1, and 70−80% complete for *Ca*HydA. Based on the extent of reaction, the affinities for formaldehyde follow the trend *Dd*HydAB > *Cr*HydA1 > *Ca*HydA. Rates of inhibition, resolved clearly with lower formaldehyde concentrations (not shown), lie in the order *Ca*HydA > *Cr*HydA1 > *Dd*HydAB, and reactions follow a clean exponential time course (for at least two half-lives). Rates of recovery lie in the order *Ca*HydA > *Cr*HydA1 > *Dd*HydAB. For *Dd*HydAB a second rinse was required to achieve nearly 100% reactivation. Binding of formaldehyde to *Dd*HydAB is thus so tight that a fraction of enzyme is still inhibited by traces remaining after the first rinse.

Analogous experiments were performed to investigate, for each enzyme, how formaldehyde inhibits H_2 oxidation. From the experiments at −58 mV, shown in Figure 3, it is clear that formaldehyde inhibits H_2 oxidation to a much lesser extent than H^+ reduction. During H_2 oxidation by the [FeFe]-hydrogenases, a fraction of the enzyme reacts rapidly with formaldehyde, seen as a fast phase of the current decrease, and this is followed by a slow phase. Upon removal of formaldehyde, some enzyme remains inactive, and the fraction of activity that is lost, after allowing for the overshoot due to temperature change, appears to correlate with the extent of the slow phase during the inhibition process. Clearly, the reaction of formaldehyde during H_2 oxidation is still fast, but binding is weaker than for H^+ reduction. For *Ec*Hyd2 [NiFe]-hydrogenase, no inhibition of H_2 oxidation activity was detected at this formaldehyde concentration.

Solution assays confirmed that formaldehyde inhibits H^+ reduction by *Dd*hydAB. Electrochemically reduced methyl viologen was used as the electron donor, and catalytic H^+ reduction was monitored over time as a decrease in absorbance of methyl viologen at 604 nm (Supporting Information). However, in contrast to the PFE experiment, reversibility could not be tested.

To further characterize the mechanism of formaldehyde inhibition, an experiment was designed that exploits the fact that *Ca*HydA releases formaldehyde rapidly. If CO binds at or near the same site as formaldehyde, removal of the latter in the presence of CO should reveal transient activity that decreases as the now-exposed site binds CO. The electrode coated with a film of *Ca*HydA was held at −558 mV vs SHE, initially under 100% N_2. In the control experiment (Figure 4A), 1 mL of N_2-saturated buffer was injected at 2500 s (black arrow). Then, at 2550 s, 0.35 mL of CO-saturated buffer was injected (red arrow); simultaneously, the gas flowing through the cell was changed to 10% CO in N_2. At 3000 s, the cell was rinsed with 50 mL of buffer pre-saturated with 10% CO in N_2.

In Figure 4B, all conditions were identical to those in Figure 4A except that the buffer injected at 2500 s contained 134 mM formaldehyde. This is a 10-fold higher concentration than used in the experiments shown in Figure 2 so that as little activity as possible remained by the time CO was introduced. At 3000 s, the cell was rinsed with buffer saturated with 10% CO in N_2. Upon rinsing the cell with a solution still containing 10% CO but no formaldehyde (interval shown in gray), the H^+ reduction activity initially recovers rapidly but then decreases. This observation demonstrates that, as soon as the formaldehyde is released from the hydrogenase, the enzyme reacts with CO that has been present throughout, thus proving that formaldehyde prevents CO binding. A related experiment with *Cr*HydA1 showed that formaldehyde also suppresses O_2 inactivation (Supporting Information).

We conclude that formaldehyde reacts rapidly and reversibly with all three [FeFe]-hydrogenases. Away from their H-domains,

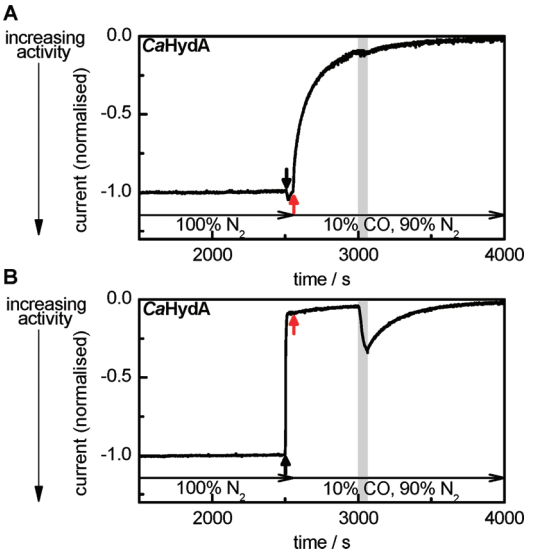

Figure 4. Experiment showing that formaldehyde protects *Ca*HydA against CO. The black arrow corresponds to injection of 1 mL of buffer in the CO-only experiment (A) and 1 mL of buffer containing 134 mM formaldehyde (B), each into 2 mL of cell solution. In both cases, the red arrow corresponds to injection of 0.35 mL of CO-saturated buffer with a simultaneous change in the gas flowing through the cell from 100% N_2 to 10% CO in N_2. In both experiments, the cell was rinsed with 50 mL of buffer (saturated with 10% CO in N_2) at 3000 s (gray stripe). Other conditions: electrode potential, −558 mV vs SHE; pH 6.0; 10 °C; electrode rotation rate, 2500 rpm.

the structures of the three enzymes diversify and the topographies differ significantly. That formaldehyde binds at or close to the active site is supported by the strong directional (potential) dependence of the inhibition, since the electronic state of the H-cluster (influenced but not set absolutely by the electrode potential under steady-state catalytic conditions) will have much less influence on a remote target site. Finally, formaldehyde protects against both CO and O_2, inhibitors that react at the active site.

We thus present compelling evidence that formaldehyde binds, reversibly, either directly at the active site or in a location that blocks access to it. Although formaldehyde can be an electron-pair donor ligand, η^2 about the C═O bond,[6] it is best characterized as an electrophile. Obvious nucleophilic targets are the bridgehead N-atom and the side chains of the conserved lysine and cysteine, as well as (unconventionally) an electron-rich Fe_d. Other possible targets are the CN^- and thiolate ligands, although these are also present in [NiFe]-hydrogenases and, as with attack on an inorganic sulfur of the [4Fe-4S]-domain, such disruption to the H-cluster would most likely be irreversible. The fact that inhibition is strongest during H^+ reduction suggests that the target is a site that becomes more available in the reduced state, H_{red}. Thus, during H_2 oxidation, where H_{ox} rather than H_{red} should prevail during the catalytic cycle, only a relatively small fraction of activity is lost rapidly and reversibly. The slow phase is barely evident under the reducing conditions used for H^+ reduction; therefore, the process responsible may be potential dependent. Although CO binds more tightly to [FeFe]-hydrogenases during H_2 oxidation, it is still a strong inhibitor of H_2 production;[5] hence, the fact that formaldehyde blocks CO binding suggests that the most likely site of attack is the distal Fe itself or the bridgehead N lying immediately above the CO binding site on Fe_d (in *Dd*HyDAB the bridgehead atom

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is 3.5 Å above Fe_d).[12] We have yet to eliminate the possibility that formaldehyde is catalytically transformed in a slow reaction.

It is most likely that formaldehyde enters the enzyme in its hydrophobic, anhydrous state (the dimensions of which are similar to a diatomic gas molecule) rather than as the hydrate: in this case the microscopic dissociation constants $K_d^{H_2CO}$ (referring to the concentration of anhydride) must be on the micromolar level, similar to that for CO.[5] The effect of formaldehyde on [NiFe]-hydrogenases is weak, possible reasons being the lack of bridgehead N-atom or conserved cysteine/lysine, or that the Fe (which remains as Fe(II)) is neither a base nor a nucleophile.

Our discovery opens up new avenues in hydrogenase research and has wider implications. One implication is that formaldehyde, as a reversible inhibitor, could be a useful mechanistic probe, such as for the role of N and S groups in proton transfer, and we are currently establishing conditions to obtain a crystal structure of the adduct. A second implication is for microbiological hydrogen metabolism, because accumulation of aldehydes could suppress H_2 evolution. In preliminary experiments with *Dd*HydAB, we have established that acetaldehyde (ethanal), an established metabolite, is also a reversible inhibitor of H_2 production, albeit with decreased affinity. The possibility of acetaldehyde inhibition of hydrogenases was investigated in the 1980s, but no conclusions were drawn.[17] Another attractive proposition is to vary the alkyl "tail length" of linear aldehydes to probe how they burrow into the enzyme. These studies are now in progress.

■ ASSOCIATED CONTENT

Supporting Information. Full Materials and Methods, un-normalized data corresponding to Figures 2 and 3 (Figures S1 and S2), assay showing formaldehyde inhibition (Figure S3), and experiment showing formaldehyde protection against O_2 inactivation (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
fraser.armstrong@chem.ox.ac.uk

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