Mechanism of Hydrogen Activation by [NiFe]-hydrogenases

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The active site of [NiFe]-hydrogenases contains a strictly conserved arginine that suspends a guanidine N-atom <4.5 Å above the Ni and Fe atoms. The guanidine headgroup interacts with the sidechains of two conserved aspartic acid residues to complete an outer-shell canopy that has thus far proved intractable to investigation by site-directed mutagenesis. Using hydrogenase-1 from *Escherichia coli*, strictly-conserved residues R509 and D574 have been replaced by lysine (R509K) and asparagine (D574N) and highly-conserved D118 has been replaced by alanine (D118A) or asparagine (D118N/D574N). Each enzyme is stable and the [(RS)$_2$Niμ(SR)$_2$Fe(CO)(CN)$_2$] inner coordination shell is virtually unchanged. The R509K variant displayed >100-fold lower activity than native enzyme. Conversely, the variants D574N, D118A and D118N/D574N where the guanidine headgroup is retained in position, showed 83%, 26% and 20% activity, respectively. The special kinetic requirement for R509 implicates the suspended guanidine group as the general base in H_2 activation by [NiFe]-hydrogenases.

INTRODUCTION

Hydrogenases catalyze the reversible oxidation of molecular H_2 with rates and efficiencies comparable to Pt, despite containing only first-row transition metals (Fe, Ni); hence their mechanisms are of great interest.[1] The active site of [NiFe]-hydrogenases (Figure 1) contains a strictly conserved arginine (R509 in Hydrogenase-1 (Hyd-1) from *Escherichia coli*, Supplementary Results, Supplementary Figure 1), the long side chain of which forms a canopy immediately above the Ni and Fe atoms of the $[(RS)_2Ni\mu(SR)_2Fe(CO)(CN)_2]$ inner coordination shell.[1,2] The guanidine N-atoms are also positioned within H-bonding distances (Supplementary Table 1) of the carboxyl groups of strictly-conserved and highly-conserved aspartic acids (D574 and D118, respectively, in Hyd-1, Supplementary Figure 1). One of the Nη atoms (N1) of R509 lies less than 4.5 Å from both Ni and Fe, a distance so short that the potentially mobile guanidine group is unlikely to simply be a spectator during catalysis (Supplementary Table 1). In fact, the metal–Nη1 distance is well suited for heterolytic H_2 activation *via* a mechanism akin to that of a frustrated Lewis pair (FLP),[3] in which H_2 is polarized by simultaneous binding to metal(s) (the acid) and N(the base). Yet, despite this close proximity, the functional role of the strictly conserved arginine has never been investigated experimentally: aside from computations that included it as part of a putative proton-transfer pathway,[4] the sidechain has long been assumed to play a stabilizing role following an early proposal[5,6] that it provides one of the hydrogen bonds to a cyanide ligand at the Fe atom. Previous attempts to substitute the equivalent arginine in hydrogenases from *Thiocapsa roseopersicina*[4] or *Desulfovibrio fructosovorans*[7] either resulted in immature enzyme or no enzyme being produced.

As we now report, we have succeeded in replacing each of the three conserved amino acids in the outer-shell canopy of the active site of Hyd-1, in all cases generating mature enzyme. Arginine-509 has been replaced by lysine, a conservative mutation that best preserves

basicity and side-chain length; aspartate-118 and 574 have been replaced by asparagine, an analogue of the protonated carboxyl form that can still form hydrogen bonds; aspartate-118 has also been replaced by alanine, a non-conservative substitution that provides a shorter, hydrophobic sidechain. The kinetic and structural results have profound mechanistic implications.

RESULTS

Production of enzymes

The C-terminal His_6-tagged Hyd-1 variants carrying the R509K, D118A, D574N or D118N/D574N substitutions were produced from the *E. coli* chromosome at its native locus at natural levels, similar to native His-tagged Hyd-1 (Online Methods). The structure of the 'Native' Hyd-1 enzyme was determined using a 'deltaTM' strain where the transmembrane domain has been removed from the *Hya* operon (Online Methods). The resulting enzyme was purified from the cytoplasmic extract, whilst other enzymes were purified from the membrane fraction, all via Ni affinity chromatography as previously described[2] (Online Methods). All variants were indistinguishable from native Hyd-1 by denaturing electrophoresis (Supplementary Figure 2), comprising large subunit (HyaB, which contains the active site) and His-tagged small subunit ($HyaA^{His}$ which contains the three FeS clusters of the electron relay and the transmembrane domain). Both subunits were proteolytically-processed correctly and the small subunit of the deltaTM strain ran at the expected lower mass.

Activities and voltammetry of enzymes

Using enzymes that had been exhaustively activated under H_2 (Online Methods), steady-state kinetic studies of methylene blue reduction by H_2 at 25 °C showed that the R509K, D574N, D118A and D118N/D574N variants have, respectively, 0.8%, 83%, 26% and 20% of the H_2 oxidation activity of native Hyd-1 (Figure 2, and Supplementary Table 2). The

electrocatalytic profiles of each enzyme, determined by protein film electrochemistry (PFE), are shown in Figure 3. In each case the enzyme film was prepared and fully activated (Online Methods), noting that some variants required longer activation times. Voltammograms were recorded under slow scan rates (0.1 mV/s) starting from a high potential at which Hyd-1 converts to the oxidised 'resting' inactive Ni^{III}-OH state,[8] (also known as 'Ready' or 'Ni-B', Online Methods) then scanning to more negative potential where the enzyme is activated rapidly by reduction. The current amplitudes have been normalized in order to focus on the potential markers, which were fully reproducible from one film to another (conversely, current amplitude is always variable, due to differences that include film quality and history). The onset potential for catalytic H_2 oxidation (signifying the most negative potential at which H_2 oxidation begins) was similar for all enzymes, as expected since this value depends mainly on properties of the FeS clusters of the electron relay.[9,10] The characteristic waveshape of native Hyd-1 was essentially unchanged for the R509K variant. In contrast, the voltammograms of D118A, D574N and D118N/D574N displayed marked negative shifts in the high-potential threshold,[11,12] thus revealing that the oxidized 'resting' Ni^{III}-OH state is stabilized in these variants, relative to that of native enzyme. Based on the Michaelis constants K_M (H_2) determined by PFE, (Supplementary Table 2), the activities of all enzymes displayed in Figure 2, with exception of D118A, refer to saturating H_2 conditions. The D118A variant showed a much higher K_M (H_2): hence the activity of D118A was underestimated.

X-ray structures

The electron density maps of the Hyd-1 variants were all of exceptional quality (Supplementary Figure 4), allowing residues 4-267 of both small subunits and 2–582 of both large subunits to be built. The resulting structures, including native Hyd-1 produced from the deltaTM strain, were very similar to those previously published[2] and it was possible to

superimpose all main chain atoms of the variant proteins with their equivalents in the native protein with rmsds in the range of 0.12–0.16 Å. Furthermore, the mutations did not alter the local conformation at the canopy above the [$(RS)_2Ni\mu(SR)_2Fe(CO)(CN)_2$] center, since the main chain atoms of residues 509, 118 and 574 of the variants could be superimposed on their equivalent residues in native Hyd-1 with an rmsds of 0.10–0.12 Å (Figure 4, panel f).

The positions of the cysteine residues directly coordinating the Ni and Fe ions were also unaffected by the mutations, with rms deviations calculated for all atoms in the coordinating cysteine residues of 0.17 Å or lower. Since the proteins were purified and crystallized under aerobic conditions some oxidation was observed at C79 (Supplementary Figure 5) and the residue was modelled as a mixture of cysteine and sulfenic acid. Similar oxidative modification has been described elsewhere[13] and is associated with so-called 'Unready' states that activate only slowly under H_2. The fraction of molecules showing oxidation at C79 was estimated to be 75% for deltaTM, 60% for R509K, 50% for D118A and 40% for both D574N and D118N/D574N. The degree of oxidation correlated with increased length of exposure to oxygen, for example, crystals of D118N/D574N and D574N were frozen immediately after crystallization, whereas deltaTM crystals were frozen approximately 10 days after crystal growth had ceased. The structural comparison of the inner coordination shell was extended to include the highly reduced catalytic centre of *D. vulgaris* hydrogenase[14] to test whether the oxidation of C79 affects the metal coordination (Figure 4, panel f). The resulting rmsd value of 0.24 Å showed that any changes due to cysteine-79 oxidation are minimal.

Calculation of Fo-Fo difference maps (which are particularly sensitive to changes in the position of Ni and Fe due to their high electron density) to compare the R509K, D118A, D574N and D118N/D574N variants with native Hyd-1 revealed only small peaks around the metal ions, confirming that the mutations do not introduce significant changes in the position

or occupancy of the metal ions. Comparisons of relevant bond distances are given in Supplementary Table 1, and the Ni-Fe bond length in all the oxidized structures varies between 2.9 and 2.7 Å, but there is no correlation with activity. All the structures contained an atom in the bridging position that was assigned as oxygen (hydroxide) which is always present in oxidized, as-isolated structures of [NiFe]-hydrogenases, but released rapidly upon reductive activation (Ni^{III}-OH $\rightarrow$ Ni^{II}).[15] All structures also showed the active site to be highly hydrated: each enzyme displayed several crystallographically ordered water molecules (Supplementary Figure 6), which could act as proton acceptors after dihydrogen lysis. The position of the water molecules is preserved between the active sites of hydrogenases from various organisms including *Desulfovibrio vulgaris Myazaki F* (4U9H.pdb), *Ralstonia eutropha* (4IUD.pdb) and *Hydrogenovibrio marinus* (3AYZ.pdb).

Although the mutations did not affect the geometry of the polypeptide backbone, subtle differences were observed in the conformations of the side chains within the active site. The side chain of lysine 509 adopts a non-standard rotamer geometry to maintain an interaction with D118 (Figure 4, panel b). The smaller lysine side chain also allows an additional water molecule to enter the active site just above the canopy (Supplementary Figure 6) and temperature factor analysis suggested the lysine residue is more flexible than the arginine in native Hyd-1. The D118A mutation removes a salt bridge partner to R509, and the loss of this interaction enables the arginine head group to move closer to the metal ions and the bridging hydroxide ion (Figure 4, panel c). The D118A mutation also reduces the size of the side chain, but no additional water molecules were observed within the active site. The most structurally conservative mutation, D574N, resulted in a small rotation of the amide head group to form a hydrogen bond between its Nδ2 atom and the Oδ1of D118 (Figure 1, panel d). Mutation of both conserved carboxylate groups in the D118N/D574N double variant caused a rotation of the N118 side chain away from R509. This rotation minimizes

repulsion between NHδ2 of N118 and NH_2 of R509 but still allows atom Oδ1 to maintain its hydrogen-bonding interaction with R509 (Figure 1, panel e). The Nδ2 atom of N118 forms a compensatory hydrogen bond to the main-chain amide group of V78 and displaces one of the structurally conserved water molecules (Supplementary Figure 6)

DISCUSSION

The structures showed that the [$(RS)_2Ni\mu(SR)_2Fe(CO)(CN)_2$] inner coordination shell of the native enzyme is preserved among all the canopy variants, and a ligand (assumed to be OH) in the bridging position, is present in all cases, as expected for as-isolated enzymes. Similarly, all enzymes showed some oxygenation of cysteine-S at the same position (C79), in full agreement with recent results[13] demonstrating that the inactive 'Unready' state (Ni-A) of [NiFe]-hydrogenases arises from aerobic oxidation of a specific cysteine-S. The successive replacements of D118 and D574 by neutral residues had surprisingly little impact on the position of the guanidinium/guanidine headgroup, an observation we return to later. The PFE results showed that the potentials for reductive activation of Ni-B (rapidly releasing the bridging OH) differed: R509K is very similar to native Hyd-1, whereas D118A, D574N, and D118N/D574N show increasing stabilization of the Ni^{III}-OH state, which may reflect the altered electrostatics, with higher affinity for hydroxide ion compensating for removal of a carboxylate. Although R509K was essentially identical to native Hyd-1 regarding the electrochemical stability of the Ni^{III}-OH state, it was by far the least active enzyme. Consequently, R509 which positions a guanidinium/guanidine headgroup immediately above the Ni and Fe atoms, could now be identified as having a catalytic role 100 times more effective than observed when a lysine primary amine is placed in an almost identical position. The large attenuation of activity is highly unlikely to result from loss of a specific hydrogen-bond that has been proposed to form between a hydrogen on argNη (N1) and one of the CN ligands to Fe: a true hydrogen bond as such cannot exist because the argNη—N-C angle is

close to 90° (in all structures solved to date, regardless of oxidation state)[1,14] rather than the 180° angle required to interact with the favored sp-hybrid acceptor orbital on the cyanide.[16,17]

It has been proposed and thereafter assumed that [NiFe]-hydrogenases use a thiolate-S ligand as the base for heterolytic H–H bond cleavage.[1] However, simple Ni complexes with terminal thiolates are not noted to be good catalysts for H_2 activation.[18] By contrast, the guanidine group is a strong base, the issue being how to exploit this property given the difficulty in deprotonating the guanidinium $[(H_2N)C(NH_2)]^+$ headgroup. There are two points to note: firstly, [NiFe]-hydrogenases behave as reversible electrocatalysts,[9,10] so microscopic reversibility will apply at each stage of the catalytic cycle for each discrete potential; secondly, a hydrido ligand bound to the most reduced state of Ni ('Ni-R', see ref[14]) is likely to be a strong base and, accordingly, a bound H_2 molecule should be a weak acid.[19]

Figure 5 shows how R509 can activate H_2 by an FLP-like mechanism in which the Ni and Fe atoms operate in conjunction with the closest guanidinium-Nη to split the H_2 molecule (bond length 1.4 Å, van de Waals length 2.8 Å). The structure of Ni-R has been established recently:[14,20] viewed in the direction of H_2 evolution (left-to-right in Figure 5) the hydrogen atom that is bound to Ni (at 1.58 Å) is expected to be strongly hydridic in character because it has been generated by successive additions of one electron, one proton, then a second electron. Therefore, the resulting hydrido ligand should be sufficiently basic to deprotonate the guanidinium group. Conversely, the deprotonation of H_2 (right-to-left Figure 5) requires a sufficiently strong base, and guanidine fulfils this role. The reversibility of H_2 activation by the [NiFe] active site contrasts strikingly with the behavior of close synthetic structural analogues, which require strong acids (HBF_4 and CF_3COOH) to produce H_2[21] or a strong base (NaOMe) to oxidize H_2.[22] Interestingly, neither carboxylate appears to play a specific proton-transfer role, an observation that is reinforced by the fact that 20% of the native activity is retained even when both aspartates are replaced simultaneously by

asparagine. Instead, several highly-conserved water molecules are available to mediate proton transfers in and out of the active site (Supplementary Figure 6). Longer range proton-transfer pathways leading from the extended active site to the protein surface have been proposed, noting that it is likely that the two protons that are ultimately produced leave by separate pathways.[4,15,23] A further intriguing issue is raised by the observation that the positioning of the headgroup of the long and potentially mobile arginine sidechain is relatively unaffected in the D118N/D574N variant by the removal of the two nearby negative charges, despite the proximity of the metal cluster. This seems a less favorable environment than expected for a positively charged guanidinium group and could increase the proportion of deprotonated, neutral guanidine.

Direct use of arginine as a catalytic base is rare but not new.[24] In the widely accepted mechanism for fumarate reductase and L-aspartate oxidase, the proton donor to the substrate is an arginine that is reprotonated by a neighboring glutamic acid.[24-26] A direct role for arginine as a base has also been proposed for Photosystem II.[27]

The mechanism we propose is similar to the current hypothesis regarding highly active functional hydrogenase analogues featuring a redox-active transition metal and a remote pendant base.[28] The concept is also similar to that emerging for [FeFe]-hydrogenases,[1] enhanced by the recent proof that the native bridging ligand is azadithiolate and the pendant bridgehead N-atom is essential for catalysis.[29] It thus follows that [NiFe]- and [FeFe]-hydrogenases and the best functional models employ a common principle in which a transition metal (acid) and a general base precisely positioned in the outer coordination shell activate H_2 like a frustrated Lewis pair.

Accession codes The coordinates and structure factors have been submitted to the protein data bank with accession codes 4UE3 (R509K), 5A4F (D118A), 5A4I (D574N), 5A4M (deltaTM) and 5ADU (D118N/D574N).

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Author Contributions

F.A.A., F.S., and R.M.E. proposed the study. R.M.E., E.J.B. and S.A.M.W. carried out all molecular biology, kinetic and electrochemical characterisations. R.M.E., E.N., E.J.B., S.A.M.W., and S.B.C., produced and purified enzymes. S.B.C. carried out all X-ray data collection, and S.B.C and S.E.V.P. were responsible for structural determinations. F.A.A., R.M.E., S.E.V.P., and S.B.C. wrote the manuscript with input from other authors.

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Figure Legends for main text

Figure 1. A stereo image of the active site of [NiFe]-hydrogenases as exemplified by *E. coli* Hyd-1 (5A4M.pdb, oxygenation sites omitted). Important outer coordination 'canopy' residues are shown, including R509, which lies directly above the inner coordination shell Ni (turquoise sphere) and Fe (orange sphere) metals.

Figure 2. Comparison of activities for Hyd-1 and variants investigated in this research. The average turnover rate (s^{-1}) is given for each enzyme, with error bars indicating the standard error of the mean of at least twenty repeats with at least two different preparations of each enzyme (see also Supplementary Table 2).

Figure 3. Protein film electrochemistry of Native and variant Hyd-1 enzymes described in this paper. Conditions: pH 6.0, 30 °C, 100% H_2. Normalization with respect to maximum current was carried out in order to optimise overlay for comparing shapes and potential markers. Linear sweep voltammograms were scanned at 0.1 mV/s under 100% H_2 from +0.39 to –0.60 V (black arrow) following anaerobic inactivation (see Online Methods and Supplementary Figure 3). All enzymes showed the same potential for onset of catalytic activity (red arrow). The negative shifts of the high-potential threshold of catalytic activity for D118A, D574N and D118N/D574N signify increasing stabilization of the inactive oxidized Ni^{III}-OH ('Ni-B') state in these variants. The poor signal-to-noise for R509K reflects the very low oxidation current always obtained for this variant (raw data shown in gray, smoothed data shown in black, 41 points per window Savitzky-Golay function).

Figure 4. The crystal structures of the active sites of as-isolated and chemically oxidized enzymes described in this paper. Native (deltaTM) Hyd-1 (a), R509K (b), D118A (c), D574N (d) and D118N/D574N (e) variants. Atoms are labelled in accordance with Supplementary Table 1. The Ni atom (turquoise), Fe atom (bronze) and the O atom of the bridging hydroxide

(red) are all shown as spheres. Oxidation of C79 to sulfenic acid was observed at the active site of each variant and is indicated with an arrow in panel a. The closest distance between the Ni and N atom of residue 509 (dashed lines) is also indicated for each variant. Panel f shows a superposition of the active site residues of the five *E. coli* enzymes with the active site of the highly reduced catalytic centre of hydrogenase from *D. vulgaris* (4U9H.pdb, orange).

Figure 5. Proposal for the H_2 activation step in [NiFe]-hydrogenases using suspended arginine as a general base, as in a Frustrated Lewis Pair mechanism. Hydrogen is polarized between the metal (acid) and deprotonated guanidine (base) of R509, oxidizing H_2 and forming the Ni-R species (left). The hydridic Ni-R species deprotonates the guanidinum headgroup of R509, forming molecular hydrogen.

ONLINE METHODS

Molecular Biology

In this study, all electrochemistry and solution assays were performed on Native and variant Hyd-1 enzymes produced from MC4100-derived *E. coli* K-12 strain FTH004 or the equivalent variant strain (Online Methods Table 1). For the crystallographic 'Native' data sets, 'deltaTM' enzyme was isolated from the supernatant of strain FTH2014; modified at the hya operon (*hyaA*) to remove the coding region for the transmembrane (TM) domain which would otherwise anchor Hyd-1 to the membrane.[30] The deltaTM enzyme was otherwise purified as previously described, but without detergent in the purification buffers.

The R509K, D118A, D118N and D574N mutations were made on the *pMAK-hyaB* plasmid construct (Online Methods Table 1) and confirmed by DNA sequencing. The codon change was transferred from the resulting *pMAK-hyaB-variant* to the FTH004 or equivalent variant strain chromosome as previously described[31,32] and again confirmed by DNA sequencing to ensure whole gene integrity.

Online Methods Table 1 – Oligonucleotide primers, plasmid constructs and *E. coli* strains used in this study

Plasmid Name	**Plasmid Function**	
pMAK-hyaB	Template plasmid for all mutations	
pMAK-hyaB-R509K	Mutated plasmid for transfer of codon change to FTH004 chromosome to create R509K variant strain	
pMAK-hyaB-D118A	Mutated plasmid for transfer of codon change to FTH004 chromosome to create D118A variant strain	
pMAK-hyaB-D574N	Mutated plasmid for transfer of codon change to FTH004 chromosome to create D574N variant strain	
pMAK-hyaB-D118N	Mutated plasmid for transfer of codon change to EJB001 chromosome to create D118N mutation	
Primer name	**Primer function**	**Primer Sequence**
hyaB_R509K_F	*hyaB* R509K mutagenesis forwards primer	5'-CCGAAGCGCCGAAAGGGGCGTTAGGC-3'
hyaB_R509K_R	*hyaB* R509K mutagenesis reverse primer	5'-GCCTAACGCCCCTTTCGGCGCTTCGG-5'
hyaB_D118A_F	*hyaB* D118A mutagenesis forwards primer	5'-CGCTCTGGTGCCACGCCCATCTGGTGCACTTC-3'
hyaB_D118A_R	*hyaB* D118A mutagenesis	5'-GAAGTGCACCAGATGGGCGTGGCACCAGAGCG-3'

	reverse primer	
hyaB_D574N_F	*hyaB* D574N mutagenesis forwards primer	5'-GTACTCTGCACAGCTTTAACCCGTGCCTCGCCTGTTC-3'
hyaB_D574N_R	*hyaB* D574N mutagenesis reverse primer	5'-GAACAGGCGAGGCACGGGTTAAAGCTGTGCAGAGTAC-3'
hyaB_D118N_F	*hyaB* D118N mutagenesis forwards primer	5'-CTGGCAACGCTCTGGTGCCACAATCATCTGGTGCACTTC-3'
hyaB_D118N_R	*hyaB* D118N mutagenesis reverse primer	5'-GAAGTGCACCAGATGATTGTGGCACCAGAGCGTTGCCAG-3'
Strain name	**Strain description**	
FTH004	Native Hyd-1 strain. Used to produce and isolated Native Hyd-1 enzyme, purified from the membrane fraction and used for solution assay kinetics and electrochemistry	
FTH2014	deltaTM Hyd-1 strain. Strain produced by removing the transmembrane domain coding region from the hya operon of FTH004 strain. Used to produce and isolate deltaTM Hyd-1 enzyme from the supernatant solution and used for crystallographic studies of un-mutated Hyd-1 enzyme	
RME011	R509K variant strain. Strain produced by codon change on chromosome of FTH004 strain using *pMAK-hyaB*-R509K plasmid	
RMESW014	D118A variant strain. Strain produced by codon change on chromosome of FTH004 strain using *pMAK-hyaB*-D118A plasmid	
EJB001	D574N variant strain. Strain produced by codon change on chromosome of FTH004 strain using *pMAK-hyaB*-D574N plasmid	
EJB002	D118N/D574N double variant strain. Strain produced by codon change on chromosome of EJB001 strain using *pMAK-hyaB*-D118N plasmid	

Protein production and purification

All strains used (Online Methods Table 1) carry an engineered *hyaA*(*his*$_6$)*BCDEF* operon to produce an enzyme with a hexa-histidine tag at the C-terminus of the small subunit. The strains were cultured anaerobically,[2] for optimal expression of the *hya* operon, cells were harvested once a stationary growth phase was attained. The growth curves for Native and variant Hyd-1 strains were indistinguishable and the yields of purified enzymes were similar at approximately 0.24 mg/L of culture. The enzymes were purified by nickel affinity chromatography as previously described[2] and were indistinguishable by SDS-PAGE (Supplementary Results, Supplementary Figure 2).

For X-ray crystallographic analysis, isolated membranes of the variant strains were mechanically homogenized prior to nickel affinity chromatography, and the resulting protein was further purified by size exclusion chromatography followed by hydroxyapatite chromatography as described elsewhere.[2,8] The deltaTM strain produces enzyme that is not

anchored to the membrane and so instead is isolated from the supernatant solution following cell lysis. The nickel affinity chromatography products of the deltaTM strain were also further purified by size-exclusion and hydroxyapatite chromatography.

Hydrogenase solution assay

The steady-state rates of oxidation of hydrogen were measured by conventional solution assay, monitoring the reduction of methylene blue (Aldrich) at 600 nm. Solution assays were performed using an Ocean Optics S2000 fibre optic spectrometer controlled with OOIBase32 software (Ocean Optics, Inc) at 25 °C in an anaerobic glove box (Belle Technologies). Methylene blue was dissolved to a stock concentration of 50 μM in pH 6.0 potassium phosphate buffer (Fisher Scientific), and diluted to a working concentration of 25 μM with phosphate buffer. Enzymes were exhaustively activated by continuous saturation with H_2 for 12–32 hours in an anaerobic glove box (MBraun or Belle Technologies). Methylene blue solution (5–10 mL) was continuously purged with H_2 in a sealed glass vial for at least 5 minutes prior to the experiment. Immediately prior to performing the experiment, 1 mL of H_2-saturated methylene blue solution was transferred to a sealed cuvette containing a stirrer bar, and the change in absorbance monitored until stable, as a negative control. Activated enzyme solutions (1–5 μL at 0.1–5 mg/mL, determined by Bradford assay[33]) were drawn into a gas-tight Hamilton syringe immediately prior to the solution assay, and injected into the sealed cuvette, continuing to monitor the change in absorbance with stirring of the solution. The change in absorbance was monitored at 600 nm for 1–10 minutes, ensuring linearity over the time course. The extinction coefficient used for methylene blue was 22400 cm^{-1} M^{-1}.[34] The results are taken from initial velocities and are expressed as μmol H_2 oxidized per minute per mg enzyme or as the apparent turnover rate, k (s^{-1}), see Supplementary Table 2.

Crystallography Methods

Native and variant proteins were concentrated to 5 mg/mL using centrifugal concentrators with a 50,000 molecular weight cut-off (Vivaspin) and protein concentration determined by Bradford assay.[33] Crystals of each Hyd-1 enzyme were obtained using the sitting-drop vapor diffusion method by mixing 1.5 μL protein solution with 1.5 μL 100 mM Bis-tris (Sigma Aldrich) pH 5.7–6.0, 20–23% (w/v) PEG 3350 (Hampton Research), 200 mM $LiSO_4$ (Sigma Aldrich), 150 mM NaCl (Sigma Aldrich) followed by incubation at 293 K. Prior to data collection crystals were cryo-protected by transferring to a solution of mother liquor containing an additional 2% PEG 3350, 5 mM $K_3[Fe(CN)_6]$ (Sigma Aldrich) and 20% (v/v) glycerol (Sigma Aldrich) followed by flash-cooling by immersion in liquid nitrogen. All chemicals used were of analytic grade.

All diffraction data were collected at 100 K at Diamond light source beam-lines I02, I04 and I04-1 at wavelengths of 0.92 or 0.9794 Å using Pilatus 2M or 6M hybrid pixel array detectors. To minimize photo-reduction during data collection all data sets were collected using the line scan mode. Data reduction was performed using XDS[35] and initial phase estimates generated using Phaser[36] with pdb file 3USC[1] as a search model. To prevent model bias in the region of the active site, all atoms in a 5 Å radius of the C_α of residue 509 were removed from the search model prior to molecular replacement. The missing residues could be manually rebuilt using COOT[37] followed by refinement using REFMAC5.[38] Hyd-1 deltaTM and R509K were refined using TLS restraints and isotropic B-factors, whereas the higher resolution data collected for Hyd-1 D118A, D574N and D118N/D574N allowed anisotropic B-factor refinement. The final molecular models displayed excellent geometry with 97% of backbone torsion angles occupying the most favored regions of the Ramachandran plot, and none in the disallowed regions. Crystals of each Hyd-1 variant are highly isomorphous (Supplementary Table 3) allowing the calculation of Fo-Fo difference

maps within COOT and all structure superpositions were performed using LSQKAB.[39] The occupancy of the SD oxygen atom, and hence the degree of oxidation at C79, was estimated by deletion of the SD oxygen atom and the carbonyl oxygen atom of R107 in the large chain followed by calculation of Fo-Fc difference density maps and comparison of peak heights for the deleted atoms.

Protein Film Electrochemistry Methods

All electrochemistry was performed in an anaerobic glove box containing a N_2 atmosphere (O_2 <2 ppm, MBraun or Belle Technologies). Measurements were carried out using an Autolab potentiostat (PGSTAT128N) controlled by Nova software (EcoChemie). The three-electrode system used comprised a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference. All potentials were converted to the Standard Hydrogen Electrode (SHE) scale using the correction $E_{SHE} = E_{SCE} + 241$ mV at 25 °C.[40] The working electrode was a pyrolytic graphite edge plane electrode (PGE) constructed in-house[11] and rotated at 1000–3000 rpm depending on the experiment in question to ensure that mass transport of substrate/products was not rate limiting.

All experiments were carried out using a mixed buffer system as previously described,[41] titrated to pH 6.0 at 30 °C unless otherwise specified. All solutions were prepared using ultrapure water (Millipore, 18 MΩ cm). Precise gas flow rates (BOC gases, high purity) were created using mass flow controllers (Sierra Instruments).

Protein films were prepared as previously described[41] after abrading the PGE electrode with P400 Tufbak Durite sandpaper. Enzyme (0.5–3 µL at 10–400 µM) was repeatedly applied and removed for ~30 seconds, then the electrode surface rinsed in a stream of purified water (Millipore, 18 MΩ cm) to remove excess enzyme. The geometric surface area of the PGE electrode was 0.03 cm^2 to 0.05 cm^2, a larger geometric surface area being used for R509K,

for example. All enzyme films were reductively 'activated' prior to every experiment by repeatedly posing the electrode at –0.659 V for 300 seconds and monitoring the H_2 oxidation activity at –0.17 to 0 V (depending on the variant) for 60 seconds until the activity level stabilized as previously described.[41]

To assess the recovery of activity from the oxidized, inactive Ni-B (Ready) state, chronoamperometry was performed using an electrode potential of +0.391 V to convert enzyme to the inactive oxidized (Ni^{III}-OH) 'resting' state known as Ni-B (Supplementary Figure 3). The reaction was facilitated using pH 9.0 buffer in the cell. The starting current was first assessed in 100% H_2, and switched to 100% Ar after approximately 1200 seconds. At approximately 8500 seconds, the gas flow was switched back to 100% H_2 for 5 minutes to check the current level was at approximately zero: at this point the buffer was changed to pH 6.0 by simultaneously adding pH 6.0 buffer whilst removing the pH 9.0 buffer under the gaseous pressure of the electrochemical cell. A 10-fold volume exchange with pH 6.0 buffer was used to ensure complete removal of the pH 9.0 buffer. The pH 6.0 buffer was allowed to equilibrate to 100% H_2 and 30 °C for 10 minutes before a scan from +0.391 V to –0.6 V was performed at 0.1 mV/s (Figure 3).

The Michaelis constant for H_2 (K_M) was assessed by monitoring the current change in response to change in [H_2] *via* protein film electrochemistry at 0 V, 30 °C. Cyclic voltammetry and chronoamperometry methods were both used and the value of K_M did not depend on the experimental method. The concentration of H_2 was varied between 0.3 and 100% and allowed to fully equilibrate and the value for K_M was determined by Hanes-Woolf analysis as previously described.[41] All data were appropriately corrected for 'film loss'[41] and are expressed as an average of at least three repeats with the error given as the standard error of the mean.

The coordinates and structure factors have been submitted to the protein data bank with accession codes 4UE3 (R509K), 5A4F (D118A), 5A4I (D574N), 5A4M (deltaTM) and 5ADU (D118N/D574N).

Methods only references

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Competing financial interests

The authors declare no competing financial interests.

Additional Information

Online Methods including molecular biology, protein production and purification, solution assays, crystallography methods, structure analysis and protein film electrochemistry and

Methods only references. Supplementary Information including Supplementary Figures 1–6 and Supplementary Tables 1–3: Analysis of key active site distances, Comparisons of kinetic data, Crystallographic data collection and refinement statistics Sequence Alignment, denaturing electrophoresis, Inactivation procedure example, Electron density maps and Supplementary References.