Mechanistic Exploitation of a Self-Repairing, Blocked Proton Transfer Pathway in an O₂-Tolerant [NiFe]-Hydrogenase

Rhiannon M. Evans, Philip A. Ash, Stephen E. Beaton, Emily J. Brooke, Kylie A. Vincent, Stephen B. Carr, and Fraser A. Armstrong

Abstract: Catalytic long-range proton transfer in [NiFe]-hydrogenases has long been associated with a highly conserved glutamate (E) situated within 4 Å of the active site. Substituting for glutamine (Q) in the O₂-tolerant [NiFe]-hydrogenase-1 from Escherichia coli produces a variant (E28Q) with unique properties that have been investigated using protein film electrochemistry, protein film infrared spectroscopy, and X-ray crystallography. At pH 7 and moderate potential, E28Q displays approximately 1% of the activity of the native enzyme, high enough to allow detailed infrared measurements under steady-state conditions. Atomic-level crystal structures reveal partial displacement of the amide side chain by a hydroxide ion, the occupancy of which increases with pH or under oxidizing conditions supporting formation of the superoxidized state of the unusual proximal [4Fe-3S] cluster located nearby. Under these special conditions, the essential exit pathway for at least one of the H⁺ ions produced by H₂ oxidation, and assumed to be blocked in the E28Q variant, is partially repaired. During steady-state H₂ oxidation at neutral pH (i.e., when the barrier to H⁺ exit via Q28 is almost totally closed), the catalytic cycle is dominated by the reduced states “Niₐ-R” and “Niₐ-C”, even under highly oxidizing conditions. Hence, E28 is not involved in the initial activation/deprotonation of H₂, but facilitates H⁺ exit later in the catalytic cycle to regenerate the initial oxidized active state, assumed to be Niₐ-SI. Accordingly, the oxidized inactive resting state, “Ni-B”, is not produced by E28Q in the presence of H₂ at high potential because Niₐ-SI (the precursor for Ni-B) cannot accumulate. The results have important implications for understanding the catalytic mechanism of [NiFe]-hydrogenases and the control of long-range proton-coupled electron transfer in hydrogenases and other enzymes.

1. INTRODUCTION

The cycling of hydrogen (H₂) by microorganisms has strong connections to biotechnology, energy and health. The metalloenzymes known as hydrogenases operate in neutral water and use a heterolytic mechanism (H₂ → H⁺ + H⁻) so it is highly significant that their active sites are deeply buried and almost completely sealed from solvent. In fact, they are remarkable among all enzymes in that H₂, the smallest of molecules, is solely formed from (or converted into) four quantum particles that must hop and tunnel through the protein. The specific routes taken by protons and electrons between solvent and active site reflect the fact that the characteristic tunneling distance for a proton is about 45 times shorter than for an electron, hence a proton pathway requires closely spaced donor–acceptor groups (mobile side chains and water molecules) whereas electron-transfer sites are typically 10–14 Å apart. Neutral gas molecules, whether substrates or inhibitors, are expected to enter and leave the active site via preferred hydrophobic tunnels. Additionally, and especially for the special ‘O₂-tolerant’ class of [NiFe]-hydrogenases that can function continuously in the presence of O₂ (and thus act as hydrogen oxidases) hydrophilic pathways must exist to ensure rapid escape of water molecules following O₂ attack.

The Escherichia coli membrane-bound [NiFe]-hydrogenase (MBH) “Hyd-1” is O₂-tolerant with a strong bias toward hydrogen oxidation at neutral pH, whereas the O₂-sensitive MBH “Hyd-2” from E. coli operates in both directions.

Received: May 10, 2018
heterodimeric protein complex, has similar architecture in all cases, the essential features being summarized in Figure 1A. The Ni and Fe atoms are anchored to the protein by four cysteine thiolates, two of which are bridging ligands while the other two are terminal to the Ni: the Fe atom is further ligated by one CO and two CN$^-$ ligands. Depending on the stage of the minimal catalytic cycle shown in Figure 1B, a fifth ligand to the Ni may be present, in a bridging position between the Ni and Fe atoms (the Fe remains low-spin Fe II throughout). Immediately above the metal atoms lies the outer coordination shell "canopy" that includes the guanidinium group of arginine 509, recently demonstrated to be essential for fast H$_2$ oxidation by Hyd-1. 

 Whereas the detailed mechanism by which the H–H bond is formed or cleaved remains to be resolved, much more is known about the pathways of electrons to and from the buried active site. In MBHs, electrons are transferred via a chain of Fe–S clusters housed in the small subunit that connects the active site to the biological membrane or redox partner protein. The Fe–S chain comprises three Fe–S clusters termed "proximal", "medial" and "distal" according to their distance from the active site. In contrast to this clearly marked pathway for electrons, our insight into long-range proton transfer owes much to the seminal work of Dementin and co-workers who showed that a highly conserved glutamate (E28 in E. coli Hyd-1, Figure 1A) located at the interface of the large and small subunits, and in direct contact with an extensive network of water molecules (see later and Figure S1) is critical for proton transport from the active site during H$_2$ oxidation. Mutation of E28 to glutamine (carboxylate-to-amide) in the [NiFe]-hydrogenase from Desulfovibrio fructosovorans resulted in a protein that could cleave H$_2$ within the active site, but was unable to exchange protons with solvent, resulting in >99.9% lowering of the H$_2$ oxidation rate. No electrochemical or structural data were presented. There has since been compelling support for a key role for this residue in proton transfer.

Figure 1. Schematic representation of the orientation of E28 in native Hyd-1 (A), in which the noncoordinating residues E28 and R509 so far established to be essential for catalysis are included. Distances (gray) between the carbonyl-O atoms of E28 and the nearest Fe atom of the [4Fe–3S]-6Cys proximal Fe–S cluster and the cysteine-S of Ni-coordinated cysteine 576 are shown (pdb.5A4M). The atom which bridges the Ni and Fe is denoted as ‘X’ to represent the varied identification of the ligand depending on the oxidation state (as shown in the catalytic cycle, B) and presence of various inhibitory small molecules. (B) In the catalytic cycle, the catalytically active (a) states are denoted "Ni a" whereas the inactive oxidized states generated anaerobically at high potential (Ni-B) or upon the binding of oxygen (Ni-B or Ni-A depending on the number of electrons immediately available to reduce the attacking O$_2$, see refs 55,69) are shown in the gray box. Each catalytic state is shown in a black box: the lower half represents the current consensus on the minimal [Ni, Fe, H] unit of the active site, including oxidation states and bonding, the upper half represents the proton acceptor(s) not associated with the minimal unit. During H$_2$ oxidation, H$_2$ binds to the Ni a-SI state (1), heterolytic activation of H$_2$ requires deprotonation by a nearby base (‘:B1’, the identity of which is still under debate$^{17,23,27,70,71}$) forming Ni a-R which has a bridging hydride (2).$^{71}$ The subsequent stage of the catalytic cycle in which the proton on B$_1$ leaves the active site is unknown at present (so we denote the protonated base in parentheses ([H$^+$]:B$_1$)), though B$_1$ must be deprotonated prior to stage 2 in order for the next molecule of H$_2$ to be activated. Following electron transfer Ni a-C is formed (3). The bridging hydride of Ni a-C migrates as a proton, the initial proton acceptor proposed to be a terminal cysteine thiolate (4),$^{27,70}$ and the resulting Ni a-L state is proposed to contain a dative metal–metal bond.$^{72}$ Finally, a second electron is transferred to the proximal Fe–S cluster to form Ni a-SI (5).
transfer (most likely via a swinging arm mechanism\textsuperscript{25}) with substitutions for glutamine greatly attenuating steady-state turnover rates. The most profound result was reported by Adamson et al.,\textsuperscript{26} who concluded (based on studies at pH 6 and below) that the E28Q variant of Hyd-1 had no discernible catalytic activity. Other studies have shown partial decreases in activity, ranging from approximately 19% (pH 7.5) for the NADP\textsuperscript{+}-linked\textsuperscript{13} soluble hydrogenase I (SHI) from \emph{Pyrococcus furiosus}\textsuperscript{27} to approximately 50% (pH 7) in the MBH “Hynn” from \emph{Thiocapsa roseopersicina}\textsuperscript{28,29}. It is important to note that the active sites of [NiFe]-hydrogenases contain many ordered water molecules (H\textsubscript{2}O or OH\textsuperscript{−}) whose positions are highly conserved:\textsuperscript{17} it is very likely that H\textsuperscript{+} can transfer easily within the active site itself.

Protein film electrochemistry (PFE) allows the H\textsubscript{2} oxidation or H\textsuperscript{+} reduction reactions of an immobilized hydrogenase to be driven and monitored in either direction depending on the electrode potential applied.\textsuperscript{29,30} Enzymes can be investigated under challenging conditions such as highly oxidizing potentials and even extremes of pH. The resulting effects on catalytic activity, measured directly as current, are immediately evident. Even enzymes with very little activity can be studied using a high-surface area working electrode coated with multiwalled carbon nanotubes (MWNT).\textsuperscript{17,23,31,32} Protein film infrared (IR) electrochemistry (PFIRE) utilizes the same principles as PFE, the important addition being that the status of the active site is monitored during turnover or under equilibrium (non-turnover) conditions, via the stretching frequencies of the CO and CN\textsuperscript{−} ligands of the active site Fe atom.\textsuperscript{33,34}

The apparent total inactivity of the E28Q variant of Hyd-1 observed previously\textsuperscript{26} suggested strongly that this enzyme depends entirely on a single route for entry and exit of at least one of the two protons produced at an “encapsulated” H\textsubscript{2} activation site. In this paper we show that the residual low activity is sufficient to allow highly informative experiments that address the distribution of catalytic intermediates under steady-state H\textsubscript{2} oxidation. We have revisited the E28Q variant (and equivalent variant in Hyd-2, E14Q) along with additional mutations in Hyd-1 in a series of studies involving X-ray structure determination, PFE and PFIRE, the latter reporting on the catalytic steps within the active site (Figure 1B) that are dependent on having an efficient escape route for protons via E28. Under steady state conditions, “stages” of the catalytic cycle (Figure 1B) that require exit of H\textsuperscript{+} via E28 should be restricted thereby allowing the preceding state or states to dominate the spectra. An unexpected result is that the H\textsubscript{2} oxidation activity increases greatly at high pH and oxidizing conditions, the origin of this enhancement being revealed in the crystal structures. The results have important significance for understanding the mechanism of enzymatic H\textsubscript{2} oxidation and how catalysis is linked to long-range proton transfer.

2. EXPERIMENTAL SECTION

2.1. Molecular Biology and Enzyme Production. Variants of Hyd-1 were produced following standard molecular biology procedures as outlined in the Supporting Information.\textsuperscript{17,23} All Hyd-1 strains (Table S1) were used for chromosomal expression of the Hyd-1 enzymes, and protein was purified from the cell membrane via detergent solubilization and Ni-affinity chromatography, as previously described.\textsuperscript{15} For structural studies, additional size-exclusion and hydroxyapatite chromatography was performed.\textsuperscript{17,22,23} The Hyd-2 enzymes were purified from the cell cytoplasm, following over-production of the HybO (Fe–S cluster-containing) subunit, as recently reported.\textsuperscript{21} The resulting native Hyd-2, previously termed “Hyd-2-NOP”, (native, “overproduced”), is simply denoted “Hyd-2”. For the Hyd-2 variant E14Q, strain HJ001-hy bHy oC was mutated as outlined in Table S1. Enzymes Hyd-2 and E14Q were purified by Ni-affinity chromatography, and for subsequent structural studies underwent additional size-exclusion chromatography.\textsuperscript{22}

2.2. Steady State Solution Assays. Turnover rates for H\textsubscript{2} oxidation were measured by anaerobic steady-state solution assays in an N\textsubscript{2}-filled glovebox (Belle Technologies, O\textsubscript{2} < 2 ppm).\textsuperscript{17,23,24} Data were collected for Hyd-1 enzymes using methylene blue (Sigma-Aldrich) and for Hyd-2 enzymes using benzyl viologen (Fluka) at 25 °C, using an Ocean Optics S2000 fiber optic spectrometer controlled with OI/OBase32 software (Ocean Optics, Inc.). The extinction coefficients were used 22.4 M\textsuperscript{−1} cm\textsuperscript{−1} (at 600 nm, methylene blue)\textsuperscript{33} and 8.4 M\textsuperscript{−1} cm\textsuperscript{−1} (at 604 nm, benzyl viologen).\textsuperscript{36,37}

Enzyme solutions were reductively activated for 24–48 h (Hyd-1 enzymes) or 12–24 h (Hyd-2 enzymes) under an atmosphere of 100% H\textsubscript{2} at room temperature (21 °C). A H\textsubscript{2}-saturated solution (i.e., 1 mL, 25 μM methylene blue, or 2 mM benzyl viologen, both in 0.1 M potassium phosphate, 0.10 M NaCl, pH 6–10.8, titrated at room temperature) was monitored for ~60 s in a sealed cuvette containing a micro magnetic stirrer ball. Enzyme solution (5 μL) was then injected using a Hamilton syringe, typically delivering 1 ng–10 μg depending on the enzyme in question.

2.3. Protein Film Electrochemistry and Protein Film Infrared Electrochemistry. Both PFE and PFIRE experiments were carried out using a potentiostat (PGSTAT 20, or PGSTAT 128N, Metrohm Autolab) controlled by Nova software (EcoChemie). A three-electrode system was used in each case, comprising a Pt wire counter, a saturated calomel reference and a working electrode. For PFE the working electrode consisted of a pyrolytic graphite “edge” (PGE, Momentive Performance Materials Ltd.) electrode, constructed in-house.\textsuperscript{36,39} For PFIRE, the working electrode was a high surface area carbon black (BP2000, Cabot Corporation) particle electrode.\textsuperscript{33,34} All potentials are quoted against the standard hydrogen electrode using the correction FE\textsubscript{H2} = E\textsubscript{SHE} + 241 mV, at 25 °C.\textsuperscript{40}

Unless otherwise stated, all experiments were carried out using a mixed buffer solution,\textsuperscript{29} prepared using ultrapure water (Millipore, 18 MΩ cm) and titrated to the desired pH at the temperature of the experiment to be performed: for PFIRE this was always pH 7.0 at 27 °C. All gases were of high purity (BOC gases) and precise mixtures were obtained using mass flow controllers (Sierra Instruments or Brooks). Protein film electrochemistry experiments were carried out using a gas-tight, water-jacketed glass electrochemical cell\textsuperscript{41} housed in an anaerobic glovebox (Belle Technologies, or MBraun, O\textsubscript{2} < 2 ppm). To avoid mass transport limitations, the working electrode was rotated at high speeds (1000–3000 rpm) as the experiment dictated. Protein film infrared electrochemistry was carried out using a spectroelectrochemical flow cell as described previously,\textsuperscript{33,34} and mass transport limitation was avoided by using a high solution flow rate (260 mL/min) through the PFIRE cell via a peristaltic pump (Whatman, 120U/D1). Infrared spectra were recorded using a custom-modified attenuated total reflectance (ATR) accessory (PIKE Technologies, GladiATR) in a Bruker Vertex 80 FTIR spectrometer, equipped with a liquid N\textsubscript{2}-cooled mercury cadmium telluride detector, housed in a dry, anaerobic glovebox (Glove Box Technology Ltd., <2 ppm of O\textsubscript{2} <85 °C dew point). Spectra were recorded using OPUS software, as an average of 256 interferograms (20 kHz) at 2 cm\textsuperscript{−1} resolution, and processed by OriginPro 2017 (OriginLab).

For PFE experiments, in the first instance, protein films were prepared for all enzymes using a PGE working electrode of geometric surface area 0.03–0.05 cm\textsuperscript{2}: the electrode surface was abraded with P400 (3 M Hookit) sandpaper and rinsed with ultrapure water. Enzyme solution (1–2 μL at 10–500 μM) was continuously applied and reapplied via pipet to the electrode surface for 30 s, then rinsed with ultrapure water to remove unadsorbed enzyme. To obtain a much higher electrode coverage and thus observe the catalytic H\textsubscript{2} oxidation activity of low-activity Hyd-1 and Hyd-2 variants, a modified electrode\textsuperscript{17,23,31,32} was used: the PGE electrode was first coated with a dispersion (32 μL) of multiwalled carbon nanotubes
(MWNT, Sigma-Aldrich, outer diameter × length = 6–9 nm × 5 μm), at a loading of 1 mg/mL in dimethylformamide (DMF, Acros Organics) and allowed to dry. A solution (32 μL) of 1-pyrenetricarboxylic acid (Sigma-Aldrich, 10 mM in DMF) was then applied for 1 h before rinsing with ultrapure water. The carboxylic acid groups were activated with a solution (32 μL) of 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC, Fluorochem, 0.4 M in ultrapure water) and N-hydroxysuccinimide (NHSS, Sigma-Aldrich, 0.1 M in ultrapure water) for 45 min. Electrodes were rinsed with ultrapure water and the enzyme solution (10 μL at 10–100 μM) was applied for 1 h at room temperature, followed by rinsing with ultrapure water. For PFE experiments, enzyme films were reductively activated under an electrochemical cell headspace of 100% H2, with application of a reducing potential (typically ~0.66 V). Progress was monitored by periodically returning to a H2 oxidation potential (~0.2 to 0 V, depending on the enzyme) and monitoring the H2 oxidation current. Activation was deemed complete when there was no significant increase in current.

"Film loss" describes a general decrease in current over time and can reflect a number of changes in the enzyme film, for example, the physical desorption of the enzyme from the electrode, global and/or local unfolding of the enzyme or rearrangement of its orientation. Film loss is exponential and the degree to which it occurs depends on the history of the film. Counterintuitively, "film loss" effects have also been reported in cases where hydrogenases are covalently attached to an electrode surface, presumably due to enzyme instability under the experimental conditions and initial removal of physically adsorbed material.32,38,39 Film loss correction, where indicated, was achieved by always returning to a standard condition; for example, after exposure to a different pH, the film was always re-exposed to pH 6.0 and the decrease in current was monitored at each potential over time. Any film loss was then corrected for by fitting the measured current at pH 6 to an exponential decay function.47

A similar modification protocol was used to obtain high coverage enzyme films for PFIRE experiments (see Supporting Information). Enzyme-loaded particles were held under a H2 atmosphere (3 bar, 4 °C) for 5 days tofacilitate adsorption and simultaneous activation of the as-isolated enzyme. Before carrying out PFIRE measurements, the immobilized enzyme was activated further in the PFIRE cell by poising at a reducing potential (~0.6 V) under 100% H2 for 12 h. Periodically (every 2 h) the atmosphere was switched to 100% Ar to ensure complete removal of oxidized, inactive species formed during aerobic purification of the enzyme.

2.4. Crystal Structure Determinations. Aerobically purified ("as-isolated") enzymes were crystallized as described previously,17,18,22,25 at pH 5.9 for E28Q (Table S4), pH 8.1 for native Hyd-1, pH 5.9 for E28D (Table S5), and pH 5.9 for E14Q-Hyd-2 (Table S6). Reduced samples were prepared using as-isolated enzyme at pH 5.9 or 8.1, purged under an atmosphere of 100% H2 in an anaerobic glovebox (Belle Technology Ltd, UK, O2 <2 ppm), at 5 °C for 40 h (E28Q) or 18 h (native Hyd-1), then crystallized as described previously22 at 23 °C (ambient glovebox temperature). Reduction of the E14Q variant by H2 was achieved by exposing an enzyme solution (5 mg/mL) to 4 bar H2 in an pressure vessel (Tinyclave Steel, Büchigasuster) for 12 h at 4 °C, then crystallized as described previously at 23 °C (ambient glovebox temperature).21 To obtain a structure at pH 10, H2-reduced crystals of E28Q (grown at pH 8.1) were soaked for 2–3 min in a solution containing 100 mM CAPS, pH 10.0, 150 mM NaCl, 200 mM LiSO4, 23% (w/v) PEG 3350. All crystals were cryo-protected by adding glycerol (15%, v/v) and 2% extra PEG 3350 to the crystallization buffer before flash-cooling in liquid N2 in the glovebox. From this point on we will refer to the pH of the crystal structures as "6", "8", or "10".

X-ray diffraction data were collected at Diamond Light Source at beamlines I24 or I03 (at wavelength = 0.98 Å, Pilatus 3 M detector), or beamline 104-I (at wavelength = 0.92 Å, Pilatus 6 M detector). Data reduction was performed with DIALS43 and AIMLESS.44 Initial phase estimates were generated by performing rigid body refinement of the native Hyd-1 structure (pdb 54AM) into the unit cell of the different variant Hyd-1 proteins using REFMAC42.

The same procedure was used for E14Q instead using the Hyd-2 structure (pdb 6EHQ). Twenty rounds of rigid-body refinement were performed with the data truncated to a maximum resolution of 6 Å, before carrying out a further 20 rounds with the data extended to the maximum resolution available. Models were manually adjusted with COOT45 followed by all-atom refinement using REFMAC, a process that was repeatedly repeated until refinement converged. The degree of oxygenation of cysteine 79 in the as-isolated structure of E28Q was estimated as described previously.17

3. RESULTS

3.1. Enzyme Production. All Hyd-1 enzymes were produced to a similar yield (~0.2 mg/L media) and were indistinguishable by denaturing electrophoresis (Figure S2A), each comprising proteolytically processed large (HybA) and small (HybA) subunits following Ni-affinity chromatography. Production of all Hyd-2 enzymes utilized the overproduction system described recently,31 raising the yield in-line with that of Hyd-1. The Hyd-2 enzymes were indistinguishable by denaturing electrophoresis (Figure S2B), both containing overproduced HybO small subunit, and processed HybC large subunit.

3.2. Characterization of E28 Variants by PFE and Discovery of Unusual Activities. Viewed with a normal PG electrode at pH 6, the E28Q variant of Hyd-1 is inactive except for a marked increase in current above +0.1 V (Figure S3). In contrast, using a modified electrode,17,25,31,32 the detailed H2 oxidation activity of E28Q becomes clearly visible, revealing a potential dependence that differs greatly from native Hyd-1 (Figure 2A). Two distinct catalytic potential regimes are clearly evident: at pH 6.0 the onset of H2 oxidation occurs at approximately −0.3 V and a second regime commences at around +0.05 V. In contrast, native Hyd-1 shows only one potential regime for H2 oxidation, which commences at approximately −0.3 V, as previously noted in several papers.17,19,30 The second catalytic regime seen with E28Q, which results in a 3-fold increase in current at +0.6 V relative to that at +0.05 V, is due to enzymatic H2 oxidation: switching to 100% Ar causes the current to drop to background levels (Figure 2B) and returning to 100% H2 at ~ +0.3 V immediately restores the current, which is retained upon subsequent scans. For both enzymes, the return scan to more negative potential shows an activation step, seen as a ridge, which appears at a more positive potential for E28Q.

The experiments were repeated over the pH range 4–10.8 for native Hyd-1 (Figure 3A) and E28Q (Figure 3B). Measured at ~0.2 V, the H2 oxidation activity of the native enzyme reaches a maximum at pH 8. The decrease in current that occurs as the potential is increased further, and which is most evident at high pH, is generally ascribed to the formation of resting state Ni-B35 (but see later), which contains a bridging OH– ligand.45 The H2 oxidation activity of E28Q shows a completely different trend with pH, particularly as the activity measured at ~0.2 V increases with pH, accelerating sharply above pH 9 (Figure 3B and Figure S4). Like native Hyd-1, the E28Q variant only becomes a reversible catalyst at low [H+] and low pH46,47 (Figure S4 and Figure S5), although the H+ reduction rate must be extremely low.

To investigate the origin of this shift in pH dependence of maximal H2 oxidation activity, the Hyd-1 E28D variant was made (Table S1). The mutation retains the carboxylate functionality but shortens the side chain by one carbon atom. Protein film voltammetry of E28D revealed only one
potential regime and a pH dependence similar to native Hyd-1 and in contrast to E28Q (Figure S6).

3.3. Steady-State H₂ Oxidation Rates of E28 Variants Measured by PFE and Solution Assays. Figure 4 compares the steady-state H₂ oxidation activities of native Hyd-1 and E28Q across a wide pH range (pH 6–12), as measured by PFE (at ~0.2 V, Figure 4A) and solution assays (Figure 4B and Table S2). The maximal activity at ~0.2 V for native Hyd-1 occurs at pH 8, whereas for E28Q it continues to rise up to at least pH 10.5, above which the current becomes very unstable, as shown in Figure 3C which includes experiments conducted up to pH 12. The general pH trend of activities for both enzymes as measured by PFE agrees very well with the steady-state H₂ oxidation activities of native Hyd-1 and E28Q, both of which can be attributed to enzymatic H₂ oxidation (B); A direct comparison is shown of the voltammetric response of native Hyd-1 (black trace, A) and E28Q (red trace, A) scanned at 5 mV/s between ~0.6 and +0.6 V in 100% H₂ (arrows indicate scan direction). The native Hyd-1 film was produced using a standard PGE electrode and the E28Q data were measured using a modified electrode, i.e., the absolute currents do not represent turnover rate as coverage is not accounted for. Other conditions: pH 6.0, 37 °C, total gas flow rate maintained at 1000 scc/min, ω = 1000–3000 rpm. The two potential regimes of E28Q report on hydrogen oxidation (B); A direct comparison is shown of the voltammetric response of native Hyd-1 (black, B) and E28Q (red, B) scanned at 5 mV/s between ~0.6 and +0.6 V in 100% H₂ (arrows indicate scan direction). The native Hyd-1 film was produced using a standard PGE electrode and the E28Q data were measured using a modified electrode, i.e., the absolute currents do not represent turnover rate as coverage is not accounted for. Other conditions: pH 6.0, 37 °C, total gas flow rate maintained at 1000 scc/min, ω = 1000–3000 rpm. The two potential regimes of E28Q report on hydrogen oxidation (B); A direct comparison is shown of the voltammetric response of native Hyd-1 (black, B) and E28Q (red, B) scanned at 5 mV/s between ~0.6 and +0.6 V in 100% H₂ (arrows indicate scan direction). The native Hyd-1 film was produced using a standard PGE electrode and the E28Q data were measured using a modified electrode, i.e., the absolute currents do not represent turnover rate as coverage is not accounted for. Other conditions: pH 6.0, 37 °C, total gas flow rate maintained at 1000 scc/min, ω = 1000–3000 rpm. The two potential regimes of E28Q report on hydrogen oxidation (B); A direct comparison is shown of the voltammetric response of native Hyd-1 (black, B) and E28Q (red, B) scanned at 5 mV/s between ~0.6 and +0.6 V in 100% H₂ (arrows indicate scan direction). The native Hyd-1 film was produced using a standard PGE electrode and the E28Q data were measured using a modified electrode, i.e., the absolute currents do not represent turnover rate as coverage is not accounted for. Other conditions: pH 6.0, 37 °C, total gas flow rate maintained at 1000 scc/min, ω = 1000–3000 rpm.

From PFE experiments (and PFIRE, see later) it was clear that Hyd-1 variants incorporating the E28Q change are extremely difficult to activate (days not hours). Because reduction of oxidized inactive states Ni-B and Ni-A (and other unready states44) requires protons as well as electrons, this observation was not surprising, but it served to emphasize the need for exhaustive activation of as-isolated enzymes.

3.4. Additional Variants to Investigate the Involvement of the Proximal Fe–S Cluster. We considered it certain that the high-potential activity commencing above
As for native Hyd-1 this is the upper limit for H$_2$ oxidation before chronoamperometry buffer exchange experiment was used at each pH in the range 6–10, while for E28Q the average current at ~0.2 V during a chronoamperometry experiment was used at each pH in the range 6–12 (Figure 3C). The potential of ~0.2 V was chosen as for native Hyd-1 this is the upper limit for H$_2$ oxidation before inactivation to Ni-B at pH 10. All fractional PFE activity and steady-state rate data are presented as a percentage relative to pH 6 (pH 6 is normalized to 100% and is 252.8 ± 27.3 s$^{-1}$ for native Hyd-1 and 1.5 ± 0.1 s$^{-1}$ for E28Q, see Table S2). For steady-state solution assays, errors represent the propagated standard error of the mean of at least three repeats.

+0.05 V at pH 6 for E28Q must be associated with a change in state of the enzyme linked to oxidation of a redox-active group. Only two site options are available, based on redox transitions previously assigned for the native enzyme at pH 6: both involve Fe–S clusters, either the medial cluster [3Fe–4S]$^{1+/0}$ ($+0.130 ± 0.015$ V) or the proximal cluster, which can perform two one-electron reactions: [4Fe–3S]$^{1+/2/3+}$ (+0.030 ± 0.030 V) and [4Fe–3S]$^{1+/2/3+}$ (+0.230 ± 0.015 V). All O$_2$-tolerant [NiFe]-hydrogenases (group 1d) so far studied possess this special proximal cluster, which is coordinated by 6 cysteines. This special function appears to be to provide an additional electron when O$_2$ attacks, becoming coordinated by a peptide-N ligand in the superoxidized state, in a process that requires a substantial conformational change at the cluster along with local proton transfer.18,22,48–52 Because of its high reduction potential and large conformational change requirements, it is unlikely that the superoxidized state is formed during normal H$_2$ catalysis by native Hyd-1. However, the proximal cluster is located just 7.7 Å (nearest atom distance) from E28 (Figure 1A), making it, and specifically a property conferred by the superoxidation transition, the prime candidate for the enhanced activity of E28Q at high-potential. The medial cluster is too far from E28 to make it a reasonable candidate.

In Hyd-2, the proximal Fe–S cluster is a standard [4Fe–4S]$^{1+/2/3+}$ cubane located 7.9 Å from the equivalent residue E14. Standard Fe–S clusters have more negative reduction potentials than the special proximal cluster of O$_2$-tolerant hydrogenases, transferring only one electron to the active site without substantial conformational change. To investigate the proximal cluster involvement in the high-potential activity of E28Q, we made the equivalent variant in Hyd-2 (E14Q), where a substantial conformational change at the cluster would not be expected upon oxidation.21

As for E28Q, PFE experiments with Hyd-2 E14Q required use of a modified electrode in order to see a substantial H$_2$ oxidation current. Within the pH range of 6–8, it was clear that there is no enhancement of H$_2$ oxidation activity up to +0.29 V (Figure 5A) as the voltammograms showed the expected reversible electrocatalysis ($H^+$ reduction at low potential) and reversible inactivation at high potential.15 Above pH 8, E14Q is unstable and the resulting decrease in activity (“film loss”) restricted further experiments. Oxidation of H$_2$ in steady-state solution assays between pH 6–9 occurs at a drastically lowered rate (>600-fold at pH 6) (Table S3). The pH dependence of the steady-state turnover rate for E14Q mirrored that of Hyd-1 E28Q, increasing with pH up to pH 9, in contrast to native Hyd-2 which is optimal at pH 8.0 (Figure S7). To investigate the involvement of the proximal cluster without the limitation of protein instability at high pH, we produced a triple C19G/C120G/E28Q variant of Hyd-1 to create a standard cubane proximal cluster incapable of forming the superoxidized state. The plan was based on previous mutagenesis work substituting the supernumerary cysteines of Hyd-1 and *Ralstonia eutropha* MBH.50,51 The triple variant displayed only low activity (noting that the C19G/C120G proximal cluster variant of Hyd-1 previously displayed approximately half the steady-state activity of the native enzyme15) and again required the use of a modified electrode. Importantly, for all pH values, there was no evidence of enhancement of activity at high-potential (Figure S5). These experiments were carried out at rotation rates up to 2500 rpm to exclude H$_2$ mass transport limitations. The current at ~0.2 V increases up to at least pH 10, as previously seen for E28Q (Figure 3B,C).

3.5. Structural Investigations. The PFE data established that replacing the highly conserved glutamate-28 of Hyd-1 by glutamine does not completely block catalytic proton transfer. Indeed, the enzyme recovers much of its activity under the special conditions of high potential and/or high pH. We thus investigated the structures of Hyd-1 variants at position 28 to identify any differences that might underlie the restoration of activity.

The hydrogenase crystals displayed a long rod-shaped morphology, as previously described,17,21,23 and diffraction data could be collected as a helical line scan to spread the radiation dose across the entire volume of the crystal, thus minimizing radiation damage resulting from the photodecomposition.
eff ect. All crystals diffracted to high resolution (1.05−1.6 Å).

Tables S4−S6 show the X-ray data collection and refinement statistics for each structure, namely, E28Q oxidized (as-isolated, Figure 6A) and reduced under H₂ at pH 6 (Figure 6B), pH 8 (Figure 6C), and pH 10 (Figure 6D), native Hyd-1 oxidized (as-isolated, Figure 6E) and reduced under H₂ (Figure 6F), E28D oxidized (as-isolated, Figure S11), and E14Q (as isolated and H₂ reduced Figures 7 and S13).

Structure of E28Q (oxidized, as-isolated). A comparison of the orientation of the Q28 side chain in the oxidized form of E28Q (at pH 6, Figure 6A) relative to native Hyd-1 (at pH 8, Figure 6E), suggests a partial disorder of Q28 by rotation about the Cα−Cβ bond: the 2Fo−Fc density for the Cγ, Cδ, Ne and Or atoms is 70% that of Cα and Cβ. An O atom, either H₂O or OH⁻, is seen very close to the amide headgroup with approximately 30% occupancy (peak height of 1 e/Å³). The proximal cluster is in the superoxidized state, characterized by Fe7 swinging away from the cluster core and coordinating the backbone peptide-N of C20 (Fe7 is separated from N by 2.1 Å).22 Appearance of the superoxidized state of the proximal cluster in the structure is consistent with data from recent EPR spectroscopic experiments.26 Nearby carboxylate-bearing residue E76 adopts two conformations, as previously described:22 in approximately half the molecules in the crystal lattice, the side chain has rotated to position its carboxylate-O atom 2.15 Å from Fe7. Two of the Fe atoms of E28Q, as-isolated (air-oxidized) structure is shown (A) with the hydroxide (red sphere) in the bridging position between Ni (green sphere) and Fe (orange sphere), and oxygenation at C79 as expected for the Ni-A state. An O atom, either H₂O or OH⁻, is seen very close to the amide headgroup with approximately 30% occupancy (peak height of 1 e/Å³). The proximal cluster is in its reduced pseudocubane state with no apparent oxidation of the Fe atoms. The conformational heterogeneity of E76 has also been removed upon reduction. The same structural features are seen with the Native enzyme in its as-isolated state (E) with the exception of the extra water molecule and rotational heterogeneity of residue 28 (see text). Upon reduction, the native structure (F) again shows a similar loss of the typical features of oxygenation as detailed for E28Q.
in the as-isolated (air-oxidized) form (A, silver) and H2-reduced form (B, blue). There is minimal change to the active site and proximal cluster upon substitution of glutamate for glutamine, or upon reduction (for an overlay see Figure S13). There is no electron density near the amide headgroup of Q14, i.e., in the position occupied by a H2O/OH− in E28Q (see text). There is no evidence of the oxidation at the proximal cluster that was previously seen for native Hyd-2.21

the proximal cluster (Fe4 and Fe7) appear to have additional ligation (Figures S8A and S9), and we note that an O-ligand at Fe4 has previously been observed in the MBH from Ralstonia eutropha.34 There is minimal change at the NiFe active site, which can be superposed on that of as-isolated native Hyd-1 with an RMSD of 0.11 Å (Figure 6A, E, and Figure S8A). Bridging ligand C79 is 40% oxygenated (50% for the native enzyme) and a hydroxide occupies the bridging position between the Ni and Fe atoms: both features have previously been seen in as-isolated native Hyd-122 and in hydrogenases from Desulfovibrio fructosovorans and Allochromatium vinosum.35

Structure of E28Q (H2 reduced). In the H2-reduced state, at pH values studied, the disorder observed in the Q28 side chain for a subpopulation of the as-isolated enzyme no longer appears (Figure 6B–D and Figure S8B). At pH 6, the low-occupancy H2O/OH− molecule close to Q28 is no longer present; however, at pH 8 and pH 10 electron density peaks with heights 0.44 and 0.7 e/Å3 respectively, appear in the same position as the H2O/OH− molecule in the as-isolated, oxidized enzyme. The distance between the side chain of Q28 and the NiFe center also increases slightly at pH 10. Additionally, an ordered CAPS molecule is observed at the surface of the large subunit (chain M) adjacent to residues 184–189. The proximal cluster and surrounding residues undergo the expected18,22,52 conformational changes upon reduction at all pH values, as also seen for native Hyd-1 (Figure 6F, and Figure S8B): Fe7 has moved back toward the body of the [4Fe–3S] cluster to form a pseudocubane structure and the bond between the peptide-N and the Fe atom, characteristic of the superoxidized state, is no longer present. The side chain heterogeneity of residue E76 is removed (Figure S8) and the oxygen-containing ligands to Fe4 and Fe7 (Figure S9) are no longer present. At the active site of E28Q, the distance between the Ni and Fe atoms has shortened from 2.98 to 2.66 Å (as observed previously56) causing terminal cysteines, C76 and C576, to rotate approximately 10° toward the Ni atom, dragging the polypeptide backbone toward the metal atoms and slightly decreasing the volume of the active site cavity. As a result the amide headgroup of Q28 moves 0.4 Å closer to the NiFe center, (coordinate uncertainty is 0.07 and 0.03 Å for as-isolated and reduced structures respectively).57 The electron density ascribed to the bridging OH− species in the as-isolated structure is removed upon reduction; however, a peak (0.94 e/Å3) in the Fo−Fc map is clearly visible close to and between the metal centers, located 1.6 Å from the Ni and 1.7 Å from the Fe atom (Figure S10). The coordination distances of the Ni and Fe atoms to the center of this peak are typical of a hydride ion and superposition of the active site of the Ni5-R form of D. vulgaris Myazaki F,58 places the associated hydride in the residual density. Interestingly, bound molecular hydrogen (i.e., the Michaelis complex) may also contribute to the density at the bridging position since the variant has been crystallized at pH 5.9 where turnover is slow; the equivalent variant in D. fructosovorans has been shown to be capable of para/ortho hydrogen conversion, but not proton exchange with solvent.24

Structure of E28D (oxidized, as-isolated). The as-isolated active site of E28D is unaffected in terms of the positions of the metal coordinating cysteines and residues in the canopy; there is a hydroxide ligand in the bridging position and C576 shows signs of significant oxygenation (Figure S11).17 Due to changes in the hydrogen bonding network formed by the truncated side chain of aspartate (relative to glutamate/glutamine), the polypeptide backbone comprising residues I27, D28 and G29 moves away from the active site relative to E28Q (Figure S12), further demonstrating the flexibility of the polypeptide backbone around residue 28 (see above). The truncation of the side chain also allows an additional H2O/OH− molecule to enter the active site cavity: the H2O/OH− occupies the same position as that described above for E28Q in the oxidized, as-isolated state. The proximal cluster of E28D is in the superoxidized conformation. Again an additional H2O/OH− ligand bound to Fe4 is observed and residue E76 adopts two, equally populated conformations allowing interaction with the mobile Fe7. atom.

Structure of Hyd-2 Variant E14Q, Oxidized (as-isolated) and H2 Reduced. The structures of the active site, proximal cluster and side chain of residue 14 in the Hyd-2 E14Q variant are unchanged relative to the native structure19 in both the as-isolated and reduced forms at pH 6 (Figure 7 and Figure S13). The proximal cluster is a standard cubane Fe4 has previously been observed in the MBH from Allochromatium vino- sum.35
3.6. Steady-State Protein Film Infrared Electrochemistry of E28Q. The aim of the PFIRE experiments was to identify catalytic intermediates (Figure 1B) for which steady-state levels are elevated or suppressed during turnover as a result of severely restricting proton transfer(s) out of the active site. By this means it was hoped to determine which steps depend on E28 for providing a proton escape route during catalytic H$_2$ oxidation.

The importance of thorough activation of E28Q has been highlighted previously for PFE experiments, and was also noted for analogous PFIRE measurements: use of a partially activated E28Q electrode revealed a much larger population of states at the Ni$_{\text{a-SI}}$ redox level, presumably both active and inactive (not shown).

Measurements were carried out at pH 7.0, under alternating 100% H$_2$ and 100% Ar atmospheres over a range of applied potentials (Figure S14). A voltammogram recorded in the spectroelectrochemical cell immediately prior to PFIRE measurements confirmed that the electrochemical response of the protein on the carbon black electrode was similar to that recorded on a MWNT-modified electrode using PFE (compare Figure 3B and Figure S15).

The difference spectrum obtained by comparing spectra at +356 and −600 mV under Ar (Figure 8A) is qualitatively similar to that measured for native Hyd-1 at pH 7.5. Electrochemical reduction results in complete conversion of the oxidized, inactive, Ni-B state (positive peak) and Ni$_{\text{a-R}}$ becomes the majority species at −600 mV (negative peak). A small amount of Ni$_{\text{a-C}}$ is evident at −600 mV, but any Ni$_{\text{a-L}}$ that might be formed is difficult to identify with certainty above the background (it is worth mentioning that native Hyd-1 is unusual in that Ni$_{\text{a-L}}$ is observed to a much greater degree at Ni$_{\text{a-C}}$ at pH 7.589). At +356 mV under Ar, a small amount of an additional species is observed with $\nu_{\text{CO}}$ close to 1900 cm$^{-1}$, similar to that reported for other [NiFe]-hydrogenases in oxidized states.55,60

As shown in Figure S16, the active site speciation of E28Q at equilibrium under Ar as a function of potential resembles that of native Hyd-1:53 under nonturnover conditions, Ni$_{\text{a-SI}}$ dominates at −100 mV (i.e., during the low potential regime, see Figure 3B and Figure S17) and Ni$_{\text{a-B}}$ appears at +100 mV (i.e., during the high potential regime, Figure 3B and Figure S17). The difference spectra in Figure 8B,C reflect the distribution of active site species at equilibrium under Ar (positive peaks) and during steady-state H$_2$ oxidation (negative peaks) in both the low (Figure 8B, −100 mV) and the high (Figure 8C, +100 mV) potential regimes. Under conditions driving steady-state H$_2$ oxidation, there is surprisingly little difference between the active site speciation in either potential regime: in both cases Ni$_{\text{a-R}}$ dominates, with Ni$_{\text{a-C}}$ being the only other active species present at an observable level. The prevalence of Ni$_{\text{a-R}}$ during steady-state H$_2$ oxidation, even when the driving force is approximately 0.5 V, is fully consistent with previous PFIRE measurements for native Hyd-1.53

For E28Q, under equilibrium conditions (Figure 8A) and during steady-state H$_2$ oxidation (Figure 8B–D), the dominance of Ni$_{\text{a-C}}$ over Ni$_{\text{a-L}}$ is in stark contrast to the native enzyme, for which the Ni$_{\text{a-L}}$ level exceeds that of Ni$_{\text{a-C}}$ by an approximately 2:1 ratio at pH 7.590 Only one form of the Ni$_{\text{a-R}}$ state, Ni$_{\text{a-R}}$(III), is observed in E28Q, again in contrast to native Hyd-1. The Ni$_{\text{a-R}}$(I) state is not seen for native Hyd-1 or E28Q.53,61 The disparity of Ni$_{\text{a}}$-R states between the two enzymes possibly reflects the role of individual Ni$_{\text{a}}$-R states in sequential proton transfers in the active site during the catalytic cycle.51,62

Poising at a potential of +356 mV under 100% H$_2$ results in a dramatic loss of catalytic H$_2$ oxidation activity (Figure S14) consistent with the PFE results for E28Q presented earlier. Importantly, the loss of activity does not correlate with Ni-B formation as defined by the IR spectra, and only a small loss of Ni$_{\text{a-SI}}$ is observed after inactivation at +356 mV for 1 h (Figure S18). This observation contrasts completely with the situation for native Hyd-1,55 where under H$_2$ Ni$_{\text{a-SI}}$ is observable at −70 mV and undergoes significant conversion of to Ni-B even after a short poise at +356 mV. For E28Q, the Ni-B signal is obtained only after replacing the H$_2$ atmosphere with Ar (Figure 8D) whereupon all remaining activity is lost (Figure S14).

![Figure 8. Protein film IR electrochemistry (PFIRE) of E28Q at pH 7.0, showing the $\nu_{\text{CO}}$ active site spectral region. Data are presented as difference spectra, showing the change in active site speciation as a result of a potential step under an inert Ar atmosphere (A) or in response to steady-state electrocatalytic turnover in the presence of 100% H$_2$ (B, C and D). Positive bands reflect the equilibrium (non-turnover) behavior of the active site at each potential, and negative bands show states present at low potential (A) or during turnover under 100% H$_2$. Labeled dashed lines indicate the $\nu_{\text{CO}}$ positions of active site states in native Hyd-1.53 Black dashed line highlights a small amount of an additional species at $\sim$1900 cm$^{-1}$ at +356 mV under Ar (see text).55,60 Scale bars represent 0.1 m.O.D. Other details: experiments carried out at 27 °C; H$_2$-saturated solution, flow rate of 50 mL/min through the PFIRE cell, such that the electrocatalytic current was maximal and independent of flow rate; gas exchange carried out in situ at each potential; linear baseline correction applied between 2150 and 1850 cm$^{-1}$. The accompanying current–time trace is shown in Figure S14, and the complete difference spectra including the $\nu_{\text{CN}}$ region are shown in Figure S17.](image-url)
4. DISCUSSION AND CONCLUSIONS

The well-documented role of residue E28 (or its equivalent) in transferring protons in and out of the active site of [NiFe]-hydrogenases has so far remained unchallenged. For Hyd-1 it is very significant that the activity of E28Q at neutral pH is not zero, but is of sufficient magnitude to allow spectroscopic measurements that reveal the speciation under conditions of steady-state catalysis. Were a variant to be completely inactive, no such information could be obtained. On the other hand, a variant which retains high activity cannot yield clearly interpretable results. The activity of E28Q at neutral pH is established to be a "base-level" condition that is relaxed upon increasing the pH or the electrode potential. Our ability to measure activity and catalytic speciation in a variant for which an essential route for proton transfer out of the active site has now been crystallographically defined over a range of conditions has important consequences for understanding the mechanism of steady-state H2 oxidation. These results and their significance are now discussed.

4.1. Case for the Active Site Being a Near-Perfectly Encapsulated System. At pH 6, H2 oxidation catalysis by E28Q is so slow that the active site of Hyd-1 can be considered to be encapsulated with regard to a key proton-transfer event during catalysis: E28 offers an essential route for proton exchange with the external environment, without which there is only a low background turnover rate (approximately 1%, Table S2). Activities reported for the equivalent variant in other bacterial systems vary considerably, making Hyd-1 E28Q an extremely valuable model system for the study of E28 as the proton gate.

Numerous crystallographically ordered waters have been previously noted among the O2-tolerant class of [NiFe]-hydrogenases. Their positions are highly conserved and they could be involved in specific proton transfers within the active site. If proton transfer in Hyd-1 occurred solely via the carboxylate side chain of E28, the Q28 variant should show zero activity. At low pH and most negative potential, where activity is minimal, the background rate of H2 oxidation lies in the same range as the rate of water molecule release demanded by the rate constant for O2 reduction by native Hyd-1 (Hyd-1 is a hydrogen oxidase). It is reasonable to attribute this low background activity to an alternative proton-transfer pathway that uses direct migration of an H2O/OH− entity and need not involve residue 28.

4.2. Unusual Increase in Activity at High pH. The stability of Hyd-1 enzymes at pH > 8 is fortunate as it allows considerable H2 oxidation current to be measured at moderate potentials, and for crystal structures to be obtained at high pH. The low rate of H2 oxidation increases with pH in all variants containing glutamine in position 28, i.e., E28Q and the triple variant of Hyd-1. Even over a limited pH range, this effect is also seen in the Hyd-2 E14Q variant. Hence, the barrier to H2 oxidation at moderate potentials depends upon a group that is ionized at high pH. A correlation is made here with the proximal cluster reduction, an action that displaces the OH− ion, suggesting a plasticity of proton-transfer groups: the glutamine side chain only adopts a native-like conformation in 70% of the molecules when the proximal cluster is super-oxidized. In the remaining 30% the side chain is disordered by rotation about the Cα−Cβ bond. Rotation of the equivalent aspartate residue about the Cα−Cβ bond has also been observed in a T18V variant of the O2-sensitive [NiFe]-hydrogenase from D. fructosovorans but with no addition of OH− near residue 28. The affinity of OH− for occupying the position near to the amide of Q28 thus depends on two factors: (1) the proximal cluster being in the superoxidized form or, (2) providing a high pH, even when the proximal cluster is reduced.

4.3. Which Catalytic Steps Are Obstructed during H2 Oxidation? A key question we sought to address in this study is: at which stage (or stages) of the catalytic cycle (Figure 1B) is a proton required to leave the active site via E28? Following thorough activation, the PFIRE data show which stages in the catalytic cycle (Figure 1B) are obstructed in the E28Q variant during steady-state oxidation of H2 (100%) at pH 7 (a pH at which the activity of E28Q is lowered to approximately 3% at 0 V, i.e., approximately 1% of the native value) when the electrochemical driving force is varied across a wide oxidizing region. Notably, in E28Q, N2−R, followed closely by N2−C, are the dominant species across the whole range of oxidizing hydroxide at high pH and the principle of conservation of charge, it is likely that the O atom entity is a hydroxide ion. In such a way, the carboxylate is replaced by an electrostatically equivalent amide-hydroxide pair.
conditions, even at $+356 \text{ mV}$, a potential at which native Hyd-1 converts to Ni-B.33

Starting the catalytic cycle (Figure 1B) at the level at which $H_2$ binds, i.e., Ni$_x$-SI, our results establish that stages 1 and 2, the binding and heterolytic cleavage of $H_2$, do not depend on E28, a result first noted by Dementin and co-workers.24 We are now in a position to extend those conclusions to formulate a description of the states dominating during steady-state catalytic $H_2$ oxidation and, therefore, the stages that depend on residue E28. Were E28 to be essential for stages 1 and 2 then we would expect to see accumulation of Ni$_x$-SI as the dominant species. Advancement of the cycle through to Ni$_x$-C also progresses unabated during turnover and hence stage 3 (the earliest stage at which a proton from $H_2$ must exit the active site) is also unlikely to involve E28. Noting that E28 is not close enough to the Ni and/or Fe atoms to be a primary base (Figure 1A), these results suggest that the primary base required for deprotonation of $H_2$ does not depend on E28 for proton exit, or else we would expect to see Ni$_x$-SI as the dominant species and considerably less Ni$_x$-C. The Ni$_x$-L state is a catalytic intermediate in Hyd-1, yet is rarely reported for other hydrogenases during turnover.24 In native Hyd-1 the Ni$_x$-L state dominates at $pH \ 7$ (Ni$_x$-C speciation is increased in the native enzyme at low $pH$) and this is consistent with the proposal that an electron from the active site must first transfer to the proximal cluster, which due to its high potential will have a high probability of already being in the reduced state.59

For E28Q, the increased speciation at the level of Ni$_x$-C (which is observed almost to the exclusion of Ni$_x$-L) suggests that E28 assists in allowing the hydride to migrate as a proton from the NiFe site in stage 4, consistent with the work of Greene et al.: in the glutamine variant of the soluble hydrogenase I (SHI) from Pyrococcus furiosus, the glutamate proton gate was implicated in the transition from Ni$_x$-C to Ni$_x$-SI, with formation of the Ni$_x$-L state only achieved upon photolysis of the bound hydride of Ni$_x$-C.27 On the basis of our work with Hyd-1 under turnover conditions, we can now conclude that residue E28 is not essential for activation of $H_2$ from the level of Ni$_x$-SI to form the Ni$_x$-C state, but is essential for the progression of $H_2$ oxidation beyond the Ni$_x$-C state. It may be significant that the persistent appearance of electron density in the bridging position in reduced structures of E28Q, that we tentatively attribute to a hydride based on the work by Ogata et al.,58 reflects the accumulation of both the Ni$_x$-R and Ni$_x$-C states that are observed spectroscopically.

The unexpected observation that the oxidized inactive state Ni-B is not produced when E28Q is subjected to a high potential in the presence of $H_2$ (Figure S18) is now easily explained: interaction of $H_2$ with the active site is maintained in E28Q, and the early stages of $H_2$ activation easily outpace electrochemical oxidation. Formation of Ni-B from Ni$_x$-SI under anaerobic conditions (Figure 1B) involves formation of a Ni–OH bond that requires not only the passage of a $H_2O$ molecule into the active site (assuming OH$^-$ does not transfer in directly) but also transfer of a $H^+$ followed by oxidation of the Ni. If a proton has to exit the active site via E28, it is much easier to start another cycle by activating $H_2$ and generating Ni$_x$-R. This competition is retained even when $H^+$ transfer is accelerated as the superoxidized form of the proximal cluster becomes dominant. The switch-off of activity of E28Q at very high potential (Figure S14) that is always observable as the potential is swept in the negative direction (Figure 3B), and is normally attributed to formation of Ni-B, must therefore have a different origin. A plausible explanation is that catalytic electron transfer from the active site to the Fe–S cluster relay is retarded due to the higher reorganization requirements associated with cycling the superoxidized proximal cluster.10,66,67 The active site remains at the level of Ni$_x$-C or Ni$_x$-R and turnover is stalled. Upon application of a more negative potential, proton-coupled electron transfer is restored and the active pool of enzyme is re-established (Figure S14).

In summary, the active site of Hyd-1 behaves as a near-perfectly encapsulated system, in and out of which proton transfer is strictly regulated, a key residue being glutamate 28. During $H_2$ oxidation, experiments with the Q28 variant show that E28 is not required for transferring protons out of the active site between intermediates Ni$_x$-SI and Ni$_x$-C. Glutamate 28 provides an essential exit route for the proton that was initially formed as a hydride upon $H_2$ cleavage, i.e., during the latter oxidative part of the catalytic cycle. As a consequence, in E28Q, the Ni$_x$-SI intermediate does not accumulate during $H_2$ oxidation. The structure of the E28Q variant has been examined in detail, the results establishing the basis by which the substituted glutamine is a poor proton-transfer site at neutral pH. A partial rescue of the proton-transfer pathway is achieved through displacement of the amide headgroup by a hydroxide ion, the occupancy of which increases with pH or as the electrode potential is raised to favor formation of the unusual superoxidized proximal cluster.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b04798.

Denaturing electrophoresis; plasmid, strains and primer details; PFIRE film preparation; initial E28Q PFE data; additional supporting PFE of E28Q on modified electrodes; PFE data for E28D variant; steady-state solution assay data; X-ray diffraction collection and crystal structure statistics; additional structural figures; PFIRE cyclic voltammograms, chronoamperometry and IR spectra of E28Q (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*stephen.carr@rc-harwell.ac.uk
*fraser.armstrong@chem.ox.ac.uk

**ORCID**

Philip A. Ash: 0000-0001-5264-464X
Kylie A. Vincent: 0000-0001-6444-9382
Fraser A. Armstrong: 0000-0001-8041-2491

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This research was supported by the UK Biological and Biotechnology Sciences Research Council (Grants BB/I022309-1 and BB/L009722/1 to F.A.A.). A studentship for E.J.B. was supported by grants from Global Innovation Initiative and the UK Engineering and Physical Sciences Research Council (EPSRC). F.A.A. is a Royal Society-Wolfson Research Merit Award holder. The work of K.A.V. and P.A.A. was supported by EPSRC grant EP/N013514/1. We are grateful to Amelia Bransett for carrying out initial PFIRE
experiments on E28Q-Hyd-1 during a summer internship. We thank Diamond Light Source for beam-time (proposal mx12346) and staff at beamlines 104-1, 103, and 124 for assistance during X-ray data collection.

### REFERENCES

(1) Olson, J. W.; Maier, R. J. Science 2002, 298 (5599), 1788–1790.
(2) Maier, R. J.; Olczak, A.; Maier, S.; Soni, S.; Gunn, J. Infect. Immun. 2004, 72 (11), 6294–6299.
(3) Woolerton, T. W.; Sheard, S.; Chaudhary, Y. S.; Armstrong, F. A. Energy Environ. Sci. 2012, 5 (6), 7470–7490.
(4) Armstrong, F. A.; Hirst, J. Proc. Natl. Acad. Sci. U. S. A. 2011, 108 (34), 14049–14054.
(5) Boralogadage, N. P.; Arachchige, R. J.; Dutta, A.; Buchko, G. W.; Shaw, W. J. Catal. Sci. Technol. 2017, 7 (5), 1108–1121.
(6) Rakowski DuBois, M.; DuBois, D. L. Chem. Soc. Rev. 2009, 38 (1), 62–72.
(7) Smith, S. E.; Yang, J. Y.; DuBois, D. L.; Bullock, R. M. Angew. Chem., Int. Ed. 2012, 51 (13), 3152–3155.
(8) Vigna, S. M. Coord. Chem. Rev. 2005, 249 (15), 1677–1690.
(9) Kliman, J. P. Trends Biochem. Sci. 1989, 14 (9), 368–373.
(10) Page, C. C.; Moser, C. C.; Chen, X.; Dutton, P. L. Nature 1999, 402, 47–52.
(11) Montet, Y.; Amara, P.; Volbeda, A.; Vernede, X.; Hatchikian, E. C.; Field, M. J.; Frey, M.; Fontecilla-Camps, J. C. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2014, XXX, XXX, XXX.
(12) Arminger, F. A.; Sargent, F.; Carr, S. B. Natl. Acad. Sci. U. S. A. 2017, 114, XXX, XXX, XXX.
(13) Wehlin, S. A. M.; Carr, S. B.; Phillips, S. E. V.; Armstrong, F. A. Catal. Sci. Technol. 2017, 761–777.
(14) Wulf, P.; Day, C. C.; Field, M. J.; Frey, M.; Fontecilla-Camps, J. C. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2011, 67 (4), 840–853.
(15) Murshudov, G. N.; Skubak, P.; Lebedev, A. V.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Vagin, A. A.;Cowtan, K. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2011, 67 (4), 355–367.
(16) Lauterbach, L.; Lenz, O. J. Am. Chem. Soc. 2013, 135, 17897–17905.
(17) Evans, R. M.; Brooke, E. J.; Wehlin, S. A. M.; Nomerotskaia, E.; Sargent, F.; Armstrong, F. A. Proc. Natl. Acad. Sci. U. S. A. 2014, 111 (18), 6606–6611.
(18) Fritsch, J.; Scheerer, P.; Frielingsdorf, S.; Kroschinsky, S.; Friedrich, B.; Lenz, O.; Spahn, C. M. T. Nature 2011, 479 (7372), 249–252.
(19) Lukey, M. J.; Parkin, A.; Roessler, M. M.; Murphy, B. J.; Harmer, J.; Palmer, T.; Sargent, F.; Armstrong, F. A. J. Biol. Chem. 2010, 285 (6), 3928–3938.
(20) Murphy, B. J.; Sargent, F.; Armstrong, F. A. Energy Environ. Sci. 2014, 7 (4), 1426–1433.
(21) Beaton, S. E.; Evans, R. M.; Finney, A. J.; Lamont, C. M.; Armstrong, F. A.; Sargent, F.; Carr, S. B. Biochem. J. 2018, 475, 1353–1370.
(22) Volbeda, A.; Amara, P.; Darnault, C.; Mouesa, J.-M.; Parkin, A.; Roessler, M. M.; Armstrong, F. A.; Fontecilla-Camps, J. C. Proc. Natl. Acad. Sci. U. S. A. 2012, 109 (14), 5305–5310.
(23) Brooke, E. J.; Evans, R. M.; Islam, S. T. A.; Roberts, G. M.; Wehlin, S. A. M.; Carr, S. B.; Phillips, S. E. V.; Armstrong, F. A. Biochemistry 2017, 56, 132–142.
(24) Dementin, S.; Burlat, B.; De Lacey, A. L.; Pardo, A.; Adryanycz-Perrier, G.; Guigliarelli, B.; Fernandez, V. M.; Rouset, M. J. Biol. Chem. 2004, 279 (11), 10508–10513.
(25) Chen, K.; Hirst, J.; Camba, R.; Bonagura, C. A.; Stout, C. D.; Burgess, B. K.; Armstrong, F. A. Nature 2000, 405 (6788), 814–817.
(26) Adamson, H.; Robinson, M.; Wright, J. J.; Flanagan, L. A.; Walton, J.; Elton, D.; Gavaghan, D. J.; Bond, A. M.; Roessler, M. M.; Parkin, A. J. Am. Chem. Soc. 2017, 139 (31), 10677–10686.
R.; Hildebrandt, P.; Friedrich, B.; Lenz, O.; Scheerer, P. Nat. Chem. Biol. 2014, 10, 378–385.

(55) Volbeda, A.; Martin, L.; Barbier, E.; Gutiérrez-Sanz, O.; De Lacey, A. L.; Liebott, P.-P.; Dementin, S.; Roussel, M.; Fontecilla-Camps, J. C. JBIC, J. Biol. Inorg. Chem. 2015, 20 (1), 11–22.

(56) Parida, P.; Kundu, A.; Pati, S. K. J. Cluster Sci. 2009, 20 (2), 355–364.

(57) Cruickshank, D. W. J. Acta Crystallogr., Sect. D: Biol. Crystallogr. 1999, 55 (3), 583–601.

(58) Ogata, H.; Nishikawa, K.; Lubitz, W. Nature 2015, 520, 571–574.

(59) Murphy, B. J.; Hidalgo, R.; Roessler, M. M.; Evans, R. M.; Ash, P. A.; Myers, W. K.; Vincent, K. A.; Armstrong, F. A. J. Am. Chem. Soc. 2015, 137, 8484–8489.

(60) Bleijlevens, B.; van Broekhuizen, F. A.; De Lacey, A. L.; Roseboom, W.; Fernandez, V. M.; Albracht, S. P. J. JBIC, J. Biol. Inorg. Chem. 2004, 9, 743–752.

(61) Ash, P. A.; Hidalgo, R.; Vincent, K. A. ACS Catal. 2017, 7 (4), 2471–2485.

(62) Ash, P. A.; Carr, S. B.; Reeve, H. A.; Skorupskaite, A.; Rowbotham, J. S.; Shutt, R.; Frogley, M. D.; Evans, R. M.; Cinque, G.; Armstrong, F. A.; Vincent, K. A. Chem. Commun. 2017, 53, 5858–5861.

(63) Pankhurst, K. L.; Mowat, C. G.; Rothery, E. L.; Hudson, J. M.; Jones, A. K.; Miles, C. S.; Walkinshaw, M. D.; Armstrong, F. A.; Reid, G. A.; Chapman, S. K. J. Biol. Chem. 2006, 281 (29), 20589–20597.

(64) Mowat, C. G.; Pankhurst, K. L.; Miles, C. S.; Leys, D.; Walkinshaw, M. D.; Reid, G. A.; Chapman, S. K. Biochemistry 2002, 41 (40), 11990–11996.

(65) Evans, R. M.; Parkin, A.; Roessler, M. M.; Murphy, B. J.; Adamson, H.; Lukey, M. J.; Sargent, F.; Volbeda, A.; Fontecilla-Camps, J. C.; Armstrong, F. A. J. Am. Chem. Soc. 2013, 135 (7), 2694–2707.

(66) Radu, V.; Frielingsdorf, S.; Lenz, O.; Jeukens, L. J. C. Chem. Commun. 2016, 52, 2632–2635.

(67) Sigurdsson, E.; Olsson, M. H. M.; Ryde, U. Inorg. Chem. 2001, 40 (11), 2509–2519.

(68) Abou-Hamdan, A.; Ceccaldi, P.; Lebrette, H.; Gutiérrez-Sanz, O.; Richaud, P.; Cournac, L.; Guigliarelli, B.; De Lacey, A. L.; Léger, C.; Volbeda, A.; Burlat, B.; Dementin, S. J. Biol. Chem. 2015, 290 (13), 8550–8558.

(69) Cracknell, J. A.; Wait, A. F.; Lenz, O.; Friedrich, B.; Armstrong, F. A. Proc. Natl. Acad. Sci. U. S. A. 2009, 106 (49), 20681–20686.

(70) Greene, B. L.; Wu, C.-H.; Vansuch, G. E.; Adams, M. W. W.; Dyer, R. B. Biochemistry 2016, 55 (12), 1813–1825.

(71) Ogata, H.; Krämer, T.; Wang, H.; Schilter, D.; Pelmschikov, V.; van Gastel, M.; Neese, F.; Rauchfuss, T. B.; Gee, L. B.; Scott, A. D.; Yoda, Y.; Tanaka, Y.; Lubitz, W.; Cramer, S. P. Nat. Commun. 2015, 6, 7890.

(72) Kampa, M.; Pandelia, M.-E.; Lubitz, W.; van Gastel, M.; Neese, F. J. Am. Chem. Soc. 2013, 135 (10), 3915–3925.