PAPER

Transforming an oxygen-tolerant [NiFe] uptake hydrogenase into a proficient, reversible hydrogen producer†

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Many hydrogenases are highly electroactive when attached to an electrode, and most exhibit reversible $2H^+/H_2$ electrocatalysis, i.e. only a minuscule overpotential is required to drive the reaction in either direction. A notable exception is an important class of membrane-bound O_2 tolerant [NiFe] hydrogenases that appear only to catalyse H_2 oxidation (the uptake reaction), at a substantial overpotential and with little activity for H_2 production, yet possess an active site that is structurally very similar to that of standard, reversible [NiFe] hydrogenases. In a discovery providing important insight into this puzzle, we show that the O_2-tolerant [NiFe] hydrogenase (Hyd-1) from E. coli converts into a reversible electrocatalyst as the pH is lowered from 8 to 3 and becomes an efficient H_2 producer below pH 4. The transformation to a reversible electrocatalyst is not due, trivially, to the higher substrate (H^+_{aq}) availability at low pH but to a large shift in the enzyme's catalytic bias. Systematic investigations provide compelling evidence that the factor controlling this behaviour is the distal [4Fe–4S] cluster, a spectroscopically elusive site that provides the natural entry point for electrons into the enzyme. In E. coli cells, Hyd-1 is located in the periplasmic (extracytoplasmic) compartment and thus, being exposed to the pH extremes of the gastrointestinal tract or the external environment, is a potential catalyst for H_2 production by these bacteria. In a wider context, the observation and proposal are highly relevant for biohydrogen production and catalysis.

Broader context

This paper highlights the importance of enzymes in understanding mechanisms of molecular electrocatalysis – in this case for interpreting the electrochemistry of $2H^+/H_2$ interconversion. Microbial O_2-tolerant hydrogenases are of particular interest as they offer the important possibility of enabling microbes to produce H_2 by modified oxygenic photosynthesis. The best characterised examples – the O_2-tolerant respiratory [NiFe]-hydrogenases – can oxidise H_2 in air without being inactivated by the action of O_2 on the fragile active site: however, a persistent puzzle has been why these enzymes do not catalyse H_2 production, i.e. why they appear to operate exclusively in one direction. The active sites of O_2-tolerant and standard [NiFe]-hydrogenases have the same ligands and similar atomic arrangements. The results described in this paper show how an O_2 tolerant [NiFe]-hydrogenase transforms smoothly into a proficient H_2 producer as the pH is lowered – an effect that is not trivially due to the increase in proton availability but to an adjustment of the enzyme's redox properties, relative to the pH-sensitive reaction equilibrium potential, to suit H_2 production. As well as being relevant for electrocatalysis and furthering the cause of renewable H_2, these results may also account for some of the biogenic H_2 produced by bacteria in gastrointestinal systems.

Introduction

The biological hydrogen cycle – the production and oxidation of H_2 by microbes – has wide relevance across biotechnology and

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† Electronic supplementary information (ESI) available: Chronoamperometric determination of the product inhibition constant, $K_{I\text{-app}}^{H_2/H^+}$ during H_2 production by Hyd-1 at pH 3. Comparison of the H_2 oxidation activity of Hyd-1 at pH 3.0 and 6.0, under 100% H_2. The onset of H_2 oxidation activity for Hyd-2 as a function of pH. Conceptual demonstration of the cyclic voltammetry trace expected if the limited activity at low potential was due to reductive inactivation of the active site. Cyclic voltammograms for Hyd-1 at low (5 mV s^{-1}) and high (100 mV s^{-1}) scan rate, pH 3.0 and pH 7.0. See DOI: 10.1039/c3ee43652g

health.[2,3] Not surprisingly, the extremely high activities of hydrogenases and the nature of their active sites, which contain Fe coordinated by CO and CN^- ligands, with or without Ni, have attracted much attention among chemists. Of the two prominent classes of enzyme, [FeFe] hydrogenases show the highest turnover rates but are notoriously oxygen sensitive, being irreversibly damaged by exposure to even traces of dissolved O_2.[4,5] In contrast, most [NiFe] hydrogenases are reversibly inactivated by exposure to O_2 and are usually able to resume catalysis upon reductive reactivation. A special subgroup of the [NiFe] enzymes, termed O_2-tolerant hydrogenases, has the unique property of catalysing H_2 oxidation in the sustained presence of O_2, which allows the host organism considerable environmental flexibility as well as identifying these enzymes as candidates for special technology applications. Oxygen-tolerant

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membrane-bound hydrogenases (MBHs) from *Ralstonia* species, *Aquifex aeolicus* and *Escherichia coli* have been extensively studied by protein film electrochemistry (PFE), crystallography and spectroscopy, from which a considerable body of mechanistic detail has emerged,[6–16] in addition to demonstrating the potential utility of O_2-tolerant hydrogen cycling catalysts in novel fuel cells.[17–22]

All studies to date[8,23–25] have found that O_2-tolerant MBHs display low or non-existent activity toward H^+ reduction (H_2 evolution). This is partly because H_2 is a strong product inhibitor, but even under N_2 with a rapidly spinning electrode (to disperse product) only a small H^+ reduction current is observed. An important mechanistic clue to the poor H_2 producing activity is the observation that a substantial overpotential must be applied before H_2 oxidation commences, as shown by comparing protein film voltammograms recorded for an O_2-tolerant and an O_2-sensitive hydrogenase from *E. coli* (Fig. 1).[24] At pH 7 and 10% H_2, the O_2-tolerant hydrogenase-1 (Hyd-1) shows negligible H^+ reduction activity, and the onset of H_2 oxidation occurs at an overpotential of approximately 0.1 V. These characteristics are conserved across O_2-tolerant MBHs studied to date and are in marked contrast to other hydrogenases, both [NiFe] and [FeFe], that behave as reversible electrocatalysts when attached to an electrode. Explanations accounting for the correlation between the additional overpotential requirement and uni-directionality are discussed further below, but at this stage it is important to be reminded that a catalyst can have no intrinsic bias for reaction direction beyond that dictated by thermodynamics. 'Bias' thus refers to comparative activities under different thermodynamic (electrode potential) conditions. It is worth noting that [NiFe]-hydrogenases are typically biased in the direction of H_2 oxidation (uptake)[26] but only for O_2-tolerant MBHs is the bias so extreme as to preclude H^+ reduction. At high potentials, the H_2 oxidation activity of both Hyd-1 and Hyd-2 is attenuated by conversion to an inactive state, known as Ni–B, which contains a Ni(III)–OH species in the active site, although this species is less stable for Hyd-1.

The [NiFe] membrane-bound hydrogenases (MBHs) are organised minimally as heterodimers, with a large subunit containing the $[(Cys–S)_2–Ni–(\mu^2-Cys–S)_2–Fe(CO)(CN)_2]$ core of the active site, and a small subunit containing three Fe–S clusters for electron transport to and from the active site. The amino acid sequences and corresponding structures of the active sites of O_2-tolerant and O_2-sensitive [NiFe] hydrogenases thus far examined are very similar, raising the likelihood that any substantial differences in behaviour must originate from elsewhere in the protein. Recent studies[6,10,14,15,27] have indeed demonstrated the importance of FeS cluster properties in determining O_2 tolerance; specifically, the FeS relay must ensure the rapid transfer of several electrons back to the active site upon its reaction with O_2, thereby forming only Ni–B and avoiding oxidative damage. The structure of Hyd-1 shown in Fig. 2 indicates the positions of the relay centres with respect to the [NiFe] active site and the region of the protein surface close to the more exposed distal cluster (D) across which electrons must enter or leave the enzyme. Mutations of the proximal (P) [4Fe–3S] and/or medial (M) [3Fe–4S] clusters of Hyd-1 severely impair O_2 tolerance but, importantly, have no effect on the onset potential for H_2 oxidation or on the low level of H^+ reduction,[6,7] thus establishing that the properties of these buried clusters are not responsible for the lack of reversibility seen for O_2-tolerant enzymes.

A relationship between onset potential and reversibility is predicted for enzymes that possess a redox relay to mediate electron transfer. According to a basic model of hydrogenase electrocatalysis proposed recently by Hexter *et al.*[28] the overpotential requirement and catalytic bias (to operate preferentially in a particular direction) of an enzyme adsorbed at an electrode depend on the reduction potential of the centre at which electrons enter or leave the catalyst, relative to the equilibrium potential of the reaction being catalysed. The closer
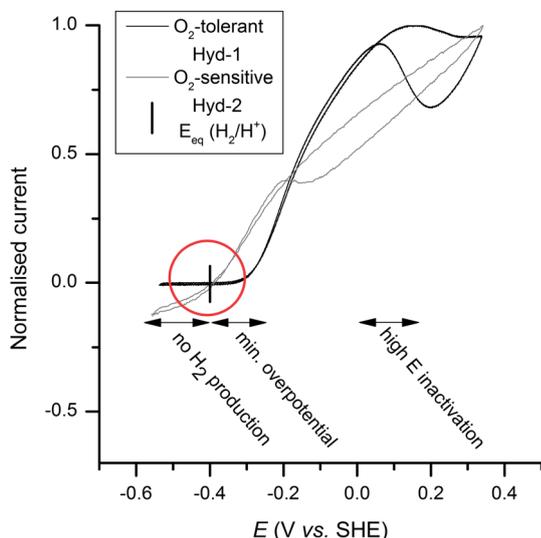
Fig. 1 Electrochemical characteristics of an O_2-tolerant (Hyd-1) *vs.* an O_2-sensitive hydrogenase (Hyd-2) at pH 7.0 under 10% H_2 at 37 °C. The $2H^+/H_2$ equilibrium potential E_{eq} is marked by the vertical bar. Scan rate 5 mV s^{-1}, electrode rotation rate 2000 rpm. The hysteresis at high potential is due to oxidative inactivation and reactivation through formation of an inactive Ni(III)–OH complex (Ni–B). Labels below voltammograms refer to properties of O_2-tolerant Hyd-1.
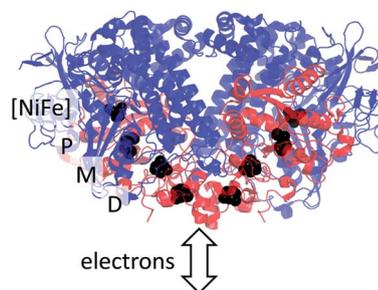
Fig. 2 The structure of Hyd-1 (PDB code 3USC[1]) labelling the redox centres in one half of the molecule (a dimer of heterodimers) and the region (arrow) that is most favoured for entry and exit of electrons. [NiFe] = active site, P = proximal [4Fe–3S]$^{5+/4+/3+}$ cluster, M = medial [3Fe–4S]$^{1+/0}$ cluster, D = distal [4Fe–4S]$^{2+/1+}$ cluster.[1]

together these potentials are, the more reversible is the electrocatalyst. Based upon structural data, the distal $[4Fe–4S]^{2+/1+}$ cluster is the natural site at which electrons enter or leave the enzyme, but a complication is revealed in recent work by Roessler et al.[12] who established that the distal [4Fe–4S] cluster in Hyd-1 is not detectable by standard EPR methods, suggesting that the reduced $[4Fe–4S]^+$ level has a ground state with $S > 1/2$: a similar observation was made for the MBH from Ralstonia eutropha.[15] Although the reduction potential of the distal [4Fe–4S] cluster in Hyd-1 could not be determined by titrations, the model for electrocatalysis predicted a value in the region of −0.19 V vs. SHE at pH 6. From studies of the O_2-tolerant Hase I from A. aeolicus, Pandelia et al.[10] reported that the midpoint potential of the distal cluster in that enzyme is −65 mV at pH 6.4, which is more than 0.2 V more positive than typical O_2-sensitive counterparts. In leading into our work, it is noteworthy that [4Fe–4S] clusters do not typically show a large pH dependence of reduction potential, whereas the $2H^+/H_2$ reduction potential shifts by −0.06 V per pH unit and by −0.03 V per order of magnitude increase in H_2 pressure ($\rho(H_2)$). Consequently, the model predicts that Hyd-1 should become a reversible electrocatalyst and proficient H_2 producer under more acidic conditions (assisted by low $\rho(H_2)$) if the distal cluster is the point of entry for electrons and responsible for the observed bias. The investigations we now describe give results that are in full accordance with this expectation and help to elucidate, in general terms, the complex factors determining electrocatalytic properties of hydrogenases. The findings not only have specific physiological and health implications in regard to H_2 production by E. coli throughout the gastrointestinal system but also have wider relevance for understanding and optimising biohydrogen production.

Methods

E. coli Hyd-1 was purified as reported previously[12] from strain FTH004,[29] carrying a 6× His affinity tag at the extreme C-terminus of HyaA, expressed from the native locus, using 0.02% TX-100 detergent. Enzyme samples typically showed initial H_2 oxidation activity of 125 s^{-1} in H_2-saturated buffer at pH 7 and 20 °C, when assayed by following the 604 nm absorbance change of benzyl viologen (BV) due to reduction by Hyd-1 in 50 mM Tris/HCl, 100 mM NaCl, 25 mM benzyl viologen ($\varepsilon_{604nm} = 9.82$ mM^{-1} cm^{-1}) buffer. This assay certainly underestimates the true activity of Hyd-1 because BV is a poor oxidant. Protein film electrochemistry was carried out using an Autolab potentiostat (PGSTAT10) controlled by Nova 1.5 software (Eco-Chemie). The electrochemical cell, featuring a standard three-electrode setup, was housed in an anaerobic glovebox (O_2 < 2 ppm). The pyrolytic graphite edge (PGE) rotating disc working electrode was used in conjunction with Pt wire as counter electrode and a saturated calomel reference electrode (SCE) that was housed in a Luggin sidearm. Potentials were converted to the standard hydrogen electrode using the formula $E_{SHE} = E_{SCE}$ + 0.241 V at 25 °C.[30] All experiments were carried out under a flow of high-purity gases (BOC) mixed using mass flow controllers (Sierra instruments). The temperature (37 °C to

mimic, to the extent possible, the typical environment under which the enzyme operates in its physiological host) was controlled through a water jacket. All experiments were carried out in mixed buffer[31] adjusted to each pH value at 37 °C. The working electrode was rotated at up to 2000 rpm to minimise effects due to H_2 mass transport, although for scans at 1% H_2 it proved impractical to overcome, completely, the diffusion limitation for H_2 oxidation.

In a typical experiment, the PGE electrode was sanded with P400 Tufbak sandpaper (Durite), rinsed and wiped with cotton wool, then 5 μL enzyme solution (approximately 1 mg mL^{-1}) was pipetted onto the surface of the graphite and left for 30–60 seconds to allow adsorption to occur. The working electrode was then introduced to the cell containing the buffer and the enzyme was activated by carrying out cyclic voltammetry scans between −0.56 V and +0.26 V vs. SHE at 10 mV s^{-1}, under 100% H_2 at pH 5, until the electrochemistry stabilised, before any experiments were carried out. Cyclic voltammograms were recorded and the forward and reverse scans were averaged to help compensate for capacitive current. Where necessary, data were smoothed using the Savitzky–Golay smoothing tool of OriginPro 8.5.1 with a 25 mV window; in all cases, the smoothed data were visually inspected to ensure they did not deviate from the raw data.

We adopted the following procedure to distinguish trace faradaic current due to low-level H^+ reduction activity above the large capacitance current of the PGE electrode. A blank scan was recorded with a bare PGE electrode under the same conditions as the experimental scans. The average slope of two blank scans (mean, ± (3× s.d.); $2.1 \times 10^7 \pm 6.85 \times 10^7$) served as a baseline when analysing onset potentials for catalytic activity. We reasoned that if reversible activity is occurring, the CV should cut steeply across the zero-current (x-) axis at the equilibrium potential E_{eq}; hence the current-axis intercept marks the onset of oxidation activity in this reversible case. For CVs in which very little H^+ reduction activity is observed, the potential at which the slope of the experimental scan exceeds the slope of a blank scan is defined as the onset potential for oxidation activity.

Results

Cyclic voltammograms recorded at pH 7.0, shown in Fig. 3A, reveal that the onset of H_2 oxidation activity is unchanged as the partial pressure of H_2 is varied over a 100-fold range.[24] Consequently, decreasing H_2 pressure has the effect of lowering the overpotential requirement by raising the equilibrium potential E_{eq} for the $2H^+/H_2$ couple (an increment of +30 mV per 10-fold decrease in $\rho(H_2)$). In contrast, the onset potential is clearly affected by changes in pH (Fig. 3B). Under 100% H_2, the onset potential decreases by approximately 40 mV per pH unit in the range pH 6 to 8, which is to be compared with the value of 61.5 mV per pH unit expected for the equilibrium potential E_{eq}. As a result, the overpotential requirement decreases substantially as the pH is lowered.

An ultimate result (Fig. 3C) is observed when cyclic voltammetry experiments are carried out at pH 3.0. Electrocatalysis at
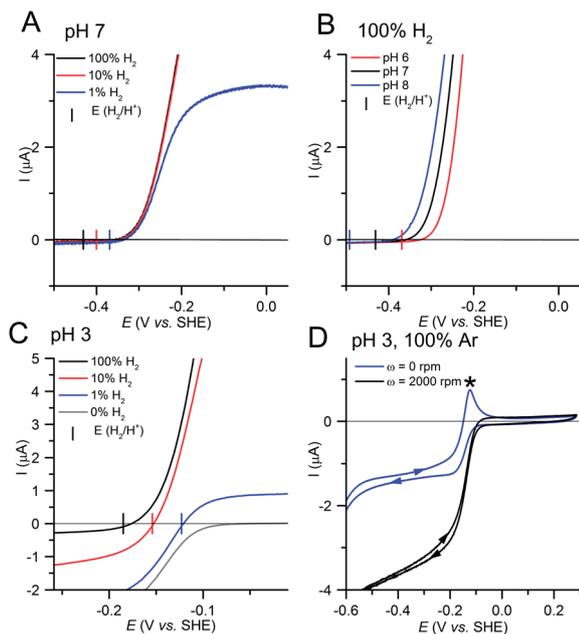
Fig. 3 Irreversible (A and B) and reversible (C and D) catalysis by *E. coli* Hyd-1. (A) At pH 7, changes in $\rho(H_2)$ have no effect on the onset potential. (B) Lowering the pH causes a 40 mV per unit shift in onset potential. (C) At low pH, the overpotential requirement for H_2 oxidation is minimised and current due to H^+ reduction becomes significant. (D) A comparison of voltammograms recorded at rotating and stationary electrodes at pH 3.0 confirm (prominent oxidation peak observed at stationary electrode) that the reductive current is due to H_2 production. All scans were recorded at 37 °C, scan rate 10 mV s^{-1}, electrode rotation rate (ω) 2000 rpm except in panel D. Scans in panels A–C have had background current subtracted.

Fig. 4 Cyclic voltammograms of Hyd-1 at 1% H_2, across a broad range of pH values. (A) Data are plotted as conventional current (*I*) *vs.* electrode potential (*E*) voltammograms. (B) Current traces are plotted as a function of overpotential. All scans were carried out at 37 °C, 1% H_2, 10 mV s^{-1}, electrode rotation rate 2000 rpm.

low $\rho(H_2)$ now reveals a large current due to H^+ reduction and the voltammetry has become highly reversible; indeed, the current–potential trace intersects the potential axis sharply at values expected for E_{eq} at 1% H_2 or 10% H_2, pH = 3.0, 37 °C. Under 100% H_2 the intersection potential is less well defined since the H^+ reduction current is much smaller, but the trend is still clearly marked, with E_{eq} becoming more positive by approximately 30 mV per decade increase in $\rho(H_2)$. A comparison of voltammograms obtained under stationary and rotating electrode conditions (Fig. 3D) confirms that the reduction current is due to H_2 production. For the stationary scan, H_2 production in the low-potential region leads to a build-up of H_2 at the electrode surface: the undisturbed H_2 is then re-oxidised as the potential crosses E_{eq} on the forward scan, giving rise to the sharp peak that is marked with an asterisk (*). This oxidation peak is not observed with rapid rotation of the electrode, which serves to disperse produced H_2 away from the electrode.

Fig. 4 gives a more complete demonstration of the changing properties of Hyd-1 as the pH is lowered. All voltammograms were recorded under 1% H_2 at a rotation rate of 2000 rpm and a scan rate of 10 mV s^{-1}. All experiments compared within a given figure were performed using the same enzyme film activated at pH 5.0 (see Methods) and then swapped between solutions of varying pH. The films were stable over time, allowing for direct comparison of the observed currents across conditions within an experiment (as

mentioned later, Fig. S1† shows the enzyme is stable for at least two hours at pH 3, 37 °C). Panel A shows the standard current *vs.* potential traces, while panel B displays the same current traces as a function of the overpotential, $\eta = E - E_{eq}$ to allow for easy comparison of the overpotential requirement across different conditions. As the pH is lowered, the catalytic current for H_2 oxidation does not change significantly (it is diffusion-controlled under these conditions of low $\rho(H_2)$) whereas progressively more reduction current is obtained. Protein film electrochemistry rarely gives absolute activities because the minute amount of adsorbed electroactive enzyme is difficult to quantify; instead the technique records the relative activities in each direction. The sample of enzyme, being immobilised on the electrode surface, can easily be transferred between solutions of different pH. Under 100% H_2 (a condition under which H_2 oxidation is not diffusion-controlled) the H_2 oxidation current measured at +0.15 V for pH 3 was still approximately 50% of that measured at pH 6 for the same film (Fig. S2†). Panel B emphasises the link between overpotential requirement and catalytic bias: all scans exhibiting a significant overpotential requirement (*i.e.* those measured for pH ≥ 6) also exhibit little to no H_2 production. Crucially, as the pH is lowered, the voltammetry increasingly resembles that of a standard [NiFe] hydrogenase or the classical reversible behavior of a Pt electrode. The tight correlation between the properties of overpotential requirement and lack of H_2 production in all scans further shows that the emergence of H^+ reduction activity is not due merely to increased H^+ availability at low pH but arises because Hyd-1 becomes a fundamentally more reversible catalyst under these conditions.

Establishing a quantitative definition for onset potential is difficult and various methods have been used:[2,20,25] some papers do not describe the method by which onset potential is assessed,[32] suggesting that it is assessed subjectively (by eye). The problem is that a PGE electrode has a high and complex resistance and capacitance, resulting in a charging current background that is very high compared to low-level Faradaic activity. We thus started from the simple observation that, in the potential region where catalytic turnover is very slow, the slope of the voltammogram is similar to the slope of a blank scan recorded under the same conditions, although the absolute value of the

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current may differ slightly due to small variations from one experiment to another. The onset of rapid catalytic turnover is accompanied by a deviation of the voltammogram from the blank scan, *i.e.*, an increase in the slope of the experimental voltammogram relative to a blank voltammogram. We therefore defined the onset potential of activity as the potential at which the slope of an experimental voltammogram deviates from the slope of a blank scan (mean plus or minus 3× s.d.). For scans in which reversible activity is observed, the CV passes steeply through the zero-current axis, and the intercept is therefore the point of 'onset'; the error due to a small current offset (*y*-axis) on the measured potential (*x*-axis) is minimised due to the steep slope of the voltammogram in this region.

Fig. 5 summarises the data collected over a range of pH values at both 1% and 100% H_2 (panels A and B). In panel C the onset potentials are portrayed as a function of pH, from which it is clear that for 1% H_2 and pH < 5 the onset potential values match the expected Nernstian equilibrium potentials, with a slope of −61 mV per pH unit. For 100% H_2 the same is true except that the intersection potential is much less clearly defined and linearity is only just being approached at pH 4. As expected, the trendline at pH < 4 for 1% H_2 lies approximately 60 mV above that for 100% H_2. As the pH is increased above 5, the onset potentials begin to deviate from the respective equilibrium potentials for the H^+/H_2 couple, the underlying trend being to become independent of H_2 concentration.

Comparative data were obtained for Hyd-2, the O_2-sensitive 'standard' MBH produced by *E. coli* (Fig. S3†). For this enzyme, reversible electrocatalysis is observed at all pH values up to pH 7; scans at pH 8 show little to no H_2 production and the onset

potential does not move below −420 mV *vs.* SHE. Compared to Hyd-1, the stability of Hyd-2 to extremes of pH, both acidic and basic, is much decreased, as observed previously.[33] For Hyd-2, we see the same trend of increasing onset potential and loss of reversibility as E_{eq} becomes more negative – the difference between Hyd-1 and Hyd-2 being the pH (and thus the value of E_{eq}) at which this trend begins.

Assessment of the product inhibition constant during H^+ reduction, $K_I^{H_2/H^+}$ (Fig. S1†) for Hyd-1 shows that although product inhibition is significant ($K_{I\text{-app}}^{H_2/H^+} = 125$ μM at pH 3, 37 °C, −536 mV *vs.* SHE), it is of the same order as the previously published value for Hyd-2 ($K_{I\text{-app}}^{H_2/H^+} = 210$ μM at pH 6, 30 °C, −600 mV *vs.* SHE).[24] This experiment also serves as an excellent demonstration of the stability of Hyd-1 catalysis at low pH; the reductive current measured at the end of the two-hour experiment was more than 98% of the current recorded at the beginning of the experiment, under identical conditions.

One explanation that we must consider for the lack of H_2 production at high pH is that the enzyme undergoes an inactive–active transformation above a certain potential. At pH 7, this 'switch' potential would be well above E_{eq}, leading to the observation that only H_2 oxidation occurs, but at pH 3 the 'switch' potential could lie below E_{eq} allowing the enzyme to operate in reverse as long as the electrode potential does not become too negative. This behaviour is observed, for example, with succinate dehydrogenase, where the current due to fumarate reduction peaks within a narrow potential range then decreases.[34] A current peak is also observed for Hyd-1 and Hyd-2 as enzyme that has become oxidatively inactivated to form Ni–B (stable at high potential) undergoes reductive re-activation. Fig. S4† shows the waveshape expected were there to be a fast, potential-dependent active–inactive transformation that allowed H^+ reduction to occur within a narrow region of potential. If this transformation were slow, hysteresis would occur and voltammograms recorded over a range of scan rates would have different waveshapes. Fig. S5† shows a comparison of scans obtained for pH 7 and pH 3 (bidirectional), at 5 mV s^{-1} and 100 mV s^{-1}. There is clearly no evidence for an active–inactive transformation occurring in the potential range of interest.

Another possible source of overpotential would be a high reorganisation energy for the centre at which electrons enter or leave the enzyme, resulting in sluggish interfacial electron-transfer kinetics. In such a situation we expect to see an increased overpotential requirement for *both* directions of catalysis. Exploiting the ability of PFE to drive reactions at extremes of potential, it was established[7] that no H^+ reduction current is evident at potentials as negative as −0.75 V (an overpotential of 0.35 V at pH 7). Therefore, a high reorganisation energy for interfacial electron transfer is not the cause of the high overpotential requirement observed for H_2 oxidation by Hyd-1.

Fig. 5 Potential of H_2 oxidation onset as a function of pH and $\rho(H_2)$. All scans were carried out at 37 °C, 10 mV s^{-1}, electrode rotation rate 2000 rpm. Panels A and B: close-up analysis of the electrocatalytic voltammograms for Hyd-1 as a function of pH at 1% H_2 (panel A) and 100% H_2 (panel B). The onset potential is defined as the zero-current intercept for scans in which significant reductive current is observable, and as the point at which the slope of the scan deviates from the average slope of a blank scan for CVs in which little to no reductive current is observed. Onset potentials are estimated to be accurate to within 0.01 V; the ovals indicating onset potential are 0.02 V wide, indicating onset value ± estimated error. Panel C shows the pH dependence of onset potentials, defined accordingly; grey diagonal lines indicate the value of E_{eq}.

Discussion

The results in this paper not only highlight the physical stability of an O_2-tolerant [NiFe] hydrogenase under acidic conditions (significant because *E. coli* experiences a wide pH variation

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during its passage through the gastrointestinal system) but also address the important question of what factors determine whether a hydrogenase is a good H_2 oxidiser or H_2 producer. In the specific case of Hyd-1, we have pursued the observation that when studied under neutral pH conditions, it acts *only* as an H_2 oxidiser and appears inactive at potentials below -0.3 V, even when no H_2 is present. Experiments undertaken over a wide range of pH values now show that *E. coli* Hyd-1 transforms into a fully reversible catalyst as the pH is lowered, with minimal overpotential requirement and high H^+ reduction activity.

An analysis of how this shift in behaviour occurs helps us to understand why the electrocatalytic properties of O_2-tolerant MBHs seem to differ so substantially from standard hydrogenases. It is well known that [NiFe] hydrogenases are less proficient at H_2 evolution because of product inhibition. While this is particularly true for O_2-tolerant [NiFe] hydrogenases, the results (particularly the comparisons of $K_{\text{I-app}}^{\text{H}_2/\text{H}^+}$ values for Hyd-1 and Hyd-2 under relevant conditions) now reveal that product inhibition is *not* the reason for either the overpotential requirement for H_2 oxidation or the lack of reversibility at neutral pH.

The data presented in Fig. 3A and B show that at and above pH 6, the pH but *not* the partial pressure of H_2 shifts the onset potential of the oxidation wave. Since H_2 must bind and be transformed at the active site, the observation that the onset potential is unchanged over two orders of magnitude in H_2 partial pressure makes it highly unlikely that the overpotential requirement for H_2 oxidation (nearly 0.1 V at pH 7, 100% H_2) is due to a property of the [NiFe] catalytic site. In contrast to the lack of variation with $\rho(H_2)$, the onset potential shows a clear positive shift with decreasing pH but by a smaller degree than the corresponding shift in E_{eq}, hence the two values are converging. Indeed, as shown in Fig. 4B, there is a smooth transformation from irreversible to reversible electrocatalysis as the pH is lowered, which shows that the overpotential requirement for H_2 oxidation and the lack of H_2 production activity observed above pH 5 are closely related.

In a recent model for electrocatalysis,[28] Hexter *et al.* proposed that an important basis for the catalytic bias exhibited by an enzyme attached to an electrode surface is the difference between the reduction potential of the substrate redox couple (in this case H^+/H_2) and the potential at which electrons enter or leave the catalytic cycle (the site at which this occurs is termed the electrochemical control centre[35]). An analogous argument should hold for the enzyme's physiological activity where, for example, an electrode is replaced by the natural redox partner such as the quinone pool.[32] The catalytic bias is minimised and reversibility maximised when the two potentials become close to one another, whereas the irreversible case is marked by a unidirectional current response with an onset defined by the reduction potential of the electrochemical control centre (ECC). The model allows us to explain the results of these experiments, because the substrate reduction potential E_{eq} shifts in a well-defined manner with pH (as required by thermodynamics) whereas the potential of the ECC is certain to have a milder pH dependence (as in this case) or no pH variation at all (as is typical for FeS clusters). The results highlight an interesting difference between enzymes and synthetic electrocatalysts:

redox enzymes usually possess relay centres that transport, trap and store charge – the ECC typically being one of these centres; in contrast, synthetic catalysts typically lack additional redox centres and the ECC must be the catalytic site itself. The *separation* of charge capture/transport and catalytic chemical conversions in enzymes suggests an important design principle for electrocatalysts in energy technologies.

Previous experiments carried out with proximal and medial cluster variants of Hyd-1 revealed that altering either cluster had a profound effect on the O_2 tolerance of the enzyme but no detectable effect on the onset potential for H_2 oxidation.[6,7] Even in the case of a mutant in which residues at both the proximal and medial clusters were altered substantially, no change was seen in the bias or onset potential.[7] We therefore consider it unlikely that the properties of either the proximal or medial clusters determine the bias and additional overpotential requirement. Having already argued that the [NiFe] site cannot be the source of the onset overpotential requirement, the simple process of elimination directs us to the distal cluster. The reduction potential of the distal [4Fe–4S] cluster should be insensitive to $\rho(H_2)$ because it does not interact with H_2, but it may be sensitive to pH depending on the extent to which acid–base equilibria involving nearby ionisable residues favour a particular oxidation level. Noting that histidine is one of the ligands to the distal [4Fe–4S] cluster it is possible that ionisation of the imidazole HN is influential, as with Rieske-type 2Fe–2S clusters.[36–38] A dilemma here is that the distal [4Fe–4S] cluster in Hyd-1 appears silent to EPR spectroscopy, precluding direct determination of its reduction potential. The distal [4Fe–4S] cluster in the MBH of *R. eutropha* is similarly elusive: this problem is not restricted to hydrogenases, for example, complex I contains FeS clusters that are not detectable by EPR.[38–41]

pH-dependent changes in the activity of various enzymes are commonly related to the pK_a value of amino acid residues directly involved in catalysis.[7] However, in such a case, a departure from the pH close to the residue's pK_a should impair catalysis in both directions because (with the exception of residues positioned close to a redox centre) amino acid pK_a values are generally redox-insensitive. We thus exclude the possibility that the emergence of Hyd-1 H_2 production at low pH is due to a simple amino-acid ionisation effect.

The different catalytic bias for Hyd-1 and Hyd-2, as reflected in the sizeable onset overpotential requirement of Hyd-1 but not Hyd-2 under neutral pH conditions, is thus traced to an enzyme-based reduction potential. In general, notwithstanding the lack of information on the distal cluster of Hyd-1, the reduction potentials of FeS clusters in O_2-tolerant [NiFe] hydrogenases appear to be significantly more positive than for their O_2-sensitive counterparts. This feature results in more stable electron occupancy and greater ability to supply electrons back to the active site when O_2 attacks. A higher reduction potential for the distal cluster in O_2-tolerant MBHs would not only supplement this protection mechanism but also result in H_2 evolution being activated only at low pH.

Important remaining questions are whether the transformation in favour of H_2 production at low pH has any physiological or biotechnological significance. Both Hyd-1 (Fig. 5) and

Hyd-2 (Fig. S3†) show reversible catalysis at sufficiently low pH and irreversible catalysis at high pH; the difference is that Hyd-1 catalysis becomes irreversible above pH 5 whereas Hyd-2 catalysis only becomes irreversible above pH 8. As to a possible physiological link, several studies[42,43] have shown that Hyd-2 is strongly expressed under mildly basic conditions (pH $\approx$ 7.5) whereas Hyd-1 is more strongly expressed under acidic conditions (a pH value at least as low as 4.7). Both Hyd-1 and Hyd-2 project into the periplasm which, unlike the cytoplasm, is exposed to the external H^+ environment. Thus if we compare these isoenzymes at their respective pH of maximal expression, they appear much more similar than they do at first glance, compared under identical conditions. The stability and H_2-evolution activity of Hyd-1 at pH 3, combined with the knowledge that Hyd-1 expression is upregulated under acidic conditions, suggests that Hyd-1 is capable of environmental deacidification provided electrons are available from the quinol pool. Evidence suggests that Hyd-1 may act as a scalar proton pump during H_2 oxidation;[29,32,44] consequently, H_2 production by Hyd-1 would require reverse electron transport and result in dissipation of the transmembrane proton gradient, an energetically costly process.

Several studies have provided compelling evidence that, in the absence of H_2 production by formate hydrogenlyase, both Hyd-1 and Hyd-2 can produce H_2 during an unusual type of anaerobic glycerol metabolism performed by *E. coli*, and H_2 production by Hyd-1 is maximised at low pH;[45–47] importantly, *E. coli* fermentations commonly attain a pH well below 5.[48] The experiments we have now described, proving that Hyd-1 converts to an effective H_2 producer upon acidification and suggesting how this occurs, raise the possibility that H_2 production activity by Hyd-1 could serve as a physiological asset, particularly as *E. coli* transits through the gastrointestinal tract or is exposed to acid stress in the external environment. Finally, the demonstration that good rates of H_2 evolution are achievable with a highly O_2-tolerant [NiFe]-hydrogenase gives fresh impetus to the quest for practical and efficient biohydrogen production.

Acknowledgements

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