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# Reversible or irreversible catalysis of H<sup>+</sup>/H<sub>2</sub> conversion by FeFe hydrogenases

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**ABSTRACT:** Studies of molecular catalysts traditionally aim at understanding how a certain mechanism allows the reaction to be fast. A distinct question, which has only recently received attention in the case of bidirectional molecular catalysts, is how much thermodynamic driving force is required to achieve fast catalysis in either direction of the reaction. 'Reversible' catalysts are bidirectional catalysts that work either way in response to even a small departure from equilibrium and thus do not waste input free energy as heat; conversely, 'irreversible' catalysts require a large driving force to proceed at an appreciable rate [Fourmond *et al.*, *Nat. Rev. Chem.* 2021, 5, 348–360]. Numerous mechanistic rationales for these contrasting behaviours have been proposed. To understand the determinants of catalytic (ir)reversibility, we examined the steady-state, direct-electron transfer voltammetry of a particular FeFe hydrogenase, from *Thermoanaerobacter mathranii*, which is very unusual in that it *irreversibly* catalyses H<sub>2</sub> oxidation and production: a large overpotential is required for the reaction to proceed in either direction [Land *et al.*, *Chem. Sci.* 2020, 11, 12789–12801]. In contrast with previous hypotheses, we demonstrate that in this particular enzyme, catalytic irreversibility can be explained without invoking slow interfacial electron transfer or variations in the mechanism: the observed kinetics is fully consistent with the same catalytic pathway being used in both directions of the reaction.

## Introduction

Hydrogenases are metalloenzymes that occur in most microorganisms, where they catalyse the oxidation or evolution of molecular hydrogen, a very important energy vector in the bacterial world. A major motivation for studying these enzymes is certainly that the knowledge we acquire will prove useful for designing the catalysts we need to produce hydrogen in a clean process and to oxidize it in affordable fuel cells. Their organometallic cofactors have inspired inorganic chemists,<sup>1</sup> and their functional diversity also defines the realm of the possible in terms of molecular catalysis of H<sup>+</sup>/H<sub>2</sub> conversion. Indeed, the hydrogenases purified from distinct microorganisms (which have the same active sites but distinct amino acid sequences, quaternary structures and cofactor contents) exhibit distinct catalytic properties, which appear to be modulated by structural elements that are remote from the active site. This diversity of catalytic properties is observed in relation to catalytic rates, but also "catalytic bias" (the ratio of the maximal rates of H<sub>2</sub> oxidation and evolution measured under very oxidizing or very reducing conditions, respectively),<sup>2–10</sup> and "catalytic reversibility". The latter is defined in relation to how much thermodynamic driving force (or overpotential, in an electrochemical experiment) is required to obtain a significant turnover frequency in either direction of the reaction.<sup>11–16</sup> The distinction between bidirectional-reversible and bidirectional-irreversible catalysis is illustrated in Fig. 1A by the blue and red traces, respectively. In addition to fast turnover, reversibility is a desirable property of any practical device that one may design and use for catalysis, because it minimizes the amount of energy that the system wastes as heat; for example, catalytic reversibility allows the voltage of fuel cells to approach the thermodynamic limit.<sup>17</sup> The molecular determinants of reversibility in molecular catalysis have been discussed only rather recently.

FeFe hydrogenases catalyse H<sup>+</sup>/H<sub>2</sub> conversion at an active site which consists of a dinuclear cluster of Fe covalently attached to a 4Fe4S cluster.<sup>1</sup> These enzymes embed a variable number of accessory FeS clusters: none in *Chlamydomonas reinhardtii* (*Cr*) HydA1 and four in *Clostridium acetobutylicum* (*Ca*) HydA,<sup>18</sup> where they mediate long range intramolecular electron transfer. The bidirectional catalytic cycle involves various states that have been

spectroscopically characterized,<sup>1,19,20</sup> mostly under equilibrium conditions; kinetic studies of the catalytic cycle are much rarer.<sup>21,22</sup>

Having the hydrogenase or any other redox enzyme adsorbed onto, and directly exchanging electrons with, a rotating disc electrode makes it possible to easily observe how the catalytic rate in either direction, measured as a current, depends on the thermodynamic driving force, which is set by the electrode potential (Fig. 1).<sup>23</sup> All FeFe hydrogenases that had been characterized using direct electrochemistry until recently demonstrated reversible H<sub>2</sub> oxidation and production. The FeFe hydrogenase HydS from *Thermoanaerobacter mathranii* (*Tam*) is unusual in that respect, since it is a rare example of an irreversible bidirectional biological catalyst.<sup>24</sup>

*Tam* HydS is a putative sensory FeFe hydrogenase.<sup>25</sup> In this enzyme, like in the sensory hydrogenase HydS from *Thermotoga maritima* (*Tm*)<sup>26</sup>, several residues that were found to be very crucial for the function of prototypical FeFe hydrogenases (those in the so-called group A<sup>25</sup>) are not conserved. Of particular note is the apparent loss of the proton transfer pathway that is strictly conserved in prototypical FeFe hydrogenases and of two conserved methionine residues that flank the H-cluster (Figure 1 in ref. <sup>24</sup>), as well as the presence of a C-terminal 4Fe4S cluster.

Hydrogenase catalytic responses less reversible than those of standard hydrogenases have also been observed for *Clostridium pasteurianum* hydrogenase CpII,<sup>27</sup> and the E144D variant of *Cr* HydA1.<sup>16</sup> Similarly, reversible or irreversible bidirectional electrochemical responses have been reported for some of the synthetic, biomimetic complexes that catalyse H<sup>+</sup>/H<sub>2</sub> bidirectional conversion.<sup>13</sup>

Regarding the origin of this (ir)reversibility, a hypothesis was that irreversibility may be a consequence of slow *interfacial* electron transfer (iET) between the electrode and the catalyst;<sup>15</sup> slow iET does flatten the electrochemical response to the point that it may become irreversible (see SI section S3), but kinetic models have explained that irreversibility is also possible when iET is Nernstian (i.e. infinitely fast).<sup>12,28</sup> In the inorganic chemistry community, catalytic reversibility is sometimes considered to result from the "energy landscape" of the reaction being ideally flat,<sup>13,29</sup> however, we have shown that rate, directionality (or "catalytic bias"<sup>2–10</sup>) and

reversibility do not obey the same design principles, and "flatness" is actually not required to achieve reversibility: on the contrary, there are examples of rough energy landscapes that allow reversible catalysis,<sup>11</sup> and under certain conditions, making the energy landscape "less flat" may actually increase reversibility.<sup>12</sup> Last, based on the observation that the substitution of a residue involved in long range proton transfer in *Cr* FeFe hydrogenase makes the catalytic response less reversible, it has been proposed that irreversibility could be the consequence of proton and electron transfers to the active site being uncoupled in the variant, with the consequence that the reaction proceeds through different mechanisms in the oxidative and reductive parts of the irreversible catalytic response.<sup>16</sup> The use of different mechanisms for the forward and reverse reactions had previously been assumed to be a characteristic of bidirectional, irreversible catalysis,<sup>13</sup> but in theory, irreversibility could also occur with an "ordered" mechanism<sup>12</sup> (according to the enzymology terminology, "ordered" means that the catalytic mechanism is defined by a unique sequence of events, which is the same, clockwise or anticlockwise, in the two directions of the reaction, whereas in the so-called "random"<sup>30</sup> or "branched"<sup>12</sup> mechanisms, distinct pathways are possible).

The vast majority of the electrochemical literature on molecular catalysis has focused on unidirectional reactions, but theoretical models of *bidirectional*, two-electron, multi-step redox catalysis have recently been developed.<sup>11,12,14,28,31</sup> Unlike kinetic models of unidirectional catalysis, they include the thermodynamic constraints that result from all steps in the cycles being bidirectional. Here we contribute to identifying the reasons catalysts function irreversibly or reversibly by using these kinetic models to quantitatively interpret the irreversible catalytic response of *Tam* FeFe hydrogenase.

## Methods

Samples of *Tam* HydS were prepared as described in ref.<sup>24</sup>.

The anaerobic electrochemical experiments were performed in a Jacomex glovebox filled with N<sub>2</sub>, using a Metrohm Autolab potentiostat and a Pine electrode rotator. The electrochemical experiments were conducted in a mixed buffer containing MES, CHES, HEPES, TAPS, Na acetate (all 5mM) and Na<sub>2</sub>SO<sub>4</sub> (0.1M). The buffers contained no chloride, to prevent any high potential inactivation of the enzyme.<sup>32</sup>

Films of *Tam* HydS were made by polishing the pyrolytic graphite edge (PGE)<sup>33</sup> surface of a homemade rotating disc electrode (RDE) with alumina (1μm, Buehler) and P1200 sandpaper. The electrode was then sonicated and 1 μL of HydS 5μM (in phosphate buffer 0.1 M pH 7) was painted on the electrode surface together with 1 μL of polymyxin 20 mg/mL. The geometric electrode surface was approximately 0.1 cm<sup>2</sup>.

To measure the H<sup>+</sup>/H<sub>2</sub> equilibrium potential, we used a platinized PGE RDE prepared as described in ref.<sup>34</sup>. All potentials were measured with respect to a saturated calomel electrode, and then corrected to the SHE using  $E_{\text{SHE}} = E_{\text{SCE}} + 244 \text{ mV}$ .

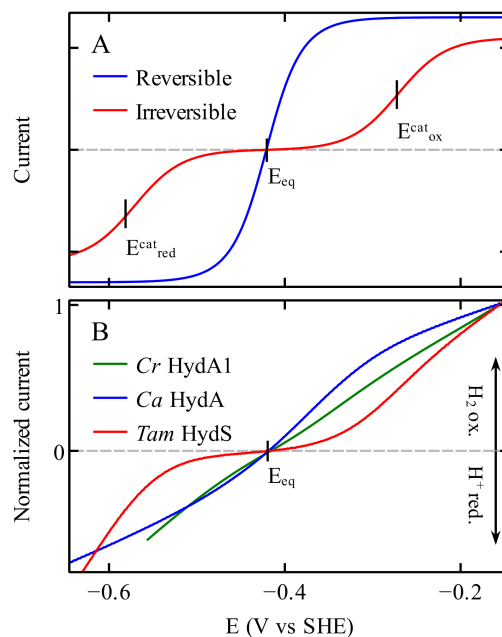
The data were analysed with the open-source, in-house program Qsoas (qsoas.org).<sup>35</sup>

## Results and discussion

Figure 1B illustrates the peculiar catalytic response of *Tam* hydrogenase by comparing the shapes of the steady-state cyclic voltammograms (CVs) obtained with three homologous FeFe hydrogenases under identical conditions of T, pH and H<sub>2</sub> concentration. In all three cases, the current is zero at the Nernst potential of the H<sup>+</sup>/H<sub>2</sub> couple ( $E_{\text{eq}}$ ) and catalysis is bidirectional. The CVs of *Cr* HydA1<sup>31</sup> (green trace) and *Ca* HydA<sup>36</sup> (blue) illustrate the "standard", reversible catalytic response, with a sharp intersection with the E-axis. The well characterized FeFe

hydrogenases from *Desulfovibrio desulfuricus* and *Megasphaera elsdenii* (including a truncated form) also give reversible responses under conditions of direct electron transfer (see e.g. refs<sup>3,17</sup>). In contrast, H<sub>2</sub> oxidation or production by *Tam* HydS (red trace) occurs only in response to a large overpotential. This is true irrespective of pH (Fig. 2 and SI Fig. 1) and temperature (from 10 to 70°C, Fig. 8A in ref.<sup>24</sup>).

This comparison shows that, at least in FeFe hydrogenases, (ir)reversibility is not simply dependent on the presence of accessory FeS clusters.



**Figure 1.** Reversible (blue) and irreversible (red) catalysis, as detected from the shape of the steady-state catalytic voltammograms (CVs) of redox catalysts. *A:* Nernstian CVs illustrating the difference between reversible and irreversible voltammetry. *B:* CVs obtained with various FeFe hydrogenases adsorbed onto a rotating disc electrode, under conditions of direct electron transfer and in the absence of mass transport limitation. Red: *Tam* HydS (this work); blue: *C. acetobutylicum* FeFe hydrogenase (*Ca* HydA, replotted from ref<sup>36</sup>); green: *C. reinhardtii* FeFe hydrogenase (*Cr* HydA1, from ref<sup>31</sup>). pH 7, 30°C, scan rate  $v = 20 \text{ mV/s}$  (*Ca* HydA and *Cr* HydA1), or  $5 \text{ mV/s}$  (*Tam* HydS), electrode rotation rate  $\omega = 3 \text{ krpm}$ .

We recorded catalytic voltammograms with *Tam* HydS adsorbed at a rotating disc graphite electrode, under one atm. of H<sub>2</sub> and over a wide range of pH (the black traces in row A of Fig. 2 show the CVs from pH 6.6 to pH 8.1, see also SI Fig. 1 for pH 5 and pH 6). To prevent interference from mass transport of H<sub>2</sub> to or from the electrode, the electrode was spun at a high rotation rate, and to ensure steady-state, the data were collected at a slow scan rate.

We fitted to all of these data the generic rate equation for two-electron catalysis by an adsorbed catalyst, which is valid for any two-electron multi-step ordered mechanism, irrespective of the exact number and the order of the steps in the catalytic cycle.<sup>12</sup> The ideal wave shape when interfacial electron transfer (iET) is very fast is a pair of Nernstian sigmoids, which is clearly not the case here; therefore, the model must take into account slow (non-Nernstian) iET and a distribution of iET rates, resulting from a distribution of orientations of the enzyme molecules on the electrode.<sup>28,31,37</sup> This generic rate equation (SI section 4) defines two "catalytic potentials",  $E_{\text{ox}}^{\text{cat}}$  and  $E_{\text{red}}^{\text{cat}}$ , that correspond to the mid-point potentials of the oxidative and reductive catalytic waves in the limit

of fast iET (Fig. 1A). These catalytic potentials *are not* the thermodynamic reduction potentials of the active site.<sup>12</sup> The difference  $E_{\text{ox}}^{\text{cat}} - E_{\text{red}}^{\text{cat}}$  measures the intrinsic irreversibility of the electrochemical response (the larger  $\Delta E$ , the less reversible the response) in the Nernstian limit. The actual separation between the two waves may increase as a result of slow iET (see SI section S3).

The hypothesis that the mechanism is ordered has a direct consequence:<sup>11,12,28</sup> the catalytic bias (defined as the ratio of the limiting currents in the two directions of the reaction) is simply related to the difference between the Nernst potential of the  $\text{H}^+/\text{H}_2$  couple and the *average* catalytic potential.

$$i_{\text{ox}}^{\text{lim}}/i_{\text{red}}^{\text{lim}} = \exp(2F/RT [(E_{\text{ox}}^{\text{cat}} + E_{\text{red}}^{\text{cat}})/2 - E_{\text{eq}}]) \quad (1)$$

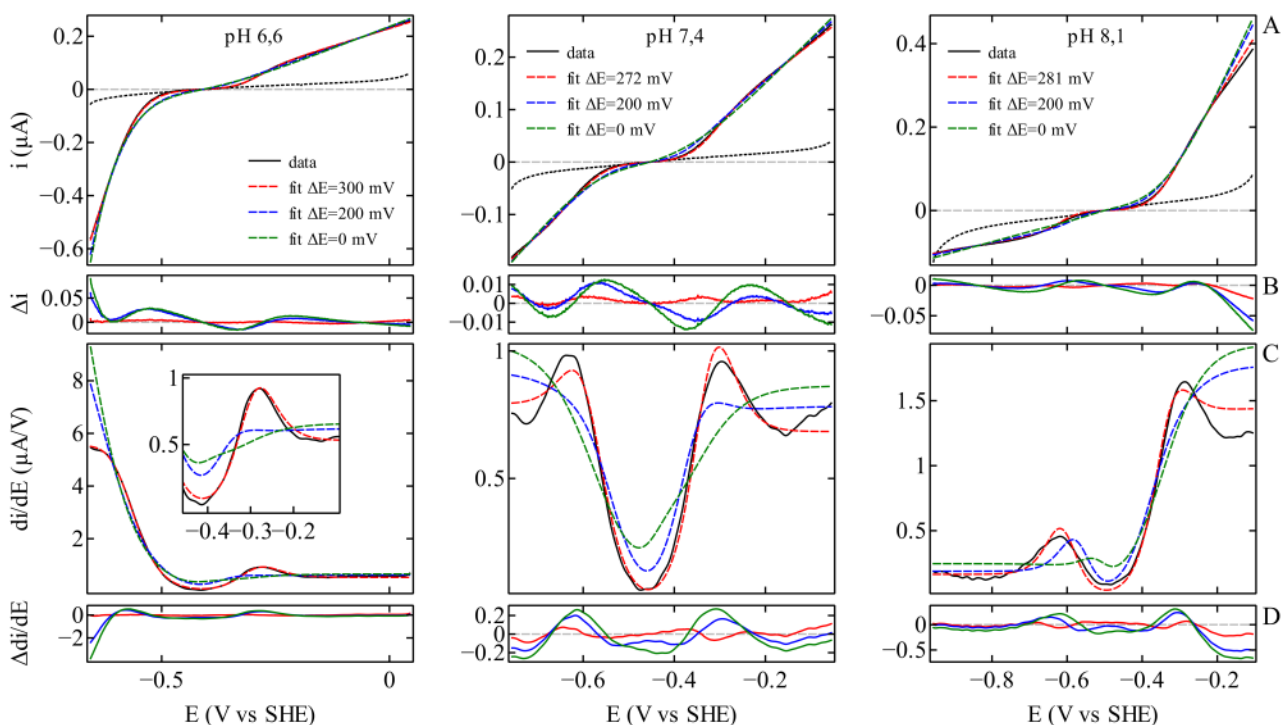
Since the catalytic current of *Tam* HydS is zero in a large potential window around  $E_{\text{eq}}$ , the catalytic CV does not indicate the value of the Nernst potential ( $E_{\text{eq}}$ ) of the  $\text{H}^+/\text{H}_2$  couple, contrary to what is seen with other, reversible hydrogenases. Thus, while recording each catalytic CV, we measured the value of  $E_{\text{eq}}$  using a platinized graphite electrode.<sup>34</sup> We then used eq. 1 as a constraint in the fitting procedure, to decrease the number of adjustable parameters (SI section 5). The actual magnitude of the current in either direction ( $i_{\text{ox}}^{\text{lim}}$  or  $i_{\text{red}}^{\text{lim}}$ ) has no relevance in this analysis, as it is dependent on the electroactive coverage of enzyme; what matters here is the *shape* of the CV, which depends on the two catalytic potentials, either  $E_{\text{eq}}$  or the ratio  $i_{\text{ox}}^{\text{lim}}/i_{\text{red}}^{\text{lim}}$  (one or the other, since the two are related by eq. 1) and the broadening that result from iET kinetics.

The goal of our analysis was to determine (1) whether or not the catalytic waveshape of *Tam* HydS is consistent with an ordered mechanism, and (2) whether the observed irreversibility arises from intrinsic properties of the catalytic cycle or from slow iET. The answers are unambiguous.

(1) At all pH values, the best fit (shown in red in Fig. 2 and SI Fig. 1) is very satisfactory (considering the simplicity of the model, the strong constraint given by eq. 1, and the fact that we

simultaneously fitted the model to the data and their derivatives). This implies that an ordered mechanism accounts for the irreversible shape of the voltammetry of *Tam* HydS; there is no reason to invoke differing catalytic mechanisms for  $\text{H}_2$  oxidation and production under oxidizing and reducing conditions, respectively. The same is true for *M. elsdenii* FeFe hydrogenase, which gives a *reversible* catalytic response at all pH (see ref.<sup>3</sup>).

(2) The shape of the catalytic wave recorded with *Tam* HydS (e.g. red trace in Fig. 1B) clearly deviates from the ideal sigmoidal waveshape that would be observed if iET were infinitely fast (Fig. 1A). Slow iET is known to flatten the catalytic response, to the point that a reversible catalyst may appear irreversible if iET is sufficiently slow (as illustrated in SI section 3).<sup>28</sup> We therefore sought to discriminate between two hypotheses: either the catalytic wave is *intrinsically* irreversible (meaning that it would also be irreversible even if iET were infinitely fast), or it is intrinsically reversible but rendered irreversible by slow iET. The answer comes from fitting the generic current equation and examining the best values of the catalytic potentials,  $E_{\text{ox}}^{\text{cat}}$  and  $E_{\text{red}}^{\text{cat}}$ , which would be the positions of the two waves if iET were Nernstian. A positive  $\Delta E = E_{\text{ox}}^{\text{cat}} - E_{\text{red}}^{\text{cat}}$  indicates that the oxidative catalytic wave is at greater potential than the reductive wave, and the current is zero in between (the catalytic response is intrinsically irreversible even in the Nernstian limit). A negative  $\Delta E$  occurs when the catalytic wave is a single two-electron sigmoid, which sharply cuts the X-axis at  $E = E_{\text{eq}}$  (reversible catalytic response). We found that at every pH, the best values of  $E_{\text{ox}}^{\text{cat}}$  and  $E_{\text{red}}^{\text{cat}}$  were such that  $\Delta E = E_{\text{ox}}^{\text{cat}} - E_{\text{red}}^{\text{cat}}$  is large and positive ( $\Delta E \approx 300$  mV, Fig. 3A). Forcing  $\Delta E$  to take a smaller value returned bad fits, where the distinctive features of the signal (the inflections of the current around  $E_{\text{cat}}$ ) are not reproduced (this is exemplified in green and blue in Fig. 2 and SI Fig. 1, see rows C and D). This proves that the irreversibility of *Tam* HydS comes from intrinsic details of the catalytic cycle, not from interfacial electron transfer being sluggish.

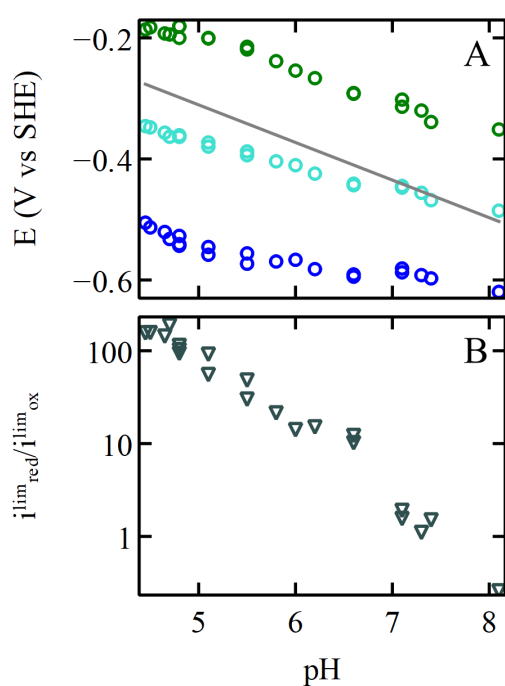


**Figure 2.** Steady-state voltammetry of *Tam* HydS from pH 6.6 to 8.1 (left to right), under one atm of  $\text{H}_2$ . The black traces in rows A and C show the data (current and  $d^2i/dE^2$ , respectively). The signals shown are the average of the forward and backward, blank-subtracted voltammetric traces. The averaged forward/backward blank traces (recorded with no enzyme) are also shown (black dotted lines). See SI



sections S1, S 2 and S5 for the raw voltammograms, additional data, and details about data analysis. The colored lines are the simultaneous fits of the two-electron, multistep, ordered model to the  $i$  and  $di/dE$  traces, either letting free the difference between the two catalytic potentials  $\Delta E = E_{ox}^{cat} - E_{red}^{cat}$  (red) or forcing  $\Delta E$  to take values lower than optimal (blue and green). The residues of the fits are shown in rows B and D. Experimental conditions  $T = 40^\circ\text{C}$ ,  $v = 2\text{ mV/s}$  (pH 6.6 and 7.4);  $v = 5\text{ mV/s}$  (pH 8.1),  $\omega = 3000\text{ rpm}$ .

As shown in Fig. 3A,  $E_{ox}^{cat}$  decreases about 58 mV per pH unit in the pH range 5 to 7.5, whereas  $E_{red}^{cat}$  is less pH dependent (-18 mV/pH between pH 5 and pH 7, -25 mV/pH over the entire pH range). This makes the difference between the average catalytic potential and  $E_{eq}$  increase about 24 mV/pH, and the catalytic bias in the direction of proton reduction increases by a factor of 6 per pH unit (Fig. 3B, calculated using eq. 1). Except for a much larger difference  $E_{ox}^{cat} - E_{red}^{cat}$ , all observations are similar to those made with the FeFe hydrogenase from *M. elsdenii*,<sup>3</sup> which functions reversibly. This suggests that the difference between the catalytic properties of the two enzymes comes from small differences in the rates of some of the steps in the catalytic cycle, not from the sequence of redox and protonation events being qualitatively different.



**Figure 3.** Dependence on pH of the two catalytic potentials of *Tam HydS* (panel A,  $E_{ox}^{cat}$  in green,  $E_{red}^{cat}$  in blue, obtained by from the fit of the wave shapes as described in Fig. 2; the average potential in cyan; the line shows the Nernst potential of the  $H^+/H_2$  couple, -62 mV/pH) and the catalytic bias (panel B).  $T = 40^\circ\text{C}$ , 1 atm. of  $H_2$ . The 21 series of data points shown here were obtained from CVs recorded with 16 different protein films (some of which used at two different pH values), and the entire data set was acquired using three different electrodes; the dispersion of the data points indicates the level of reproducibility of the results.

In previous spectroscopic investigations of both *Tm*<sup>26</sup> and *Tam*<sup>24</sup> HydS hydrogenases, it has been observed that the reduction of the most oxidized form of the active site ( $H_{ox}$ ), occurs at higher potential than in standard hydrogenases, resulting in an increased thermodynamic stability of the one-electron reduced state. This stabilization may contribute to the larger separation between the two catalytic potentials, compared to standard hydrogenases. However, one should refrain from thinking that the two catalytic potentials in Fig. 3A are the thermodynamic redox potentials  $E^0$  of the active site.<sup>12</sup> The catalytic potentials that define the catalytic waveshape take apparent values which depend on the two  $E^0$  of the active site but potentially also on the *kinetics* of all steps in the catalytic cycle

(see e.g. Table 1 in ref. <sup>12</sup>). The proton transfer pathway in *Tam HydS* is completely different from that of prototypical (and reversible) FeFe hydrogenases.<sup>24</sup> These structural differences should affect the kinetics of long range proton transfer, which is probably enough for shifting the catalytic potentials and making the response irreversible. In support of this assumption is the previous observation that modifying the proton transfer chain in *Cr HydA1* hydrogenase impairs reversibility,<sup>16</sup> and the extensive work on the Dubois bio-inspired catalysts, which also demonstrated the importance of the proton relays for achieving reversible catalysis.<sup>13</sup> Alternatively, the two catalytic potentials may be affected by the kinetics of long range electron transfer along the chain of accessory FeS clusters, on condition that intramolecular ET is rate determining in at least one direction of the reaction,<sup>31</sup> but the pH-dependencies of the catalytic potentials of *Tam HydS* and the truncated form of *M. elsdenii* hydrogenase being the same argues against slow intramolecular ET causing irreversibility in HydS.

## Conclusion

From the above analysis of the catalytic waveshape, we conclude that the irreversible voltammetry of *Tam HydS* is fully consistent with a single, ordered, clockwise/anticlockwise mechanism, and although slow iET does affect the shape of the voltammogram, it is not the reason the response is irreversible. The reversible catalytic waveshape of the FeFe hydrogenase from *Megasphaera elsdenii* is also well described by the ordered mechanism. However, in ref. <sup>12</sup>, we observed that eq. 1 is not obeyed with certain reversible bidirectional synthetic catalysts of  $H_2$  production and oxidation (which implies that their mechanisms involve multiple competing pathways). It is therefore clear that (ir)reversibility is not simply related to whether or not the catalytic cycle is ordered.

Understanding which details of the catalytic cycle of FeFe hydrogenases make their responses (ir)reversible will require that the values of the catalytic potentials be understood in relation to the thermodynamics of the catalytic intermediates, and the nature and rates of each of the bidirectional steps in the catalytic cycles.<sup>12</sup> Tackling this challenge will be critical to unravelling Nature's design principles for  $H_2$  gas processing.

## Associated content

### Supplementary information

The Supporting Information is available free of charge on the ACS Publications website. Section S1: Catalytic voltammetry of HydS at low pH. S2: CVs before and after blank subtraction. S3: Discussion of the effect of the rate of iET on the reversibility of the catalytic signal. S4: the generic current equation for two-electron catalysis by an adsorbed catalyst undergoing direct ET with the electrode. S5 and S6: data analysis.

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