Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Reactivation of Sulfide-Protected [FeFe] Hydrogenase in a Redox-active Hydrogel

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1. Methods

1.1 Polymer synthesis

The synthesis of the viologen modified polymer P1 (Scheme S1) was carried out according to ref [1].

1.2 *Dd*HydAB sample preparation

*Dd*HydAB was prepared heterologously in *E. coli* BL21(DE3) ΔiscR cells, purified by Strep-tag affinity chromatography, and artificially maturated with diiron precursor complex as described previously.[2] The sulfide-protected enzyme was generated by oxidizing the active enzyme with hexaammine-ruthenium (III) chloride under an N_2 atmosphere, in the presence of Na_2S, as described previously.[3]

1.3 Electrode preparation

Prior to electrode drop-casting, glassy carbon working electrode (GCE) was polished with 1, 0.3 and 0.05 µm Al_2O_3 slurry then rinsed with ultrapure water and sonicated in water for 10 min. For the preparation of P1/*Dd*HydAB redox hydrogel, a solution of *Dd*HydAB (1 µL, 200 µM in 50 mM Tris-HCl buffer, pH 8) was mixed with an aqueous solution of P1 (5 µL, 6 mg mL^{-1}). The mixture was drop-cast on a GCE and left to dry at 4 °C for 16 h. For DET measurements, a pyrolytic graphite electrode was polished with sand paper (P280) then with alumina paste (1 µm grain size) in the glovebox. Afterwards, *Dd*HydAB (5 µL, 50 µM in 50 mM Tris-HCl buffer, pH 8) was placed on the electrode surface and left for 10 min. The electrode was then rinsed with water and placed in the electrochemical cell for measurements.

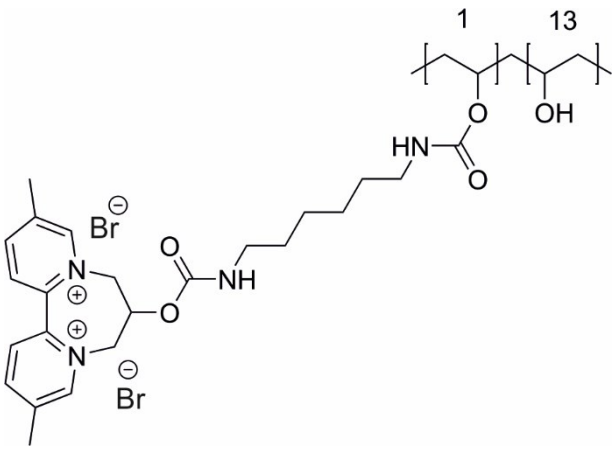
1.4 Electrochemistry

Mediated electron transfer electrochemical measurements were performed under ambient lab conditions. The direct electron transfer experiment as well as the control experiments in Figure S1 were performed in a glovebox filled with N_2 (MBraun). All electrochemical experiments were recorded on a Gamry Reference 600™ potentiostat. A rotating disk glassy carbon working electrode with a diameter of 3 or 5 mm was used in a three-electrode setup. Ag/AgCl 3M KCl was used as reference electrode and Pt wire as counter electrode. Commercial Tris base (Sigma Aldrich), citric acid (Sigma Aldrich) and KI (Fluka Analytics) were used to prepare the electrolyte containing Tris-citrate buffer (50 mM, pH 8.8) and KI (0.1 M).

1.5 Spectroelectrochemistry

FTIR spectroelectrochemical titrations were performed using a homebuilt transition mode electrochemical cell based on a design by Moss and coworkers.[4] A 50 µm thick gold mesh was used as a working electrode, deposited on a CaF_2 window. A mixture of 22 µL of 1.78 mM sulfide-protected

*Dd*HydAB and 63.3 µL of 15.8 mg mL^{-1} redox polymer P1 was prepared. 80 µL of this mixture was deposited on the gold mesh in 20 µL portions and allowed to dry for 1 h after each addition at room temperature. The window was then fixed into the electrochemical cell and 40 µL of Tris-citrate buffer (50 mM pH 8.8) with KI (0.1 M) was pipetted over the film. A platinum counter electrode and a Ag/AgCl (sat. KCl) reference electrode completed the three-electrode system. The reference electrode was calibrated before and after each measurement using (hydroxymethyl)ferrocene (Aldrich, +420 mV *vs* SHE) as a reference, to ensure the potential stability during the course of the experiment. The potential was controlled by an Autolab PGSTAT101 potentiostat using Nova software. Each potential was applied for 30 minutes prior to measuring IR spectra, in order to obtain equilibrium. All potentials referred to in the text are quoted versus the standard hydrogen electrode (SHE). The temperature of the cell was maintained at 10 ^{o}C using a water circulator system (Huber, Offenburg). Spectra were measured on a Bruker IFS 66v/S FTIR spectrometer equipped with a nitrogen cooled Bruker mercury cadmium telluride (MCT) detector. Spectra were collected in the double-sided, forward-backward mode with a resolution of 2 cm^{-1}, an aperture setting of 2.5 mm and a scan velocity of 20 KHz. Spectra are the average of 1000 scans. Data were processed using home-written routines in the MATLAB™ environment.



Scheme S1. Structure of the redox polymer P1

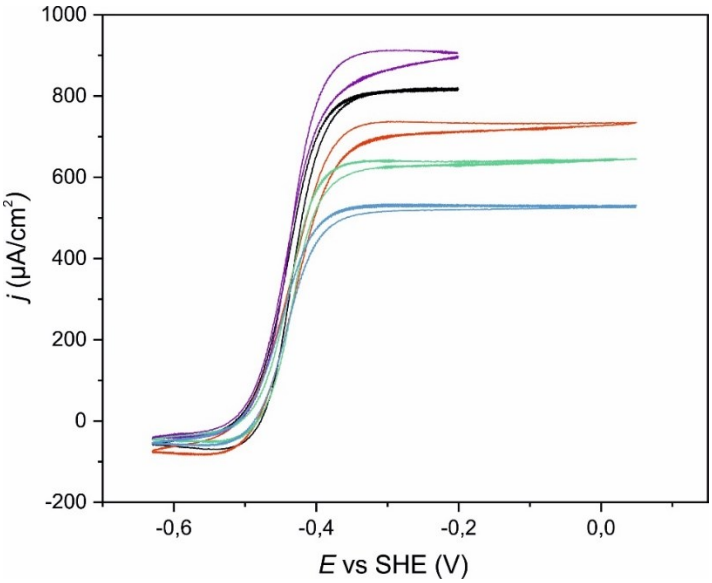


Figure S1. Cyclic voltammetry experiments of five different GCEs modified with protected *Dd*HydAB embedded in the redox polymer P1. Conditions: Tris-citrate buffer (50 mM, pH 8.8) with KI (100 mM), 2000 rpm, 20 mV s^{-1}, room temperature.

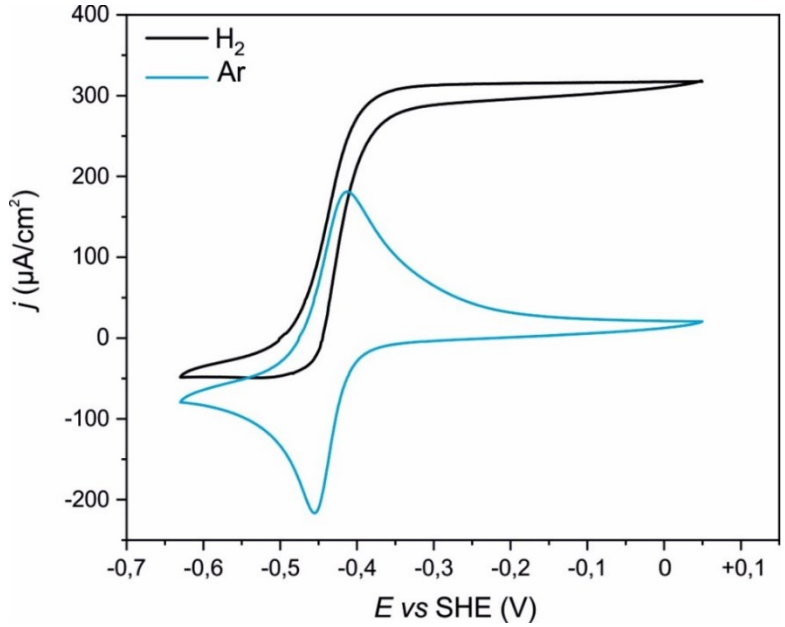


Figure S2. Cyclic voltammetry experiments under Ar (blue trace) and under H_2 (black trace) of unprotected *Dd*HydAB embedded in the redox polymer P1. The electrode was prepared and measured in an anaerobic glovebox. Conditions: 50 mM Tris-citrate buffer pH 8.8 with 100 mM KI, 2000 rpm, 20 mV s^{-1}, room temperature.

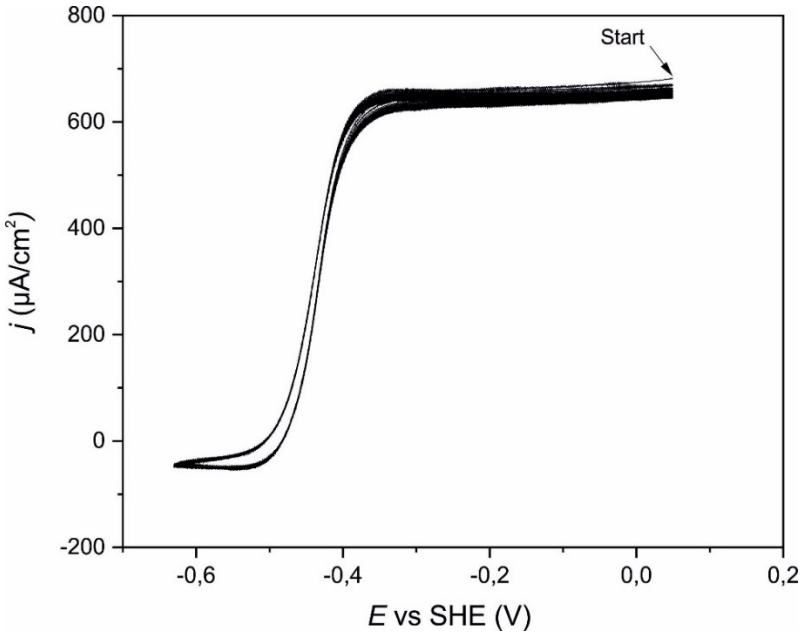


Figure S3. Successive cyclic voltammetry scans (15 scans) of protected *Dd*HydAB embedded in the redox polymer P1 under hydrogen. Scanning was started from positive potentials. Conditions: Tris-citrate buffer (50 mM, pH 8.8) with KI (100 mM), 2000 rpm, 20 mV s^{-1}, room temperature.

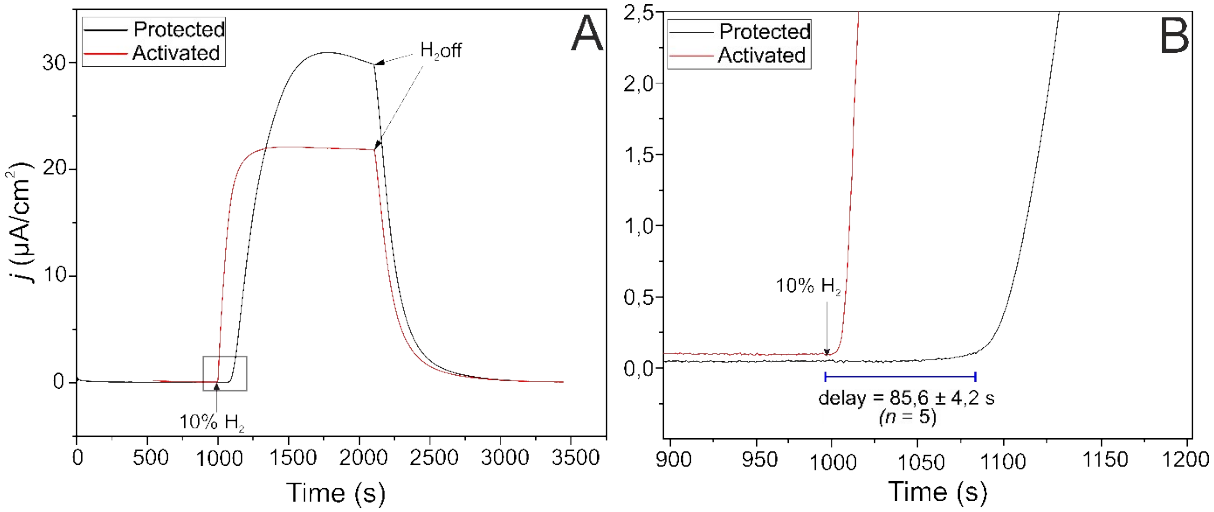


Figure S4. Chronoamperometry experiments of protected *Dd*HydAB embedded in redox polymer before (black) and after (red) activation. A potential of +199 mV *vs* SHE was applied under Ar. After the current stabilized close to 0, 10% H_2 was added to the gas flow then switched off again upon reaching maximum current (indicated by arrows) (A). Zoom-in view of H_2 addition section (B). Conditions: Tris-citrate buffer (50 mM, pH 8.8) with KI (100 mM), 2000 rpm, room temperature.

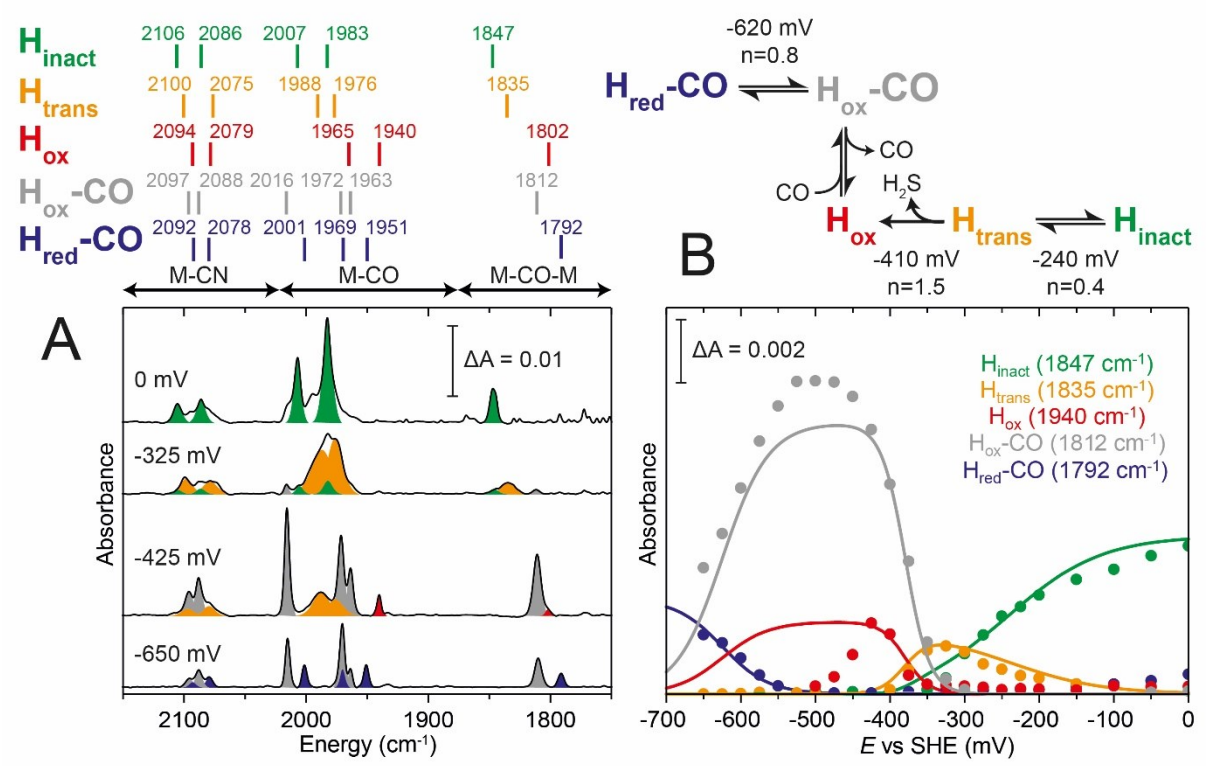


Figure S5. FTIR spectroelectochemistry of reactivation of sulfide-protected *Dd*HydAB in a redox polymer hydrogel matrix. A) FTIR spectra of *Dd*HyAB at various applied potentials during the redox titration, highlighting the main species observed: H_{inact} (green peaks), H_{trans} (orange peaks), H_{ox} (red peaks), H_{ox}-CO (gray peaks), H_{red}-CO (purple peaks). B) The intensities of the bridging CO band (between 1820 cm^{-1} and 1790 cm^{-1}) for the H_{inact} (green circles, 1847 cm^{-1}), H_{trans} (orange circles, 1835 cm^{-1}), H_{ox}-CO (gray circles, 1812 cm^{-1}), and H_{red}-CO (purple circles, 1792 cm^{-1}), and the terminal CO band from the H_{ox} state (red circles, 1940 cm^{-1}) are plotted against the applied potential. The data were fitted with curves based on the Nernst equation, and the scheme above the figure shows the *E* and n values used for fitting. The sample preparation is described in the supplementary methods. Measurements were carried out at 10 °C, and with 2 cm^{-1} resolution. The applied potential was swept from positive to negative.

Due to the overlap on the most intense bands of H_{inact}, H_{trans}, H_{ox}-CO and H_{red}-CO (between 2020 cm^{-1} and 1950 cm^{-1}), which convolutes the analysis, we chose to follow the intensities of the more well-separated bridging CO bands (between 1850 cm^{-1} and 1790 cm^{-1}). However, the most intense band for H_{ox} (1940 cm^{-1}) is well separated from all the other peaks of the various states, and so the intensity of this peak was followed for the H_{ox} state. It was previously reported for the enzyme in solution in the presence of soluble redox mediators, that the H_{inact} state converts to the H_{trans} state at -140 mV vs SHE with n = 1.[3] For the enzyme embedded in a redox polymer (Figure S5), the conversion of the H_{inact} state to the H_{trans} state occurs with a lower midpoint potential (-240 mV) and n = 0.4 behavior. This can be explained in part by the higher pH (8.8 vs 8) used in the current redox titration compared with that performed with the enzyme in solution. All the potentials are pH dependent and accordingly

shifted to more negative values for pH 8.8 compared with pH 8. Electron transfer rates through the polymer are likely to be extremely poor at applied potentials much more positive than the redox midpoint potential of the viologen moieties in the polymer (-440 mV). This further explains the more negative midpoint potential of the H_{inact} to H_{trans} transition, as well as the n = 0.4 behavior. In the solution measurements, soluble mediators including anthraquinone-1,5-disulfonic acid ($E_{m,7}$ = –234 mV) and anthraquinone-2-sulfonate ($E_{m,7}$ = –277 mV) were used to facilitate rapid electron exchange between the working electrode and the enzyme.

As the applied potential approaches that of the viologen potential, the conversion of H_{inact} to H_{trans} goes to completion and shortly afterwards the H_{trans} state is converted to a mixture of H_{ox} and H_{ox}-CO, with a midpoint potential of -410 mV and n = 1.5. Presumably, at this point the active sites of some enzyme molecules have degraded involving the release of the CO ligands, which then bind to the activated enzymes in the H_{ox} state.[5] It does not require much of the enzyme to be damaged to inhibit a large number of the active enzymes because *i)* each active site contains three CO ligands, *ii) Dd*HydAB has a high affinity for CO[6], and *iii)* the small volume of the spectroelectrochemical cell enables accumulation of CO. Other than this, the behaviour is similar to that reported for the solution measurements, but with a slightly more negative potential, reflecting the higher pH in the current experiments. The high value of n reflects the fact that this step is irreversible: there is no H_2S in solution available to rebind the active site, so the reaction proceeds to completion in a kinetic fashion, where the kinetics depend on the applied potential. Interestingly, below the redox midpoint potential of the polymer, at around the potential that H_{ox} should be converted to the H_{red}/$H_{red}H^+$ states, the H_{ox} state is lost completely and replaced by an increase in the H_{ox}-CO state. As this step cannot not involve a simple one-electron reduction of the enzyme, it cannot be fit with the Nernst equation.

Finally, at very low potential (-620 V vs SHE) the H_{ox}-CO state is converted to the H_{red}-CO state, with a potential slightly more negative than that previously reported at pH 8.[7] Overall, the spectroelectrochemistry experiments demonstrate that the behaviour of the enzyme in the redox polymer is similar to the behaviour in solution, and that the reactivation of the enzyme follows the same pathway. The main difference, the formation of a large amount of the H_{ox}-CO state observed in the spectroelectrochemical cell, is unlikely to occur in the electrochemical experiments during purging of the electrochemical cell with H_2.

References

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