Supporting Information for:

Spectroscopic Evidence of Reversible Disassembly of the [FeFe] Hydrogenase Active Site

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Supplementary Discussion – General remarks on FTIR spectroelectrochemistry

In an FTIR spectroelectrochemical experiment, the spectral features of each redox state of the enzyme are followed as the applied potential is altered. A series of potentials are chosen and stabilized in a three-electrode FTIR spectroelectrochemical cell, which is based on the design of Moss and co-workers.[1] The sample is loaded on a semitransparent (70% transparency) gold mesh that, together with a platinum foil counter electrode and an Ag/AgCl reference electrode, complete the three-electrode system. Radiation passes through the FTIR spectroelectrochemical cell in the typical transmission mode. [FeFe] hydrogenases contain CO and CN^- ligands in their active sites in order to stabilize the low oxidation states of the iron ions. The IR stretching vibrations of the CO and CN^- ligands occur in a region free from other vibrations associated with the protein and the buffer components. The iron bound CO stretch for the hydrogenases is shifted to lower frequencies relative to the free ν_{CO} stretch (2155 cm^{-1}) and can be commonly observed in the range of 1800-2100 cm^{-1}. On the other hand, the ν_{CN} (2080 cm^{-1}) in hydrogenases is shifted to higher frequencies (2200-2000 cm^{-1}). In [FeFe] hydrogenases, the CO and CN^- ligands are sensitive to the change in the oxidation state of the iron ions (which modulates the back-bonding strength to the CO ligands) as well as changes in their interactions with the surrounding (which also affects the CN^- vibrations). This is the reason why unique spectra for each redox state of the active site can be observed. Changes in the hydrogen bonds or in the movement of the ligands will influence the recorded spectra.

Figure S1 - Chronoamperometry during the spectroelectrochemical redox titration.

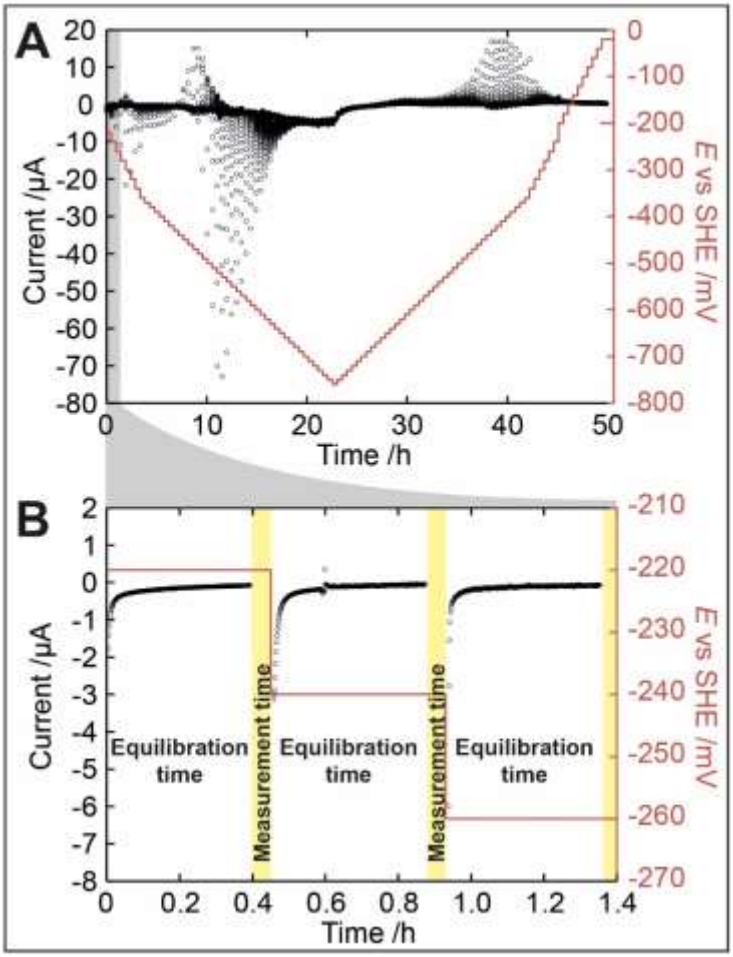

Figure S1. Associated chronoamperometry measurements for Figure 3. The spectroelectrochemical cell was connected to a potentiostat coupled to a spectrometer, and potentials are applied in steps from less negative to more negative potential and back again (red curve). The current passing through the spectroelectrochemical cell (black circles) was followed over time (chronoamperometry) until it reached a plateau indicating that the sample is at equilibrium (or very close to equilibrium) with the applied potential. At this point the FTIR spectrum was measured while the potential was still being applied. Then the potential was stepped and the process repeated. **A:** the chronoamperometric data from the entire titration shown in Figure 3. **B:** close up view of the first three steps (gray region) from **A** indicating the

equilibration phase (white area) and the measurement phase (yellow area)**.** The experimental conditions are the same as in Figure 3 of the main text.

Figure S2 - FTIR spectroelectrochemistry from -300 mV to -700 mV

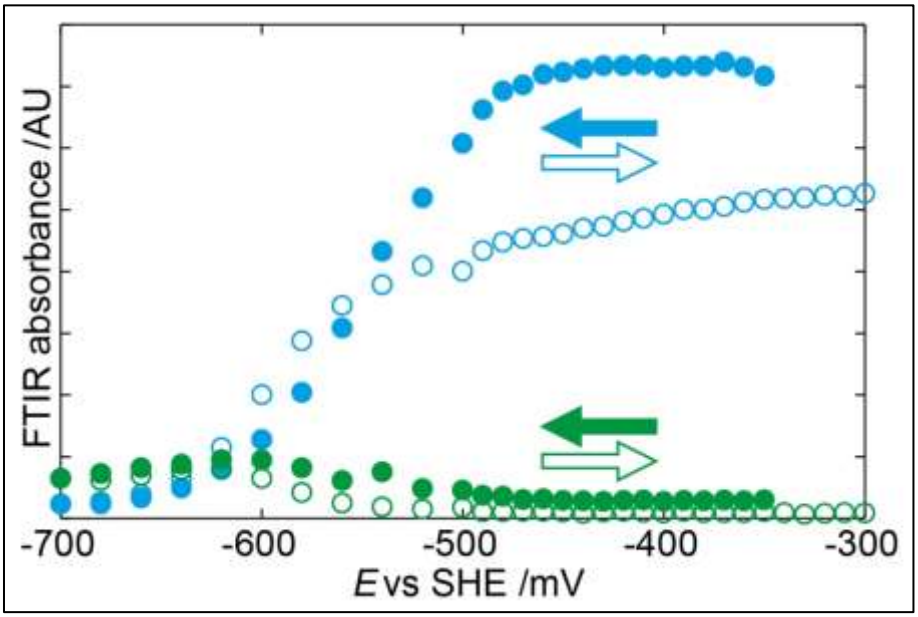

Figure S2. Titration curves obtained from the spectroelectrochemical redox titration of CO-inhibited *Dd*HydAB in 50 mM MES, 50 mM HEPES at pH 8 and 150 mM KCl (as supporting electrolyte) containing redox mediators (see experimental section). The titration curves represent the intensities of the main peaks in each state (H_{ox}-CO = 2017 cm^{-1} (light blue circles) and H_{red}-CO = 2002 cm^{-1} (green circles)) plotted against the applied potential. Filled circles indicate reductive titrations and open circles indicate the subsequent oxidative titrations. All other experimental conditions are the same as in Figure 3 of the main text.

Figure S3 - Chronoamperometry during spectroelectrochemistry.

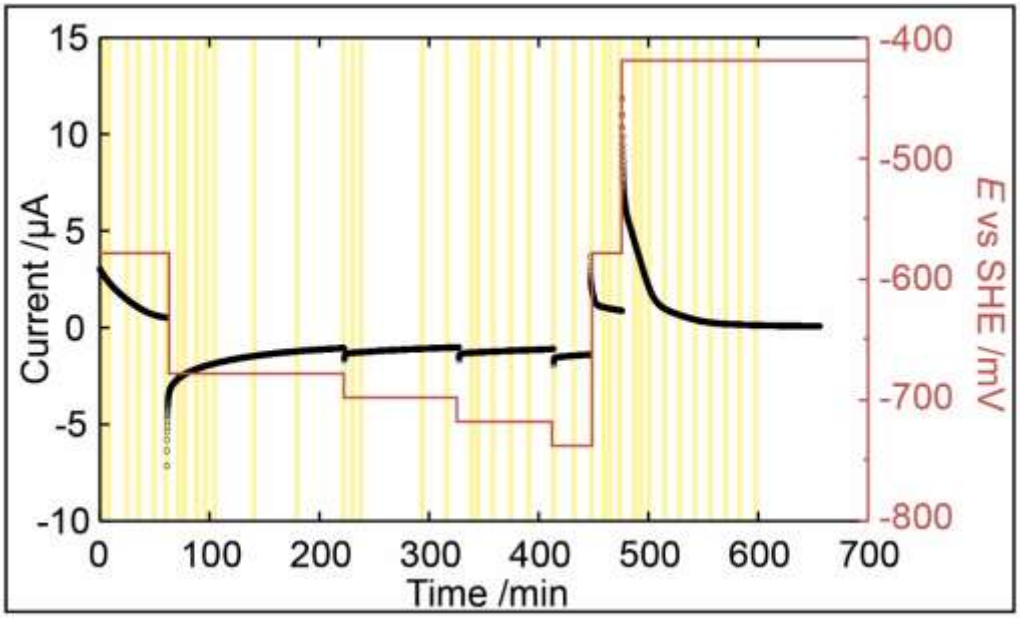

Fig. S3. Associated chronoamperometry measurements for Figure 4. The spectroelectrochemical cell was connected to a potentiostat coupled to a spectrometer, and potentials are applied in steps (red curve). The current passing through the spectroelectrochemical cell (black circles) was followed over time (chronoamperometry) and FTIR spectra were measured (yellow areas). The experimental conditions are the same as in Figure 4 of the main text.

References:

1. Moss, D.; Nabedryk, E.; Breton, J. L. J.; Mäntele, W., Redox-Linked Conformational Changes in Proteins Detected by a Combination of Infrared Spectroscopy and Protein Electrochemistry. Evaluation of the Technique with Cytochrome *c*. *Eur. J. Biochem.* **1990**, *187*, 565-572.