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SUPPORTING INFORMATION

Axial iron coordination and spin state change in a heme *c* upon electrostatic protein-SAM interaction

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Materials and Methods

Materials. All chemicals were reagent grade. 11-mercapto-1-undecanoic acid (MUA) and 11-mercapto-1-undecanol (MU) (Sigma-Aldrich) were recrystallized from hexane before use. MILLIQ water was used throughout.

Protein expression and purification. *Sb*-DHC from *Shewanella baltica* OS155 was cloned, overexpressed in *E. coli* and purified as reported previously.[1]

Electrochemical Measurements. Cyclic voltammetry (CV) experiments were performed with a Potentiostat/Galvanostat PAR mod. 273A using a cell for small volume samples (0.5 ml) under argon. Experiments were carried out using a 1 mm-diameter polycrystalline gold wire as working electrode. A Pt sheet and a saturated calomel electrode (SCE) were used as counter and reference electrode, respectively. The electric contact between the SCE and the working solution was obtained with a Vycor set. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methylviologen). All the redox potentials reported here are referred to SHE.

The cleaning procedure for the gold working electrode started with flaming it in oxidizing conditions and subsequent heating in concentrated KOH for 30 min. After rinsing in MILLIQ water, the electrode was dipped in concentrated sulfuric acid for 30 min. To minimize residual adsorbed

impurities, the electrode was then subjected to 20 voltammetric cycles between +1.5 and -0.25 V at 0.1 V s^{-1} in 1 M H_2SO_4. Finally, it was rinsed in water and anhydrous ethanol. The Vycor set was treated in an ultrasonic pool for about 5 min. Electrode coating with the mixed SAM (self-assembled monolayer) of MUA/MU was made by dipping the cleaned electrode into a 1 mM ethanolic 1:1 solution of both substances for 12 h at 5 °C and then rinsing it with MILLIQ water. SAM alignment was achieved performing 10 CV cycles from +0.2 to -0.3 V (vs SCE) with the MUA/MU-coated electrode in a 0.1 M sodium perchlorate solution outgassed with argon. The resulting CV was taken as the background and checked for the absence of spurious signals. Protein adsorption on the MUA/MU SAM-coated Au electrode was achieved dipping the functionalized electrode into a 0.05 mM protein solution at 4 °C for 15 s.

All electrochemical experiments were performed in 5 mM sodium perchlorate plus 5 mM phosphate buffer at pH 7. The current intensity is linearly dependent on the scan rate, as expected for a diffusionless electroactive species (Fig. S1). The surface coverage Γ_0 was calculated from the overall charge Q_{tot} exchanged by the protein (determined upon integration of the baseline-corrected cathodic peaks) and the area A of the gold electrode by applying the relationship:

$$\int i(V)\, dV = \nu(nFA\Gamma_0) = \nu Q_{tot}$$

where ν is the sweep rate (in Vs^{-1}), n (=1) the number of electrons exchanged in the redox center half reaction, and F is the Faraday constant. The area of the electrode was determined electrochemically by applying the Randles-Sevçik relationship to the CV signal obtained for aqueous solutions of ferrocenium tetrafluoborate of known concentration in a diffusion-controlled regime, in which the bare electrode was dipped at exactly the same depth as for the measurements with adsorbed *Sb*-DHC. A coverage of 16±1 pmol cm^{-2} was determined. The formal reduction potentials E°’ for the proteins were calculated from the average of the anodic and cathodic peak potentials. The experiments were performed at least two times and the reduction potentials were found to be reproducible within ±2 mV.

CV experiments at different temperatures were carried out with a cell in a "nonisothermal" setting,[2-4] namely in which the reference electrode is kept at constant temperature (21±0.1 °C) whereas the half-cell containing the working electrode and the Vycor® junction to the reference electrode is under thermostatic control with a water bath. The temperature was varied from 5 to 45 °C. With this experimental configuration, the reaction entropy for protein reduction ($\Delta S°'_{rc}$) is given by: [2-4]

$$\Delta S°'_{rc} = S°'_{red} - S°'_{ox} = nF\frac{dE°'}{dT} \qquad (1)$$

thus, $\Delta S°'_{rc}$ was determined from the slope of the plot of E°' *vs*. temperature which turns out to be linear under the assumption that $\Delta S°'_{rc}$ is constant over the limited temperature range investigated (Fig. S2). With the same assumption, the enthalpy change ($\Delta H°'_{rc}$) was obtained from the Gibbs-Helmholtz equation, namely as the negative slope of the E°'/T vs. 1/T plot. The nonisothermal behavior of the cell was carefully checked by determining the $\Delta H°'_{rc}$ and $\Delta S°'_{rc}$ values of the ferricyanide/ferrocyanide couple.[3-5]

The proposed 'perpendicular' orientation of the protein toward the electrode and the resulting vectorial ET, regulated by the proximity of the heme centers to the electrode and their reduction potentials, is ultimately demonstrated by the evidence gained for the "two-electron" nature of anodic component of peak I. In particular, we have carried out repeated CV scans at different scan rates (from 0.02 to 1 V s^{-1}) also allowing different elapsing times between the reductive scan and the subsequent anodic scan (up to 60 s). Independently of the scan rate and the elapsing time, the current of the first oxidation peak is lower than that of the reductive peak (Fig. 1, main text). However, in the subsequent cycles, the anodic peak area is close to the sum of the area of the two cathodic peaks (Fig S3). Therefore, the lower area of the anodic return of couple I is due to partial desorption of the reduced protein (which is less firmly bound because of the lower positive charge of the C-terminal domain). This behaviour can be explained as we are using a polycrystalline gold electrode on which adsorption

sites of different affinity could exist (as previously observed for cytochromes c)[6] Therefore, likely most of the weakly adsorbed protein in the reduced form is lost after the first scan. Afterwards, we are monitoring the redox response of the firmly bound protein.

Rate constants of heterogeneous ET for immobilized *Sb*-DHC. Cyclic voltammograms at variable scan rate were recorded to determine the rate constant k_s for interfacial electrochemical ET process for the adsorbed protein, according to Laviron.[7] The k_s values were averaged over five measurements and found to be reproducible within 6%, which was taken as the associate error. The separation between the anodic and the cathodic peak increases with increasing the scan rate as expected for systems adsorbed in both oxidation states, while the E°' values remain unchanged. The *ks* values were also measured at 5, 10, 15, 20, 25, 30 °C to determine the activation enthalpies ($\Delta H^{\#}$) using the Arrhenius equation, namely from the slope of the plot of ln k_1 versus 1/T (Fig. S4).

Raman and SERR spectroscopy. Raman spectra were collected in back-scattering geometry using an InVia Raman microscope (Renishaw plc, Wotton-under-Edge, U.K.) equipped with a BluePhoton 405 nm diode laser (Omicron GmbH, Rodgau-Dudenhofen, Germany), and with a spectrograph having a 2400 l/mm grating (spectral resolution of approximately 4 cm^{-1}) and a thermoelectrically cooled (-70°C) CCD camera. The spectrograph was calibrated using the lines of a Ne lamp. For RR measurements, a 10× microscope objective (NA = 0.25) was used to focus the laser and collected the scattered light from a rotating glass capillary containing 15 μL of protein solution. The capillary was kept rotating to prevent photo-induced sample degradation. RR spectra were acquired in 120 s, using a laser power of 5 mW at the sample. For SERRS measurements, a 60× water immersion microscope objective (NA = 1.00) was used to focus the laser on the surface of the MUA/MU-coated Ag electrodes on which the *Sb*-DHC protein had been adsorbed (contact solution: 5 mM sodium perchlorate plus 5 mM phosphate buffer at pH 7). The electrode was housed in a modified version of a spectro-electrochemical cell previously described,[8] and designed to be used with a Raman microscope. A ring

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of Ag co-axial with the working electrode was left in a saturated KCl solution overnight was used as a Ag/AgCl reference electrode. A ring of Pt co-axial with the working electrode and with the reference electrode was used as counter-electrode. During each SERRS measurement, the cell was kept moving using a motorized microscope stage (Prior Scientific Instruments Ltd., Cambridge, UK) to renew the part of the electrode surface illuminated by the laser, thus avoiding overheating and/or sample photodegradation. SERRS spectra were acquired in 30 s, using a laser power of 0.5 mW at the sample. SERRS measurements were performed in three independent replicas (i.e. on three different electrodes).

Supplementary References

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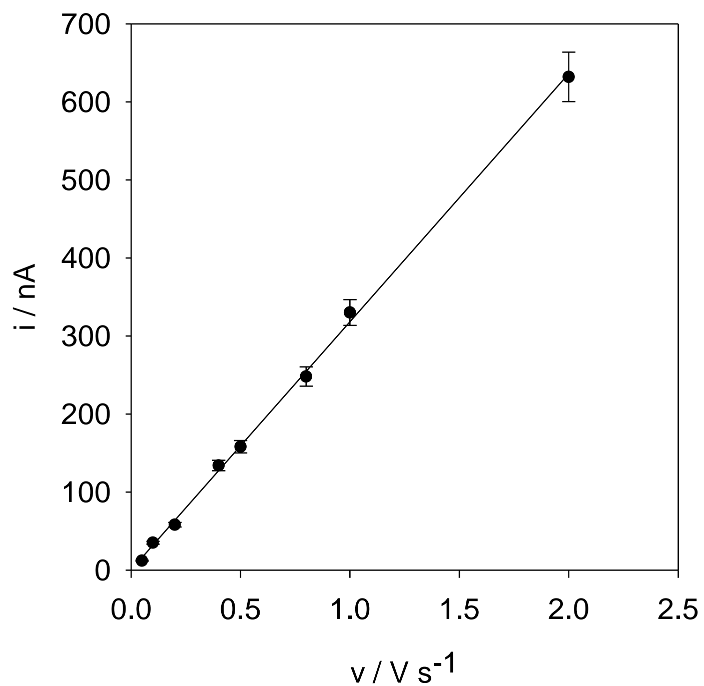

Figure S1. Current intensity for signal I as a function of the scan rate for *Sb*-DHC electrostatically bound on a polycrystalline gold electrode coated with a SAM of MUA/MU in 5 mM phosphate buffer, 5 mM sodium perchlorate in H_2O at pH 7. Sweep rate: 0.05 V s^{-1}, T = 20 °C.

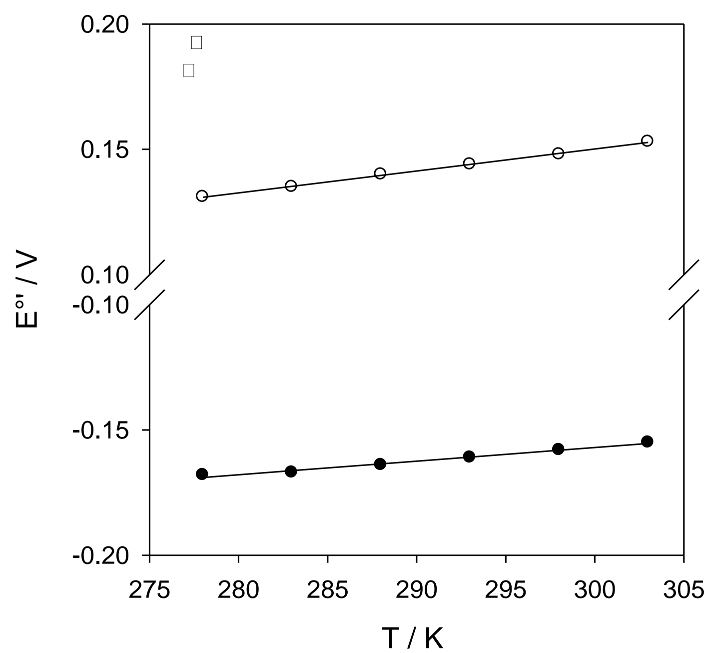

Figure S2. Plot of E°' versus temperature for *Sb*-DHC electrostatically bound on a polycrystalline gold electrode coated with a SAM of MUA/MU in 5 mM phosphate buffer, 5 mM sodium perchlorate in H_2O at pH 7. (●) peak I; (○) peak I'. Sweep rate: 0.05 V s^{-1}, T = 20 °C.

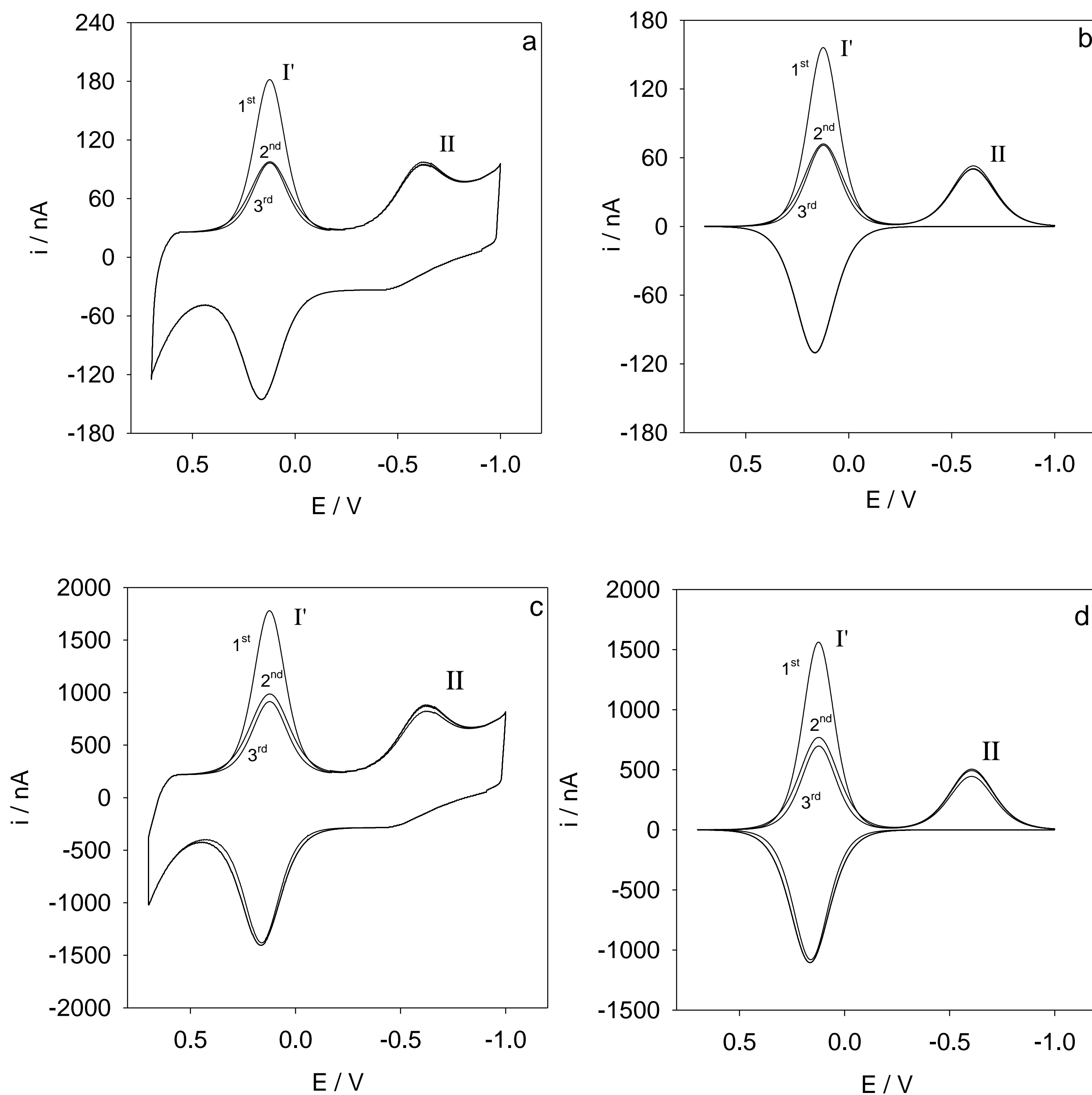

Figure S3. First three scans of the cyclic voltammograms for *Sb*-DHC electrostatically bound on a polycrystalline gold electrode coated with a SAM of MUA/MU recorded at (a, b) 0.05 Vs^{-1} (c, d) 0.5 Vs-1. CVs in b) and d) are subjected to background suppression. CVs were recorded in 5 mM phosphate buffer, 5 mM sodium perchlorate, pH 7. T = 20 °C.

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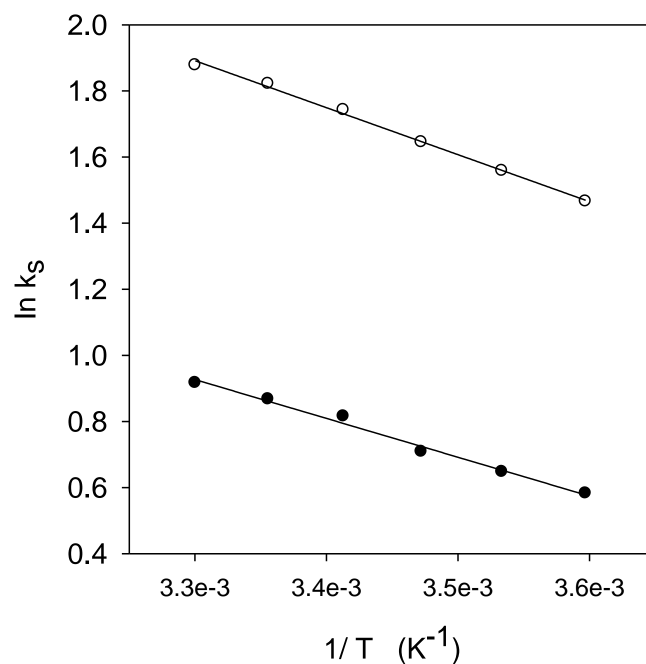

Figure S4. Arrhenius plot for the low-potential heme center of *Sb*-DHC electrostatically bound on a polycrystalline gold electrode coated with a SAM of MUA/MU recorded 10 s (signal I) (●) and 6 min (signal I') (○) in 5 mM phosphate buffer, 5 mM sodium perchlorate in H_2O at pH 7. Sweep rate: 0.05 V s^{-1}, T = 20 °C. Solid lines are least-squares fits to the data points.