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Electronic Supporting Information

A Decahaem Cytochrome as Electron Conduit in Protein-Enzyme Redox Processes

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Contents

Experimental Section

Material and reagents

All chemicals: 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich), tris(hydroxymethyl)amino-methane (Tris, Sigma-Aldrich), KCl (Alfa Aesar), KOH (Breckland Scientific), NH_4OH (30%) solution (Fisher Scientific), H_2O_2 (30%) solution (Fisher Scientific), polystyrene beads (Polysciences, Inc.), indium tin oxide (ITO) nanoparticles (Sigma-Aldrich), ITO- and fluorine-doped tin oxide (FTO)-coated glass (Sigma-Aldrich), MeOH (Fisher Scientific), acetone (Fisher Scientific) and isopropanol (Sigma-Aldrich) were purchased from commercial suppliers and used without further purification unless otherwise noted. Reagents for the analytical part of the work were of the highest available purity.

Protein Preparation and Characterisation

[NiFeSe]-hydrogenase from *D. baculatum* (H_2ase) was purified using a previously published method.[1] The pure enzyme was dialysed against 20 mM Tris/HCl at pH 7.6. The enzyme integrity was verified spectrophotometrically at λ = 604 nm by measuring its specific activity for H_2 oxidation with an aliquot of the H_2ase under H_2 in the presence of methyl viologen (1 mM) for 30 min at 30 °C. The preparation has a specific activity of 2115 μmol H_2 min^{-1} mg^{-1}, and the stock enzyme solution was diluted with 20 mM Tris/HCl buffer in an anaerobic glovebox to obtain a concentration of 8 μM before adsorption on the electrodes.

A soluble form of MtrC was purified and judged to be >95% pure by SDS-PAGE with protein visualised by Coomassie Brilliant Blue as previously described.[2]

FccA was purified from *S. oneidensis* MR-1 grown anaerobically in LB media with 20 mM lactate as electron donor and 20 mM fumarate as electron acceptor. Initial steps of the purification were as described previously.[3] After addition of $(NH_4)_2SO_4$ to 50% saturation the precipitated proteins were removed by centrifugation. Soluble proteins were concentrated and exchanged in to 20 mM Hepes, 100 mM Na_2SO_4 pH 7 .4 by ultrafiltration with a 50 kDa cut off membrane. SDS-PAGE with protein visualised by Coomassie Briliant Blue showed the protein to be >95% pure and as a consequence no further purification steps were performed. The purified sample coupled oxidation of dithionite reduced methyl viologen to fumarate reduction at a rate of 18.3 mM succinate min^{-1} mg^{-1} FccA.

Preparation of inverse opal mesoITO electrode

The inverse opal mesoITO electrodes were prepared according to a previously reported procedure.[4] In brief, a mixed dispersion of ITO nanoparticles (<50 nm diameter) and polystyrene beads (750 nm diameter, 2.6% w/v suspension in water) was prepared as follows: ITO nanoparticles (35 mg) were dispersed via sonication in a MeOH/water mixture (300 μL, 6:1 v/v) for 3 h. The dispersion of polystyrene beads (1 mL) was centrifuged (10,000 rpm, 3 min), the supernatant removed, and the polystyrene pellet redispersed in MeOH (1 mL). The polystyrene dispersion was centrifuged again (10,000 rpm, 0.5 min), the supernatant removed and the ITO nanoparticles dispersion was added to the polystyrene pellet. This mixture was further diluted with a MeOH/water mixture (1200 μL, 6:1 v/v) and thoroughly sonicated for 10 min in ice-cold water (<10 °C) to give a homogeneous polystyrene-ITO dispersion.

FTO-coated glass slides (2x1 cm^2) were cleaned by sonication in two 30 min steps in 2-propanol and absolute ethanol. The polystyrene-ITO dispersion was drop-cast (4.2 µL) onto a FTO slide on the area pre-defined by a Parafilm ring (0.25 cm^2 geometrical surface area) giving a 2 µm thick film. The electrodes were annealed at a 1 $^oC\ min^{-1}$ ramp rate from room temperature to 500 ^oC and then sintered for 20 min. The electrodes were cleaned with a mixture of H_2O_2, NH_4OH and water (1:1:5 v/v/v) at 70 °C for 15 min, rinsed with water, and then heated for 1 h at 180 °C before deposition of the proteins.

Preparation of the mesoITO electrodes

The mesoITO electrode were prepared as previously reported.[5] Briefly, ITO slides (1 × 2 cm^2) were sonicated in 2-propanol for 20 min followed by acetone for 20 min, and dried at room temperature in air. A mixture of 20 wt% of ITO nanopowder was sonicated for 20 min in an acetic acid solution in EtOH (5 M). The resulting ITO dispersion was spread on ITO-coated glass slides with Scotch® tape (3M) as spacers to maintain a constant surface area (Ø = 0.2 cm^2) and to control the film thickness. After drying the ITO slides in air, the tapes were removed, and the resulting slides annealed in a furnace under atmospheric conditions with the following temperature programme: the slides were heated from 25 °C to 450 °C (4 $°C\ min^{-1}$), kept at 450 °C for 20 min, and then slowly cooled to room temperature in the furnace chamber. The obtained mesoITO were subject to an additional washing step. The mesoITO electrodes were placed in a mixture containing H_2O, H_2O_2 (30%) and NH_4OH (30%) in a ratio of 5:1:1 at 70 ºC for 1 h and then washed with H_2O and dried at 180 °C for 1 h.

Enzymes immobilization on the electrode

For the preparation of IO-mesoITO|MtrC|FccA or IO-mesoITO|MtrC|H_2ase electrode, 3 µL of 40 µM MtrC (in MES buffer 0.1 M pH 6.5) were first drop-casted onto a cleaned 2 µm IO-mesoITO electrode (geometrical surface area of 0.25 cm^2) and the electrode was left to dry for 10 min before being rinsed with electrolyte solution. This was followed by the drop-cast of a 3 µL of 32 µM FccA (in MES buffer 0.1 M pH 6.5) or a 3 µL of 8 µM H_2ase (Tris/HCl buffer 20 mM pH 7.6) and the electrode was left to dry for 10 min before being rinsed with electrolyte solution. The IO-mesoITO|FccA and IO-mesoITO|H_2ase were prepared in a similar way but without MtrC. The same procedure was used for the preparation of the mesoITO electrode used in the SEC experiments.

Electrochemical and spectroelectrochemical measurements

Protein film voltammetry and spectroelectrochemistry were performed in a three-electrode configuration with the Ag/AgCl (3M NaCl) (Bioanalytical Sys.) reference electrode and the platinum wire as a counter electrode. A CompactStat potentiostat (Ivium Technologyies B.V.) was used for the electrochemical experiments. All redox potentials are quoted against the standard hydrogen electrode (SHE) and the potentials were obtained by using the following correction factor: $E_{SHE} = E_{Ag/AgCl} + 0.209$ V (25 °C). All experiments were performed in a mixed buffer at pH 7.0, consisting of 25 mM each of MES, TAPS, HEPES and K^+ acetate. All experiments except spectroelectrochemistry were carried out in an anaerobic MBraun glovebox with an O_2 concentration of less than 1 ppm.

Spectroelectrochemistry experiments were carried out using a Varian Cary 50 Bio UV-Visible spectrometer. Typically, a three-necked electrochemical cell was used and the working electrode was

placed perpendicular to the beam of the UV-spectrometer. UV-vis spectra were recorded while applying different set potentials with the potentiostat. Alternatively, absorbance at a fixed wavelength (420 nm) was recorded while running CV at 10 mV s^{-1}.

Supporting Table and Figures

***Table S1**: Quantitative summary of the cofactors (haems and flavins) and proteins surface coverage, UV-Vis absorbance values obtained from MtrC, FccA and MtrC|FccA immobilized onto the mesoITO electrodes.*

Electrodes	cofactors (nmol cm^{-2})	Γ [a] (nmol cm^{-2})	Ratio Γ_{film} [b] / $\Gamma_{MtrC-FccA}$	Abs. 410 nm	Abs. 419 nm
mesoITO|MtrC	1.5	0.15	0.62	0.26	0.35
mesoITO|FccA	0.6	0.1	0.41	0.09	0.12
mesoITO|MtrC|FccA	1.9	0.24	1	0.35	0.44

[a] Γ = Enzyme surface coverage. [b] Γ_{film} = Γ_{MtrC}, Γ_{FccA} or $\Gamma_{MtrC|FccA}$.

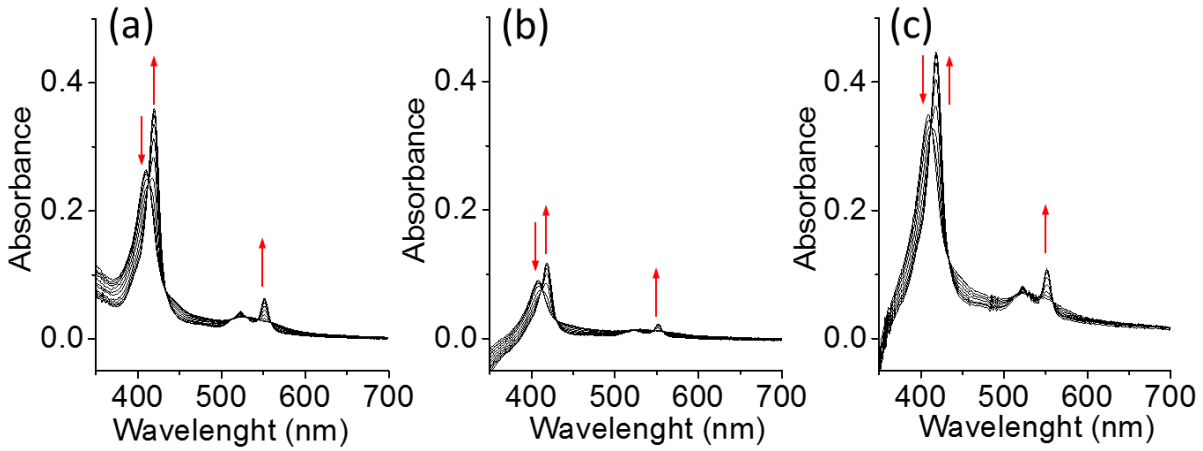

***Figure S1.** UV-vis spectra of the mesoITO electrodes modified with (a) MtrC (b) FccA and (c) FccA/MtrC recorded at different applied potentials (0.1; 0.05; 0; −0.05; −0.10; −0.15; −0.20; −0.25; −0.3; −0.35; −0.4 and −0.45 V vs. SHE) in mixed buffer pH 7.*

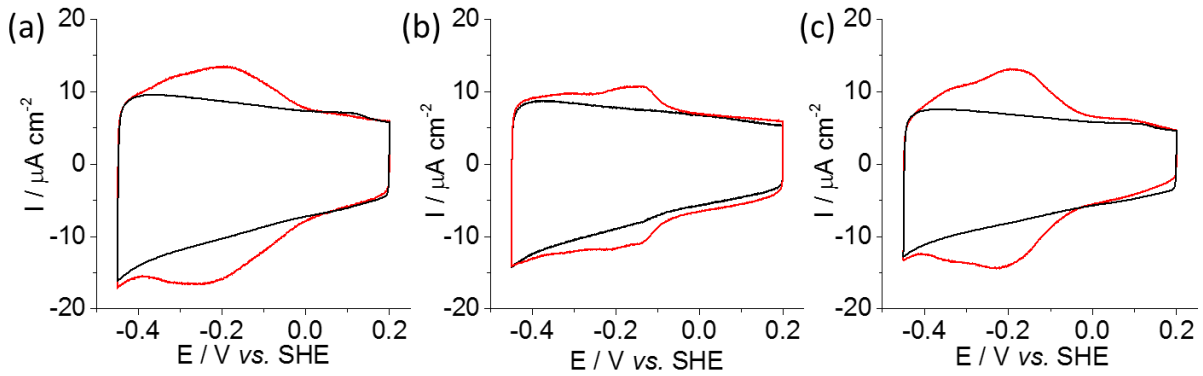

***Figure S2**. PFV of (a) bare mesoITO (black trace) and mesoITO|MtrC (b) bare mesoITO (black trace) and mesoITO|FccA (c) bare mesoITO (black trace) and mesoITO|MtrC|FccA in mixed buffer pH7 (v = 10 mV s^{-1}).*

Supporting References

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