IMMOBILIZED UNFOLDED CYTOCHROME *C* ACTS AS A CATALYST FOR DIOXYGEN REDUCTION

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Electronic Supplementary Information

Figure S1

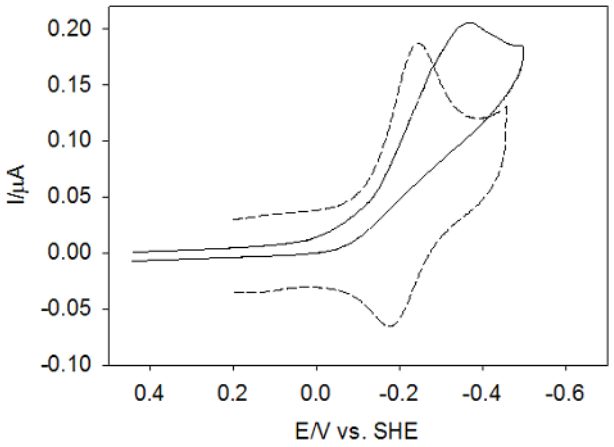

Fig. S1 Cyclic voltammograms for the unfolded His-His form of yeast cytochrome *c* adsorbed on a polycrystalline gold electrode coated with MP (– – –) and for reduction of O_2 on a polycrystalline gold electrode coated with MP (———). Working solution: 10 mM sodium perchlorate and 5 mM phosphate buffer at pH 7, urea concentration 9 M. Sweep rate 0.05 V s^{-1}, T = 293 K. Similar results are obtained on a polycrystalline gold electrode coated with MUA/MU.

EXPERIMENTAL S2

1. Materials

All chemicals were of reagent grade. Yeast cytochrome *c* (ycc) and urea were purchased from Sigma-Aldrich. ycc was purified on a cation exchange column (Whatman, CM52, carboxymethyl cellulose). 11-mercapto-1-undecanoic acid (MUA, Sigma-Aldrich), 11-Mercapto-1-undecanol (MU,

Sigma-Aldrich) and 4-mercaptopyridine (MP, Sigma-Aldrich) were re-crystallized from hexane before use. Water was purified through a Milli-Q Plus Ultrapure Water System coupled with an Elix-5 Kit (Millipore). The water resistivity was over 18 MΩ cm.

2. Electrochemical measurements

A Potentiostat/Galvanostat mod. 273A (EG&G PAR, Oak Ridge, USA) was used to perform cyclic voltammetry (CV). Experiments were carried using a cell for small volume samples (0.5 mL) under argon atmosphere. A 1 mm-diameter polycrystalline gold wire as working electrode was used and a Pt sheet and a saturated calomel electrode (SCE) as counter and reference electrode, respectively. The electric contact between the SCE and the working solution was achieved with a Vycor® (PAR) set. All the redox potentials reported here are referred to the standard hydrogen electrode (SHE). The working gold electrode was cleaned as reported elsewhere [S. Monari, D. Millo, A. Ranieri, G. Di Rocco, G. van der Zwan, C. Gooijer, S. Peressini, C. Tavagnacco, P. Hildebrandt, M. Borsari, J. Biol. Inorg. Chem. 15 (2010) 1233-1242]. The SAM covalently attached onto gold electrode was obtained by dipping the polished electrode into a 1 mM ethanolic solution of both MUA and MU for 12 hrs and then rinsing it with water. The MP SAM was performed by dipping the polished electrode into a 1 mM aqueous solution of 4-mercaptopyridine for 120 s, then rinsing it with water. Protein solutions were freshly prepared before use in 5 mM phosphate buffer at pH 7 and their concentration was carefully checked spectrophotometrically (Jasco mod. V-570 spectrophotometer). Protein adsorption on all the SAM-coated Au electrodes was achieved dipping the functionalized electrode into a 0.2 mM protein solution at 4 °C for 5 hrs. CV experiments on the protein coated electrodes were performed in 10 mM sodium perchlorate and 5 mM phosphate buffer at pH 7, the urea concentration was 9 M and T = 293 K. The gas supplied to the electrochemical cell was a mixture of O_2/N_2 whose composition was finally tuned by means of calibrated mass flow controllers (Alltech Digital Flow Check-HR) and gas mixing valves [A. Melchior, S. Peressini, R. Portanova, C. Sangregorio, C. Tavagnacco, M. Tolazzi, Inorg. Chim. Acta 357 (2004) 3473-3482]. The electrocatalytic reduction of dioxygen by the immobilized cytochrome *c* was studied varying the O_2

partial pressure (from 2000 to 20000 Pa). Surface coverages of cytochrome *c* on MP and MUA/MU were calculated from the overall charge exchanged (determined by integration of the baseline-corrected cathodic peaks observed in CV) and the area of the gold electrode (determined from the diffusion controlled CV of the electrochemical standard ferricinium tetrafluoboride in water). They were found to be $\Gamma^{MP} = 12.3 \pm 0.7$ pmol/cm^2 and $\Gamma^{MUA/MU} = 18.5 \pm 0.8$ pmol/cm^2.

3. Detection of the superoxide ion

Proper electrolysis experiments were carried out in an homemade Optically Transparent Thick-Layer (spectro)Electrochemical cell (OTTkLE cell, 1 mm lightpath) in order to verify the presence of superoxide ion using the method based on its reaction with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (4-chloro-7-nitrobenzofurazan, NBD-Cl, BioReagent Fluka) [R. O. Olojo, R. H. Xia and J. J. Abramson, *Anal. Biochem.*, 2005, **339**, 338. G. Bartosz, *Clinica Chimica Acta*, 2006, **368**, 53. J. Heo and S. L. Campbell, *Biochemistry*, 2006, **45**, 2200.]. In fact the superoxide ion eventually (electro)catalically generated at the protein coated working electrode reacts with NBD-Cl to give a specific product easily identified by a characteristic UV-vis band at 470 nm. The threee-lectrode configuration consisted of a gold minigrid working electrode (Buckbee-Mears, Chicago, IL), an homemade Ag/AgCl/KClsat microreference electrode and a platinum wire as counter electrode, both separated from the working solution by a Vycor set [G. Battistuzzi, M. Borsari, A. Ranieri and M. Sola, *J. Am. Chem. Soc.*, 2001, **124**, 26]. The reference electrode was calibrated against a saturated calomel electrode before each set of measurements. Potentials were applied across the OTTkLE cell with an Amel model 533 potentiostat/galvanostat. Constant temperature was maintained by a circulating water bath, and the OTTkLE cell temperature was monitored with a Cu-costan microthermocouple. UV-vis spectra were recorded using a diode-array Hewlett-Packard HP 8452-A spectrophotometer. The gold minigrid was cleaned and coated with MP and MUA/MU SAM as already reported for the working gold electrode used for CV measurements. Protein adsorption on the SAM-coated gold minigrid was achieved dipping it into a 0.2 mM protein solution at 4 °C for 5 hrs. All experiments were carried out under a mixture of O_2/N_2 (as above

reported for the electrochemical measurements) to have a partial O_2 pressure of 20000 Pa at 293 K. A 0.2 mL samples containing 200 μM NBD-Cl made up in 10 mM sodium perchlorate and 5 mM phosphate buffer at pH 7 was used throughout, an urea concentration of 9 M was employed to have the unfolded His-His ligated ycc onto the SAM. The spectrophotometic measurements were obtained using as a blank the minigrid coated with SAM and protein and the same working solution but without NBD-Cl. UV-vis spectra were recorded after fixed times of electrolysis at the cathodic peak potential (at about -0.230 V vs. NHE) of the CV of the unfolded His-His ligated protein in order to verify the presence of the band at a wavelength of 470 nm, characteristic of the product of the reaction between superoxide ion and NBD-Cl [R. O. Olojo, R. H. Xia and J. J. Abramson, *Anal. Biochem.*, 2005, **339**, 338. G. Bartosz, *Clinica Chimica Acta*, 2006, **368**, 53. J. Heo and S. L. Campbell, *Biochemistry*, 2006, **45**, 2200.]. Both on MP and MUA/MU SAM, at the different times of electrolysis a single, a well defined band at 470 nm is observed, it increases with the electrolysis time to the detriment of that of NBD-Cl (wavelength 344 nm) (see Figure 2 in the text). For long-lasting electrolysis (>1000 s) the spectra loose definition and are less reproducible, probably due to secondary reaction in the spectro-electrochemical cell. On the opposite, UV-vis spectra performed after electrolysis on the same system but in absence of urea in solution, i.e. when the working electrode is coated by native His-Met ligated ycc do not show any band at 470 nm (see Figure 2 in the text). Also the spectra performed after electrolysis on SAMs in absence of adsorbed protein do not show the band at 470 nm. This fact confirm the presence (also) of the superoxide ion as a product of the reduction of O_2 (electro)catalically induced by the unfolded His-His form of ycc and the absence of (electro)catalytic activity of the native His-Met ligated ycc.