

## Electronic Supplementary Information (ESI) for the manuscript: A surface-immobilized cytochrome *c* variant provides a pH-controlled molecular switch.

Carlo Augusto Bortolotti,<sup>\*a</sup> Licia Paltrinieri,<sup>a</sup> Stefano Monari,<sup>a</sup> Antonio Ranieri,<sup>a</sup> Marco Borsari,<sup>a</sup> Gianantonio Battistuzzi,<sup>a</sup> and Marco Sola<sup>a</sup>

<sup>a</sup>Department of Chemistry, University of Modena and Reggio Emilia, Via Campi 183, I-41125, Modena, Italy.

\*carloaugusto.bortolotti@unimore.it

## Materials and Methods

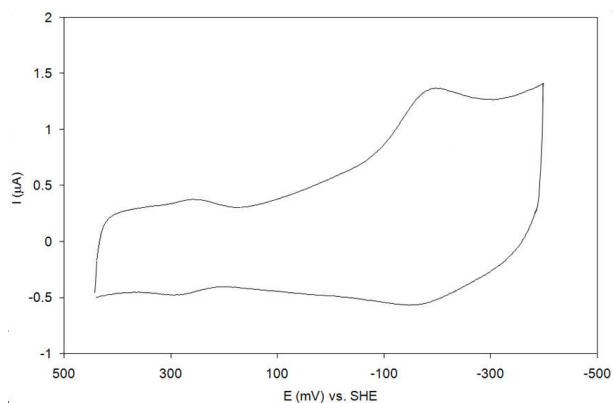
### Protein production and purification

The K72A/K73H/K79A was produced and purified following previously published procedures<sup>1-4</sup>. The QuikChange XL site-directed mutagenesis kit (Stratagene) was used for the Lys to Ala and Lys to His substitutions, starting from two synthetic oligonucleotide primers carrying the desired mutations and using as DNA template the plasmid pMSV1, as described elsewhere<sup>5-10</sup>. This plasmid expresses the C102T variant of yeast iso-1-cytochrome *c* from the pTrc promoter and confers ampicillin resistance. Therefore, the K72A/K73H/K79A variant carries a threonine in place of the native Cys102. This substitution prevents dimerization and minimizes autoreduction while resulting in retention of the spectral and functional properties of the protein<sup>1,5-10</sup>.

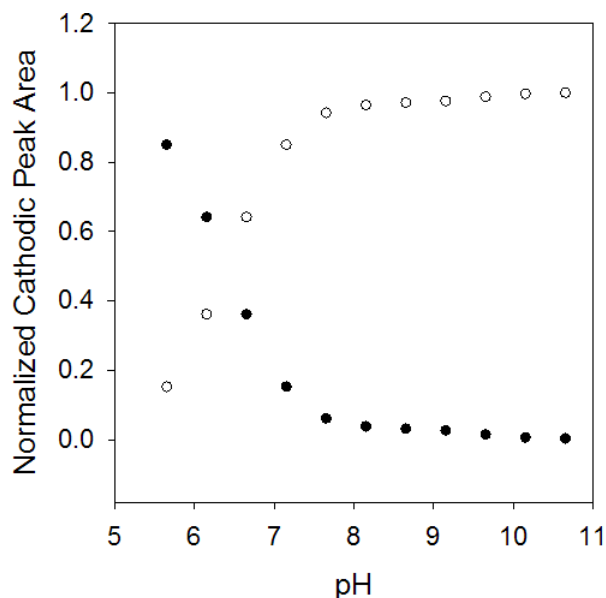
### Electrochemical Measurements

Cyclic voltammetry (CV) experiments were carried out with a Potentiostat/Galvanostat mod. 273A (EG&G PAR, Oak Ridge, USA) at different scan rates (0.02-1 V s<sup>-1</sup>) using a cell for small volume samples (0.5 ml) under argon. Experiments on immobilized and freely diffusing cytc were carried out using a 1 mm diameter polycrystalline gold wire as working electrode and a Pt sheet and a saturated calomel electrode (SCE) as counter and reference electrode, respectively<sup>6-10</sup>. The electric contact between the SCE and the working solution was obtained with a Vycor set. Potentials were calibrated against the MV<sup>2+</sup>/MV<sup>+</sup> couple (MV = methylviologen)<sup>11</sup>. The same experimental setup was used for the CV measurements of freely diffusing cytc. All the redox potentials reported here are referred to the standard hydrogen electrode (SHE). The working gold electrode for both diffusionless and diffusion-controlled experiments was cleaned by dipping it in concentrated nitric acid for 10 min, then flaming it in oxidizing conditions. Afterward, it was heated in KOH 2.5 M for 4 h then, after rinsing in water, in concentrated sulfuric acid for 2 h. To minimize residual adsorbed impurities, the electrode was subjected to 10 voltammetric cycles between +1.5 and -0.2 V at 0.1 V s<sup>-1</sup> in 0.1 M H<sub>2</sub>SO<sub>4</sub>. The Vycor (PAR) set was treated in an ultrasonic pool for about 1 min. The CV measurements on immobilized cytc were performed using a gold electrode, whose surface was modified with a covalently attached SAM obtained by dipping the polished electrode into a 1 mM ethanolic solution of both 11-Mercapto-1-undecanoic acid (MUA) and 11-Mercapto-1-undecanol (MU) for about 15 h and then rinsing it with MILLIQ water<sup>6-10</sup>. Different SAMs (hydrophobic, hydrophylic, positively- or amine-terminated) were screened for cytc adsorption. They all yielded lower currents compared to the mixed 11-Mercapto-1-undecanoic acid (MUA) and 11-Mercapto-1-undecanol (MU) SAM, most likely due to protein desorption or denaturation, as previously reported.<sup>12</sup> Protein solutions were freshly prepared before use in 5 mM phosphate buffer at pH 7, and their concentration was checked spectrophotometrically. Protein adsorption on the SAM-coated Au electrode was achieved dipping the functionalized electrode in 100 μl of protein solution at 4°C for 4 h. CV experiments were performed in 5 mM phosphate buffer containing 5 mM sodium perchlorate. The CV measurements on freely diffusing cytc were performed using a gold electrode, whose surface was modified with a covalently attached SAM obtained by dipping the polished electrode into a 1 mM solution of 4-mercaptopyridine for 30 s, then rinsing it with Nanopure water<sup>6-10</sup>. For both regimes, either immobilized or diffusion-controlled, equilibrium reduction potentials E<sup>0'</sup> for cytc were calculated from the average of the anodic and cathodic peak potentials<sup>9</sup> and found almost independent in the range of the scan rate used. For each species, the experiments were performed at least two times and the reduction potentials were found to be reproducible within

$\pm 0.002$  V. The pH of the working solutions was adjusted with small additions of concentrated  $\text{HClO}_4$  or  $\text{NaOH}$  under fast stirring. The electrocatalytic reduction of hydrogen peroxide by the cytc variants was studied by gradually adding aliquots of  $\text{H}_2\text{O}_2$  solution at known concentration to the  $\text{O}_2$ -free cell solution at  $20^\circ\text{C}$ <sup>6,8-10</sup>.



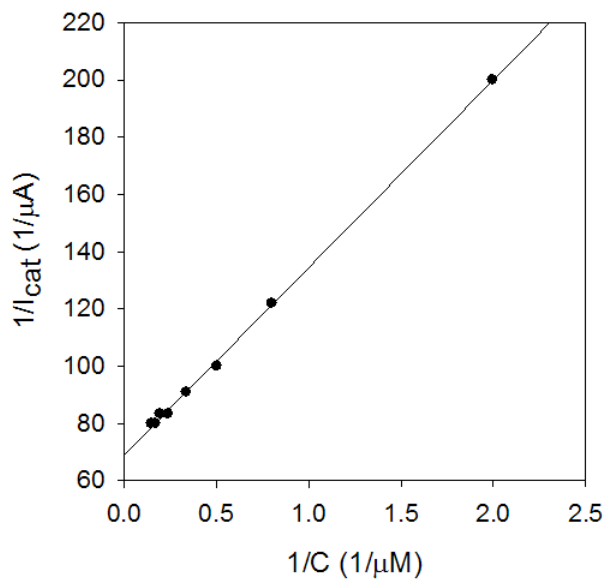
**Figure 1** Diffusion controlled cyclic voltammetry of yeast cytochrome *c* mutated K72A/K73H/K79A at pH 6.  $T = 293$  K, scan rate  $0.05 \text{ V s}^{-1}$ . Working solution: phosphate buffer 5 mM and sodium perchlorate 50 mM, pH 7.



**Figure 2** pH dependence of the intensity (measured as the normalized area) of the baseline-corrected cathodic peaks for the His, Met ( $\bullet$ ) and His, His ( $\circ$ ) forms of the freely diffusing K72A/K73H/K79A mutant of cytochrome *c*.

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**Figure 3** Lineweaver-Burk plot made with the electrocatalytic currents yielded by the K72A/K73H/K79A mutant immobilized on a polycrystalline gold electrode coated with a SAM of MUA/MU in 5 mM phosphate buffer and 5 mM sodium perchlorate, pH 7 and 298 K, in the presence of increasing concentrations of H<sub>2</sub>O<sub>2</sub>. Solid line is least-squares fit to the data points.

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