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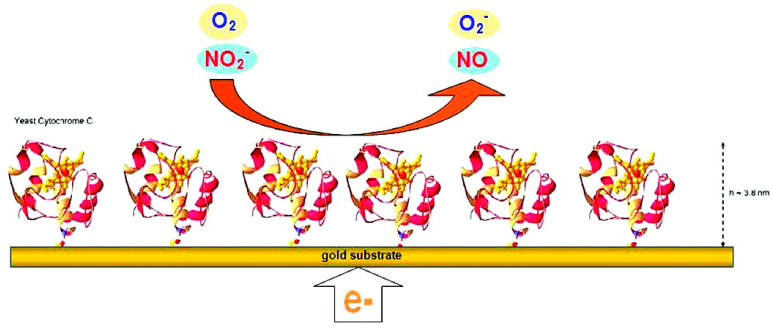

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Catalytic Reduction of Dioxygen and Nitrite Ion at a Met80Ala Cytochrome *c*-Functionalized Electrode

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Abstract: The Met80Ala variant of yeast iso-1-cytochrome *c*, immobilized on a gold electrode, is found to exchange electrons efficiently with it in nondenaturing conditions and to provide robust and persistent catalytic currents for O_2 and nitrite ion reduction from pH 3 to 11. Direct covalent protein linkage to gold yields the best electrochemical and electrocatalytic performances without drastically affecting the structural properties of the bound protein compared to the freely diffusing species. Therefore, this biocatalytic interface can be of use for the amperometric detection of the above species, which are of great environmental, industrial, and clinical interest, with particular reference to the exploitation in nanostructured biosensing devices. This work shows that the use of a small engineered electron transfer (ET) protein, featuring an axial heme iron coordination position available for the binding of exogenous ligands, in place of a large heme enzyme is a viable strategy for the improvement of the heterogeneous ET rate and the stability and efficiency of sensing gold−protein interfaces over a wide range of *T* and pH.

Introduction

Redox proteins and enzymes immobilized on solid electrodes in an electrochemical environment may provide an unmediated transduction of a chemical event into an electric signal. Therefore, they are recognized as important constituents of biocatalytic interfaces for molecular recognition to be exploited in nanostructured biosensing devices.[1−4] The latter are attracting much interest as a new class of multifunctional tools for genetic, biological, and chemical analysis with an enhanced sensitivity and selectivity and higher rates of recognition with respect to current means. In this respect, heme-containing proteins and enzymes are appealing candidates because of the large catalytic versatility of the heme group.[5] We are exploring the utilization of engineered electron transfer (ET) heme proteins, particularly cytochrome *c*, as electrode-immobilized biocatalysts in place of larger heme enzymes.[6−8] This approach, which exploits a functionally versatile and catalytically tunable molecular system, would allow improvement of the heterogeneous protein−electrode ET rates and of the stability of the hybrid interface in hostile conditions in terms of pH, temperature, and the presence of organic solvents.

In this work, the Met80Ala (M80A) variant of iso-1-cytochrome *c* from *Saccharomyces cerevisiae*, covalently attached to a gold electrode through a Au−S(Cys) linkage, was studied as electrocatalyst for the amperometric biosensing of molecular oxygen and the nitrite ion. These are analytes of great clinical, environmental, and industrial relevance. M80A is a stable cytochrome *c* variant that features an axial heme iron coordination position available for the binding of exogenous ligands (Figure 1).[9−12] A water molecule and a hydroxide ion serve as a "sixth" axial ligand below and above pH 6, respectively.[9,10,12] This species represents a sort of "minimal" mutant serving as starting point for modeling chimeric heme enzymes utilizing the cytochrome *c* scaffold.

The M80A/C102T untrimethylated recombinant iso-1-cytochrome *c* from *S. cerevisiae* (cytc) variant (the latter mutation is carried by the plasmid used as DNA template for protein expression in *Escherichia coli*)[13] adsorbed on a polycrystalline gold electrode coated with different self-assembled monolayers (SAMs) (4-mercaptopyridine, 4-MP, and 11-mercapto-1-undecanoid acid/11-mercapto-1-undecanol, MUAc/MUAl) was previously shown to catalyze the reduction of dioxygen.[7] However,

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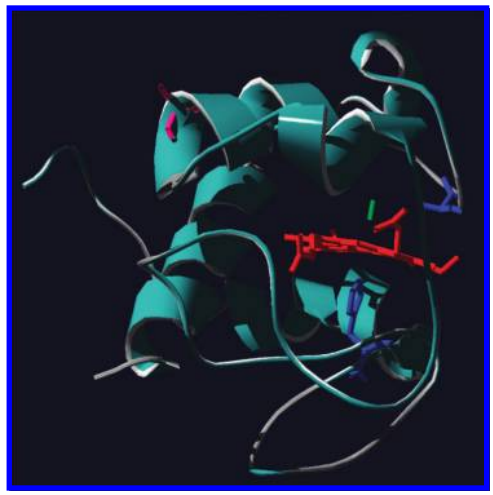

Figure 1. Three-dimensional representation of the averaged MD structure of the M80A/C102T/N62C variant of *S. cerevisiae* iso-1-cytochrome *c*.[10] The heme is in red, the proximal histidine and the alanine80 are in blue, the hydroxide ion axially bound to Fe(III) is in green, and the cysteine62 is in magenta.

the lifetime of the electrocatalytic effect was severely reduced by the loss of protein, likely as a result of the oxidative disruption of the SAM brought about by the products of oxygen reduction (superoxide and/or hydroxide radicals). Since production of a stable biocatalytic interface is crucial to the efficiency and durability of the biosensing device, here we explored the unmediated covalent attachment of the above mutant to the electrode surface. In particular, we focused on the M80A/C102T/N62C mutant of yeast iso-1-cytochrome *c*, which, thanks to the newly engineered cysteine, has been shown to bind firmly to the gold electrode through a Au−S(Cys) linkage, without undergoing severe denaturating effects and to efficiently exchange electrons with it.[7] In particular, this attachment site to gold induces a perpendicular orientation of the heme group toward the electrode, which, compared to the parallel one of the native form, makes the electron transfer process 20 times faster.[6,7] In this work, we report on the reductive electrocatalytic performances of this variant toward dioxygen and the nitrite ion. Heme-based electrocatalytic reduction of the nitrite ion has been obtained previously with cytochrome P-450 and myoglobins adsorbed onto glassy carbon leading to nitric oxide and even to ammonia.[14,15] Our goal is to produce an engineered, highly tunable, bioelectrocatalytic core amenable of miniaturization at the nanoscale for use in sensing devices. This would be of great use for the detection of these chemicals in a cellular environment or in physiological fluids.

Materials and Methods

Materials. The M80A/N62C/C102T mutant of *S. cerevisiae* iso-1 cytochrome *c* was produced using the QuikChange XL site-directed mutagenesis kit (Stratagene) starting from two synthetic oligonucleotide primers carrying the desired mutation and using as DNA template the plasmid pMSV1, as described elsewhere.[6,7,13,16] The protein is stored at −20 °C in TRIS 50 mM and $(NH_4)_2SO_4$ 1.8 M at pH 7. All chemicals were reagent grade. Nanopure water was used throughout.

Electrochemical Measurements. Cyclic voltammetry (CV) experiments were carried out with a potentiostat/galvanostat PAR model 273A at different scan rates (0.02−5 V s^{-1}) using a cell for small volume samples (0.5 mL) under argon. All experiments 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. The electric contact between the SCE and the working solution was obtained with a Vycor (PAR) set. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methylviologen).[16] All the redox potentials reported here are referred to as standard hydrogen electrode (SHE). The working gold electrode was cleaned by flaming it in oxidizing conditions; afterward, it was heated in concentrated KOH for 30 min, then after rinsing in water, in concentrated sulfuric acid for 30 min. To minimize residual adsorbed impurities, the electrode was subjected to 20 voltammetric cycles between +1.5 and −0.25 at 0.1 V s^{-1} in 1 M sulfuric acid. Finally, the electrode was rinsed in water and anhydrous ethanol. The Vycor set was treated in an ultrasonic pool for about 5 min. Covalent protein linkage to the gold electrode was achieved through the following steps: (i) ammonium sulfate was eliminated from the protein solution through a gel filtration column and protein solutions were made up in 10 mM phosphate buffer, 100 mM NaCl, at pH 7, and their concentration (typically 50 μM) was checked spectrophotometrically, (ii) protein was reduced with a 5-fold excess of tris[2-carboxyethyl]phosphine (TCEP), (iii) TCEP was then removed through dialysis against 2 L of 50 mM TRIS buffer, previously outgassed (with Ar for 3 h), (iv) a cleaned 1-mm-diameter gold wire was dipped in the above 50 μM M80A/N62C/C102T cytc solution for 48 h at 5 °C, and (v) finally, the functionalized electrode was washed with bidistilled water and subjected to CV measurements in a 10 mM phosphate, 200 mM NaCl working solution. The transfer coefficient α is found to be approximately 0.5, and the equilibrium reduction potentials ($E^{\circ\prime}$) for cytc, calculated from the average of the anodic and cathodic peak potentials, are almost independent of the scan rate in the range 0.01−2 V/s. Experiments were repeated at least two times, and the reduction potentials were found to be reproducible within ±0.002 V. To estimate the % surface coverage, the area of the immersed portion of the gold wire was carefully calculated after each CV session by dipping the bare electrode at exactly the same depth into a solution of an electrochemical standard, ferricenium tetrafluoborate, recording the CV signal for the standard, and then applying the Randles−Sevcik relationship.[17] Cyclic voltammograms at variable scan rate were recorded to determine the electron-transfer rate constant k_s for the adsorbed protein, according to the Laviron method.[18] The k_s values were averaged over five measurements.

Variable-temperature CV experiments were carried out using a "nonisothermal" cell,[19,20] in which the reference electrode was kept at constant temperature (21 ± 0.1 °C), whereas the half-cell containing the working electrode and the Vycor junction to the reference electrode were kept under thermostatic control with a water bath. The temperature was varied from 5 to 35 °C. With this experimental configuration, the reaction entropy for reduction of the oxidized protein for heme Fe(III) to Fe(II) reduction ($\Delta S^{\circ\prime}_{rc}$) is given by:[19−21]

$$\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox} = nF(dE^{\circ\prime}/dT) \quad (1)$$

Thus, $\Delta S^{\circ\prime}_{rc}$ was determined from the slope of the plot of standard reduction potential for the heme Fe(III)/Fe(II) couple ($E^{\circ\prime}$) versus temperature, which turns out to be linear under the assumption that

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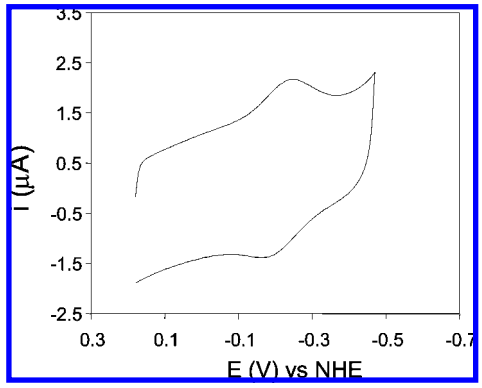

Figure 2. Cyclic voltammogram for M80A/C102T/N62C *S. cerevisiae* iso-1-cytochrome *c* covalently linked to a polycrystalline gold electrode through a Au−S(Cys) bond in 10 mM phosphate buffer, 0.2 M sodium chloride, pH 7. Sweep rate, 0.05 V s^{-1}. $T = 20$ °C.

$\Delta S^{\circ\prime}_{rc}$ is constant over the limited temperature range investigated. With the same assumption, the enthalpy change for heme Fe(III) to Fe(II) reduction ($\Delta H^{\circ\prime}_{rc}$) was obtained from the Gibbs−Helmholtz equation, namely as the negative slope of the $E^{\circ\prime}/T$ versus $1/T$ plot. The nonisothermal behavior of the cell was carefully checked by determining the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values of the ferricyanide/ferrocyanide couple. The electrocatalytic reduction of O_2 by covalently bound M80A/C102T cytc at different pH values was studied by gradually adding air to the O_2-free solution at normal atmospheric pressure at 20 °C.[7] The electrocatalytic reduction of nitrite by covalently bound and SAM-adsorbed M80A/C102T cytc was studied by adding to the solution small aliquots of a 1 mM $NaNO_2$ solution prepared in the same buffer used for the electrochemical experiments (previously outgassed). The adsorption of M80A/C102T on 1:1 mixed SAMs of 11-mercapto-1-undecanoic acid and 11-mercapto-1-undecanol (MUA) and 4-MP SAMs was achieved as reported in ref 7.

Results and Discussion

Thermodynamics and Kinetics of Heterogeneous ET for Covalently Immobilized M80A Cytochrome *c*. The voltammetric response of M80A/N62C/C102T cytc covalently linked to a polycrystalline gold electrode (Figure 2) shows the typical one-electron reduction/oxidation reaction of the heme iron of class I cytochrome *c*.[22] The anodic/cathodic peak current ratio is approximately 1 at all temperatures and scan rates investigated. The peak current linearly increases with increasing scan rate, as expected for an adsorbed electroactive species (not shown). The $E^{\circ\prime}$ value of −0.205 V (vs SHE) determined at pH 7 and 25 °C is lower by 0.575 V than that of native cytochrome *c* covalently immobilized on gold in the same conditions ($E^{\circ\prime} = +0.370$ V).[6] This effect is mostly the result of axial heme iron coordination by a hydroxide ion in the mutant, which strongly stabilizes the oxidized state, in place of the methionine thiother sulfur ligand in the wt species. The present $E^{\circ\prime}$ value is very similar to those reported previously for the M80A/C102T cytc variant adsorbed on SAMs made of MUA and 4-MP (−0.201 and −0.194 V, respectively).[7] Thus, apparently the immobilization strategy does not affect appreciably the reduction potential of the protein. This indicates that the structural integrity of M80A/C102T/N62C cytc directly bound to the gold surface is largely preserved, as confirmed by the analysis of the reduction thermodynamics (see below). The surface coverage of the covalently linked protein is 15.9 ± 0.8 pmol/cm^2, which, as above, is comparable to those found for M80A/C102T cytc chemisorbed on 4-MP and MUA (17.5 and 16.8 ± 0.8 pmol/cm^2, respectively)[7] and corresponds to 84% of a full densely packed monolayer (19 pmol/cm^2, as estimated from the crystallographic dimensions of the protein).[23,24] Consistent with the absence of relevant structural changes in the heme environment due to direct protein−electrode covalent linkage, a pH decrease to 5 induces a cathodic shift in $E^{\circ\prime}$ of approximately 0.150 V ($E^{\circ\prime}_{pH5} = -0.054$ V), which can be confidently ascribed to the protonation of the axially bound hydroxide ion to water with the consequent stabilization of the ferrous state of the heme iron, as previously discussed for M80A/C102T cytc adsorbed on the SAM-coated gold electrode.[7] Also, the behavior at alkaline pH values parallels that found previously for the protein adsorbed on the SAM-coated electrode. The $E^{\circ\prime}$ decrease by 0.022 V ($E^{\circ\prime}_{pH11} = -0.228$ V) can be attributed to the electrostatic effect on the heme center exerted by deprotonation of one or more lysines onto the protein surface, with no involvement of these residues in axial heme binding, as discussed previously.[7] Most notably, the pH-induced $E^{\circ\prime}$ changes in the pH range 3−10 are to a large extent reversible, being accompanied by only a slight peak enlargement. Therefore, it is apparent that in these conditions the bound protein does not suffer the permanent pH-induced structural changes detected for horse heart cytochrome *c* covalently bound to mixed carboxylic acid and hydroxyl-terminated SAM-coated gold electrodes.[25]

The thermodynamics for heme Fe(III) to Fe(II) reduction in the M80A/C102T/N62C cytc variant covalently bound to the gold electrode are listed in Table 1. The enthalpic and entropic terms differ from those determined for M80A/C102T adsorbed on the SAM-modified gold electrode, especially for the MUA SAM. However, these differences are compensatory (the E° values being very similar). This behavior suggests that reduction-induced solvent reorganization effects in the solvation sphere of the immobilized proteins (which are known to feature exact enthalpy−entropy compensation)[26−28] are mainly responsible for the observed differences. This is conceivable because of the different nature of the interaction between the solvated protein and the SAM-coated or bare electrode. The large reduction enthalpy on the MUA SAM is likely to be also determined by the enthalpic stabilization of the more positively charged ferric form of the protein due to the electrostatic interactions with the carboxylate groups of the SAM.[7] Comparison with the reduction thermodynamics of the His,Met-ligated form of cytc covalently attached to gold with the same cysteine engineered in position 62[6] shows that the $E^{\circ\prime}$ decrease by 0.575 V is almost totally enthalpic in origin, consistent with the fact that the ligand exchange [OH^- replacing S(Met)] is the main determinant of the observed difference. The small change in reduction entropy also confirms that the covalently immobilized state replacement of the Met ligand with a nonbonding Ala residue scarcely affects the protein structure and the hydrogen bonding network within the solvation sphere in both redox states.

The values of the kinetic rate constant, activation enthalpy, and reorganization energy of the heterogeneous electron transfer

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Table 1. Thermodynamic Parameters at Different pH Values for Fe(III) to Fe(II) Reduction for the M80A/C102T/N62C Variant of *S. cerevisiae* Iso-1-cytochrome *c* Covalently Linked to a Polycrystalline Gold Electrode through a Au−S(Cys) Bond

protein	pH	$E^{\circ\prime}$ [a,b] (V)	$\Delta H^{\circ\prime}_{rc}$ [b] (kJ mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ [b] (J K^{-1} mol^{-1})	$-\Delta H^{\circ\prime}_{rc}/F$ (V)	$T\Delta S^{\circ\prime}_{rc}/F$ [a] (V)
M80A/C102T/N62C[c]	3.0	−0.056	+3	−9	−0.028	−0.028
M80A/C102T/N62C[c]	7.0	−0.205	+16	−11	−0.170	−0.035
M80A/C102T/N62C[c]	10.5	−0.227	+17	−15	−0.180	−0.047
M80A/C102T[d]	7.0	−0.194	+13	−21	−0.132	−0.064
M80A/C102T[e]	7.0	−0.201	+42	+78	−0.435	+0.241

[a] At 25 °C. [b] Average errors on $E^{\circ\prime}$, $\Delta H^{\circ\prime}_{rc}$, and $\Delta S^{\circ\prime}_{rc}$ values are ±0.002 V, ±1 kJ mol^{-1}, and ±2 J mol^{-1} K^{-1}, respectively. [c] Values were obtained in 10 mM phosphate buffer, 0.2 M sodium chloride. [d] M80A/C102T immobilized on a polycrystalline gold electrode coated with a SAM of 4-MP. Electrolyte: 10 mM phosphate buffer, 0.1 M sodium chloride. From ref 7. [e] M80A/C102T immobilized on a polycrystalline gold electrode coated with a 1:1 mixed SAM of 11-mercapto-1-undecanoic acid (11-MUAc) and 11-mercapto-1-undecanol (11-MUAl). Electrolyte: 5 mM phosphate buffer, 5 mM sodium perchlorate. From ref 7.

Table 2. Kinetics Constants Measured at Different Temperatures and pH Values and Calculated Activation Enthalpies for the Heterogeneous Electron Transfer between the Protein and the Electrode for the M80A/C102T/N62C Variant of *S. cerevisiae* Iso-1-cytochrome *c* Covalently Linked to a Polycrystalline Gold Electrode through a Au−S(Cys) Bond[a]

protein	pH	k_s (5 °C) (s^{-1})	k_s (20 °C) (s^{-1})	k_s[a,b] (35 °C) (s^{-1})	$\Delta H^{\#}$[a,b] (kJ mol^{-1})	λ[a,b] (eV)
M80A/C102T/N62C[c]	7.0	1.05 (0.09)	1.25 (0.09)	1.3 (0.08)	7.63	0.32
M80A/C102T/N62C[c]	10.5	0.48 (0.05)	0.53 (0.04)	0.73 (0.08)	9.95	0.41
M80A/C102T[c]	7.0	1.8	2.2	2.8	10.5	0.435
M80A/C102T[d]	7.0	1.4	1.9	2.2	10.8	0.448

[a] Standard deviation for k_s values are given in parentheses. Average errors on $\Delta H^{\#}$ and λ values are ±0.6 kJ mol^{-1} and ±0.03 eV, respectively. [b] Values were obtained in 10 mM phosphate buffer, 0.2 M sodium chloride. [c] M80A/C102T immobilized on a polycrystalline gold electrode coated with a SAM of 4-MP. Electrolyte: 10 mM phosphate buffer, 0.1 M sodium chloride. The relative error on k_s is ±10%. From ref 7. [d] M80A/C102T immobilized on a polycrystalline gold electrode coated with a 1:1 mixed SAM of 11-MUAc and 11-MUAl. Electrolyte: 5 mM phosphate buffer, 5 mM sodium perchlorate. The relative error on k_s is ±10%. From ref 7.

process of immobilized M80A/N62C/C102T cytc mutant, the latter calculated from the Marcus equation for heterogeneous ET, as shown by Bowden and co-workers,[7,29−32] are listed in Table 2. Kinetic data could be obtained only at neutral and alkaline pH values. At pH 3, the electrochemical response was affected by the presence of residual O_2, which could not be removed, that decreased signal quality and hampered the kinetics analysis. The kinetic parameters at pH 7 and 10.5 are comparable to those obtained for M80A/C102T cytc immobilized on the SAM-coated gold electrode.[7] The distance of ET between the electrode surface and the heme edge can be determined from the linearized form of the Marcus equation:[7,29,30]

$$\ln k_s = \ln \nu_0 + [-\beta(r - r_0)] - \Delta G^{\#}/(RT) \qquad (2)$$

(where, since the activation entropy in these systems is in general negligible,[30,33] $\Delta G^{\#}$ can be assumed to correspond to $\Delta H^{\#}$) using a β value of 1.4 Å^{-1}, which is applicable to electron tunneling through the protein matrix,[34−39] and an r_0 value of 3 Å.[29,30,32] The tunneling distance is 21.2 Å, independent of pH. This value compares well with the distance of 19.85 Å between the heme iron and the sulfur atom of Cys62 calculated from the 3D protein structure.[24] This can be taken as a further indication that the overall structure of the protein does not suffer severe unfolding upon binding to the gold surface.

Reductive Electrocatalysis of Dioxygen and the Nitrite Ion. M80A/N62C/C102T cytc covalently bound to gold is able to catalyze dioxygen reduction, as shown in Figure 3. Cathodic currents increase with increasing dioxygen concentration of the solution up to the maximum value achievable at atmospheric pressure (cell open to air). Compared to the electrocatalytic behavior shown by M80A/C102T adsorbed on SAMs of 4-MP and MUA,[7] we observe that (i) the same amount of electrode-immobilized protein (which is related to the electrode surface exposed to the solution and the surface coverage of the protein film) originates a remarkably larger increase in catalytic current upon increasing dioxygen concentration, indicating that the covalently linked protein has an higher catalytic efficiency, (ii) the electrochemical response (curve shape) is of better quality, and, most notably, (iii) no current decrease is observed upon repeated scans at all dioxygen concentrations.

Progressive current fading was the main drawback affecting the electrocatalytic performance of adsorbed M80A/C102T cytc, which was attributed to the formation of some radical species of dioxygen that oxidize the SAMs, thereby inducing the release of the immobilized cytochrome *c* into the bulk solution.[7] It is also noteworthy that the pH range of electrocatalytic activity is increased: with SAM-adsorbed M80A/C102T cytc, no experiments could be performed below pH 5 because of protein layer disruption due to SAM protonation, whereas in the present case the Au−S(Cys) bond and the electrochemical response turn out to be unaffected at pH values as low as 3 and up to pH 10 (Figure 3).

The voltammograms obtained with the M80A/C102T variant adsorbed on 4-MP or MUAc/MUAl SAM-coated gold and with the covalently immobilized M80A/N62C/C102T variant in the presence of increasing concentrations of nitrite ion at pH 7 are shown in Figure 4. In all cases, cathodic currents grow catalytically (with no appreciable changes in Fe(III)/Fe(II)

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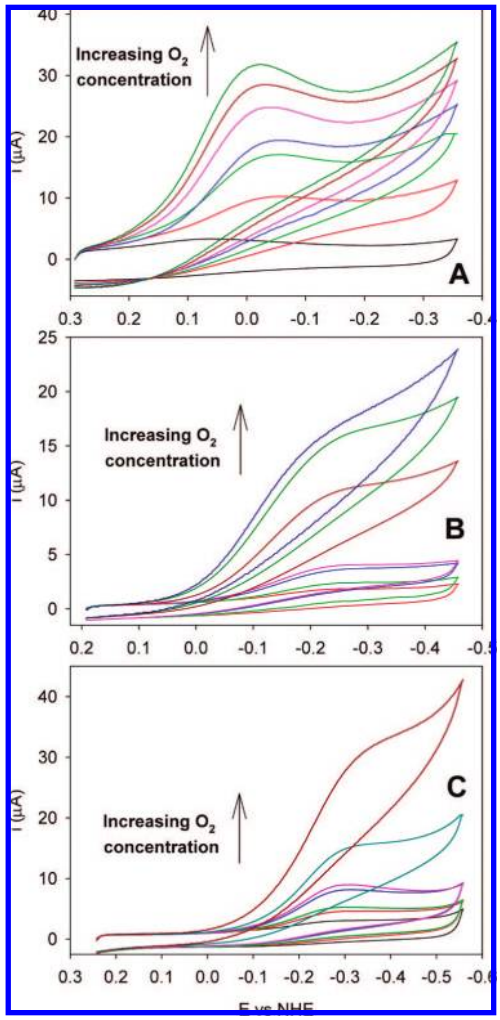

Figure 3. Cyclic voltammograms for M80A/C102T/N62C *S. cerevisiae* iso-1-cytochrome *c* bound to a gold electrode through a Au−S(Cys) bond recorded in 10 mM phosphate buffer, 0.2 M sodium chloride, at pH 3 (A), 7 (B), and 10 (C) at different exposure times of the electrochemical cell (initially under argon) to air at normal atmospheric pressure. Red, 0 s; green, 60 s; blue, 2 min; pink, 3 min; brown, 4 min; dark green, 7 min; dark blue, 14 min. Sweep rate, 0.02 V s^{-1}. $T = 35$ °C.

potential) displaying the typical decrease of the anodic return up to a nitrite concentration of 20 μM. The performances in terms of current intensity and layer stability are poorer for the protein adsorbed on SAMs with respect to the covalently linked species, for which, at each nitrite concentration, the voltammogram is unchanged after several repeated cycles. The proposed reaction mechanisms

$$\text{cytFe(III)}-\text{OH}^- + \text{e}^- \rightarrow \text{cytFe(II)} + \text{OH}^- \quad (3)$$

$$\text{cytFe(II)} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{cytFe(III)}-\text{OH}^- + \text{NO} \quad (4)$$

imply reduction of the ferric heme iron, which favors dissociation of the hydroxide ion, axially bound to the iron atom at pH 7,[8,9,11] followed by a bimolecular reduction of nitrite to NO with the consumption of one proton. Since no other peaks at more negative potentials are observed (up to −0.8 V vs SHE), it is likely that NO does not bind to Fe(III), at variance with myoglobin and cytochrome P450.[15]

Regardless of the immobilization strategy, the catalytic currents decrease at nitrite concentrations above 20 μM and do not recover upon lowering anion concentration. This effect can

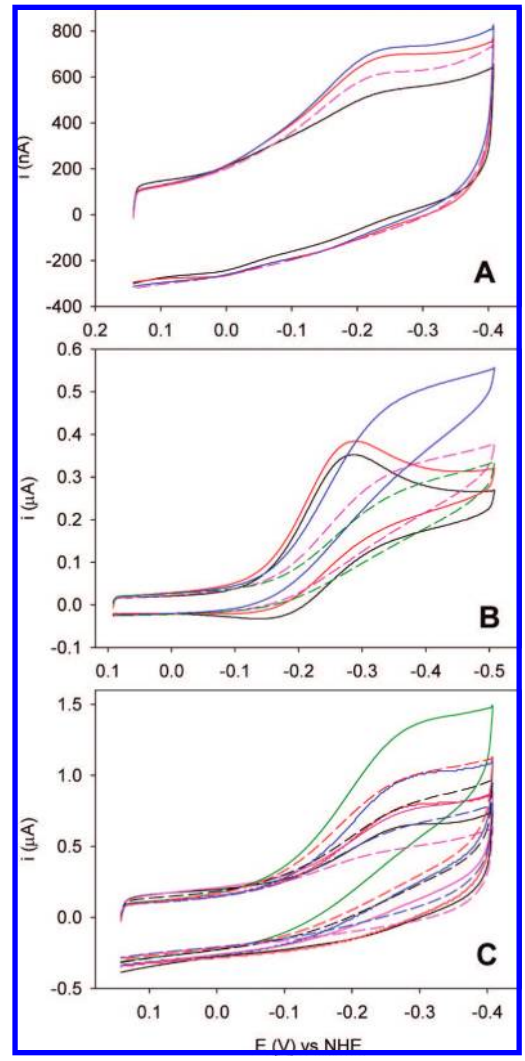

Figure 4. Cyclic voltammograms for the M80A variant of *S. cerevisiae* iso-1-cytochrome *c* immobilized with different procedures on a polycrystalline gold electrode at pH 7 recorded in the presence of increasing concentrations of sodium nitrite. (A) M80A/C102T on a 4-MP SAM. Black, no nitrite; red, 10 μM; blue, 20 μM; magenta, 100 μM. (B) M80A/C102T on a SAM of 1:1 11-MUAc and 11-MUAl. Black, no nitrite; red, 1 μM; blue, 5 μM; magenta, 15 μM; green, 20 μM. (C) M80A/C102T/N62C covalently linked to the electrode through a Au−S(Cys) bond. Black, no nitrite; red, 1 μM; magenta, 2 μM, blue, 5 μM; green, 10 μM; dashed red, 20 μM; dashed black, 30 μM; dashed magenta, 50 μM. Sweep rate, 0.02 V s^{-1}. $T = 20$ °C.

be tentatively ascribed to an irreversible protein conformational change with loss of catalytic activity due to specific NO_2^- binding to the positively charged patches formed by surface lysine residues.[22] Consistently, the current of the Fe(III)/Fe(II) couple measured in the absence of nitrite (buffer only) after incubation of the functionalized electrode in a 30 μM nitrite solution decreases with increasing incubation time, with no potential shift. With the MUAc/MUAl SAM-coated gold electrode, also a deformation of the electrochemical response occurs. Possibly, in this case the ionic strength increase also depresses the electrostatic interaction between the protein molecules and the carboxylate groups of the SAM, thus leading to a partial disruption of the immobilized layer and release of protein molecules in the bulk solution. This could also be accompanied by a change in the folding and/or orientation of the adsorbed proteins with a consequent lowering of the heterogeneous ET rate constant.

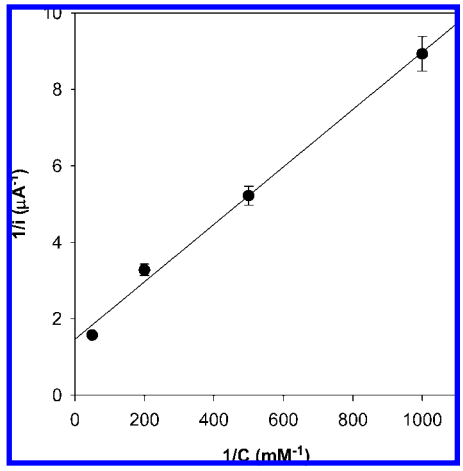

Figure 5. Lineweaver−Burk plot made with the electrocatalytic currents yielded by the M80A/C102T/N62C variant of *S. cerevisiae* iso-1-cytochrome *c* covalently linked to the electrode through a Au−S(Cys) bond, pH 7, in the presence of increasing nitrite ion concentrations.

The Michaelis−Menten equation for the electrocatalyzed reduction of nitrite can be expressed in terms of current as:

$$1/i_{cat} = 1/i_{max} + K_M/(i_{max}[NO_2^-]) \quad (5)$$

where i_{cat} is the electrocatalytic current and i_{max} is the maximum current at substrate saturation. The Lineweaver−Burk plot (Figure 5) yields i_{max} and K_M values of 2.14 μA cm^{-2} (at v = 0.02 V s^{-1}) and 5.1 μM, respectively. K_M value results are much lower than those measured for immobilized myoglobin and cytochrome P450 (3.3 and 11.5 mM, respectively)[14] and comparable to that for freely diffusing nitrite reductase (NiR) accepting electrons from an electrode-immobilized cytochrome c_{551} (20 ± 5 μM).[40] Also, i_{max} is similar to that measured in the latter case: a value of approximately 1.1 μA cm^{-2} was calculated from the data in ref 27 (at v = 0.01 V s^{-1}).

These values indicate that the kinetic affinity of nitrite for immobilized M80A is larger than that for myoglobin and cytochrome P450 and that the catalytic efficiency of immobilized M80A toward nitrite reduction is very similar to that for freely diffusing NiR subjected to heterogeneous ET from immobilized cytc$_{551}$.

Conclusions

The M80A mutant of yeast iso-1 cytochrome *c* immobilized on a gold electrode either covalently or electrostatically is able to catalytically reduce dioxygen with a good signal stability and durability. This is an important achievement for the fabrication of nanostructured bioelectrocatalytic interfaces acting as core constituents of miniaturized dioxygen biosensing devices to be used in physiological fluids (blood) and cells (e.g., for investigation of hypoxia). The ability of immobilized M80A cytc to catalytically reduce nitrite, despite the presently limited response to an anion concentration lower than 20 μM, is an important result as well, due to the importance of this chemical as a pollutant and in the food industry. Provided the sensitivity and stability of the electrocatalytic layer are improved, this result opens the way for efficient nitrite biosensing utilizing a low-cost and stable engineered protein.

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