Elsevier Editorial System(tm) for Electrochimica Acta
Manuscript Draft

Manuscript Number: EF15-186

Title: Thermodynamics and kinetics of reduction and species conversion at an hydrophobic surface for mitochondrial cytochromes c and their cardiolipin adducts

Article Type: Research Paper

Keywords: cytochrome c; cardiolipin; electron-transfer; voltammetry; adsorption

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Abstract: Cytochrome c (cytc) and its adduct with cardiolipin were immobilized on a hydrophobic SAM-coated electrode surface yielding a construct which mimics the environment experienced by the complex at the inner mitochondrial membrane where it plays a role in cell apoptosis. Under these conditions, both species undergo an equilibrium between a six-coordinated His/His-ligated and a five-coordinated His/- ligated forms stable in the oxidized and in the reduced state, respectively. The thermodynamics of the oxidation-state dependent species conversion were determined by temperature-dependent diffusionless voltammetry experiments. CL binding stabilizes the immobilized reduced His/- ligated form of cytc which was found previously to catalytically reduce dioxygen. This effect would impart CL with an additional role in the cytc-mediated peroxidation leading to programmed cell death. Moreover, immobilized cytc exchanges electrons more slowly upon CL binding possibly due to changes in solvent reorganization effects at the protein-SAM interface.

Cover Letter (including Suggested Referees)

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DIPARTIMENTO DI SCIENZE CHIMICHE E GEOLOGICHE

Modena, April 29, 2015

To **Prof. A.R. Hillman**,
Editor in Chief, Electrochimica Acta
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University Road,
Leicester, LE1 7RH,
UK

Manuscript Title: Thermodynamics and kinetics of reduction and species conversion at an hydrophobic surface for mitochondrial cytochromes *c* and their cardiolipin adducts
Name of the Corresponding Author: Marco Borsari
Name of all Other Authors: Antonio Ranieri, Giulia Di Rocco, Diego Millo, Gianantonio Battistuzzi, Carlo A. Bortolotti, Lidia Lancellotti, Marco Sola
Type of Manuscript: Research Paper

Dear Prof. Hillman,

We have submitted the above manuscript, which I would like you to consider for publication in Electrochimica Acta as a Full Paper.

In this work, we have studied the electron transfer properties and the speciation of the adducts formed by cytochrome *c* (cytc) with cardiolipin (CL) immobilized on a self-assembled monolayer (SAM) of decane-1-thiol. This construct would reproduce the motional restriction and the nonpolar environment experienced by the complex at the inner mitochondrial membrane. The axial heme iron ligands are found to be oxidation state-dependent and different from those observed in solution under the same conditions. These findings indicate that restriction of motional freedom due to interaction with the membrane is one additional factor playing in the mechanism of cytc unfolding and cytc-mediated peroxidation functional to the apoptosis cascade.

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Partita IVA.: 00427620364

per Creditori Privati: Conto Unicredit: IT40H0200812930000102063651 – SWIFT CODE: UNCRITMM - Codice Bilancio UNIMORE: A.005

per Creditori Pubblici: Conto Banca d'Italia: IT23E0100003245243300037150 (indicare nella causale il Codice Struttura: A.005)

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As the interest toward the electrochemistry involved in the interaction of cytochrome *c* with the inner mitochondrial membrane and its physiological implications is expanding, we thought to Electrochimica Acta as to the best medium for this paper.

The Authors state that this manuscript, or its content in some other form, has not been published previously by any of the authors and it is not under consideration for publication in another journal.

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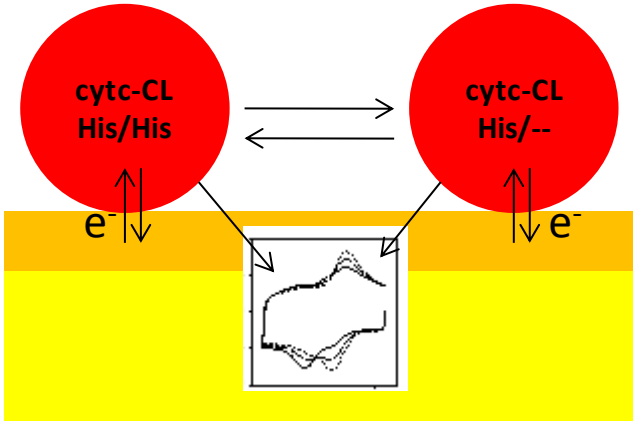
GRAPHICAL ABSTRACT



Thermodynamics and kinetics of reduction and species conversion at an hydrophobic surface for mitochondrial cytochromes *c* and their cardiolipin adducts

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Abstract

Cytochrome *c* (cytc) and its adduct with cardiolipin were immobilized on a hydrophobic SAM-coated electrode surface yielding a construct which mimics the environment experienced by the complex at the inner mitochondrial membrane where it plays a role in cell apoptosis. Under these conditions, both species undergo an equilibrium between a six-coordinated His/His-ligated and a five-coordinated His/- ligated forms stable in the oxidized and in the reduced state, respectively. The thermodynamics of the oxidation-state dependent species conversion were determined by temperature-dependent diffusionless voltammetry experiments. CL binding stabilizes the immobilized reduced His/- ligated form of cytc which was found previously to catalytically reduce dioxygen. This effect would impart CL with an additional role in the cytc-mediated peroxidation leading to programmed cell death. Moreover, immobilized cytc exchanges electrons more slowly upon CL binding possibly due to changes in solvent reorganization effects at the protein-SAM interface.

Keywords

cytochrome *c*; cardiolipin, electron-transfer, voltammetry, adsorption.

1. Introduction

The involvement of mitochondrial cytochrome *c* (cytc) in apoptosis upon binding of the phospholipid cardiolipin (CL) at the inner mitochondrial membrane (IMM) [1-9] endows cytc with the status of a multifunctional protein whose role goes beyond that of an electron shuttle between membrane protein complexes in respiration [10-13]. The structural changes induced by CL binding include swapping of the sixth (axial) methionine iron ligand by a His or Lys residue or a hydroxide ion, in an oxidation state-dependent event [3-5, 14, 15, 16]. In a previous study, we have shown that this coordination change at the heme iron and likely the mechanism of cytc unfolding and cytc-

mediated peroxidation in the initial stages of apoptosis is influenced by protein immobilization on IMM during CL recognition and binding [14]. In particular, cyclic voltammetry and surface enhanced resonance Raman scattering (SERRS) studies were carried out on the cytc-CL adduct (cytc-CL hereafter) immobilized on an electrode coated with a hydrophobic self-assembled monolayer (SAM) of decane-1-thiol (DT). This construct reproduces the motional restriction experienced by cytc upon binding to CL at IMM [17, 18]. We found that immobilized cytc-CL experiences an equilibrium between a low-spin six-coordinated (6c) His/His and a high-spin five-coordinated (5c) His/- ligation states prevailing in the oxidized and reduced form, respectively. The six-coordinated His/His species differs from the low spin 6c His/Lys and 6c His/OH^- states observed in solution [3]. Here, we further explored this system for two main reasons: *i)* to gain a deeper insight into the above oxidation-state dependent species conversion, measuring the reaction thermodynamics through temperature dependent E° measurements and *ii*) to measure the effect of CL binding on the ET kinetics and the reduction thermodynamics of adsorbed cytc. We focused on three cytc species: the recombinant *Saccharomyces cerevisiae* yeast iso-1 protein (ycc), its triple K72A/K73A/K79A variant (KtoA) and the native protein from beef heart (bcc). This choice was dictated because *in vivo* bcc is proapoptotic, while ycc is not, and because the three clustered lysines located at the rim of the heme crevice are heavily involved in several binding/recognition events of cytc with redox partners and contribute to several physicochemical properties of the protein (surface electrostatic potential, anion binding, pH-induced axial heme coordination changes *in vitro*) [15, 19-26]. In this context, their deletion should therefore be informative on the role of these residues in the studied physicochemical events.

2. Experimental

2.1. Materials

Wild type (wt) recombinant untrimethylated *Saccharomyces cerevisiae* iso-1-cytochrome *c* (ycc) and its variant K72AK73AK79A were expressed in *E. coli* and purified following the

procedures described elsewhere [27, 28]. In both species, Cys102 was replaced by a threonine to avoid dimerisation and minimize autoreduction without affecting the spectral and the functional properties of the protein [29, 30]. Beef heart cytochrome *c* was purchased from Sigma-Aldrich and purified as ycc. All chemicals were of reagent grade. Decane-1-thiol (DT) and cardiolipin were purchased from Sigma-Aldrich. Doubly distilled water was used throughout.

2.2. Electrochemical measurements

A Potentiostat/Galvanostat mod. 273A (EG&G PAR, Oak Ridge, USA) was used to perform cyclic voltammetry (CV). Experiments were carried out at different scan rates (0.02 - 40 V s^{-1}) using a cell for small volume samples (0.5 mL) under argon. A 1 mm-diameter polycrystalline gold wire, a Pt sheet, and a saturated calomel electrode (SCE) were used as working, counter, and reference electrode, respectively. The electrical contact between the SCE and the working solution was achieved with a Vycor® (from PAR) set. All the redox potentials reported here are referred to the standard hydrogen electrode (SHE), unless otherwise specified. The working gold electrode was cleaned by flaming it under oxidizing conditions; afterwards, it was heated in concentrated KOH for 30 min, rinsed with water and subsequently cleaned by 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 V (vs. SCE) at 0.1 V s^{-1} in 1 M H_2SO_4. Finally, the electrode was rinsed in water and anhydrous ethanol. The Vycor® set was treated in an ultrasonic pool for about 5 min. SAM coatings on the gold electrode were obtained by dipping the polished electrode into a 1 mM ethanol solution of DT for 12 hrs and then rinsing it with MILLIQ water. Protein solutions were freshly prepared before use in 10 mM HEPES buffer at pH 7 and their concentration (typically 10 μM) was carefully checked spectrophotometrically (Jasco mod. V-570 spectrophotometer). Protein adsorption on the DT SAM-coated Au electrode was achieved by dipping the functionalized electrode into a 10 μM protein solution at 4°C for 1 hrs. Cytc-CL adducts were obtained by mixing cytochrome *c* and CL to obtain a 10 μM protein and 300 μM CL solution (cytc/CL molar ratio 1:30)

in 10 mM Hepes at pH 7, which was allowed to stand for 30 min. In these conditions cytc is fully bound to CL [3, 14, 31]. Adduct adsorption on the DT-coated electrode was achieved by dipping the functionalized electrode into the above solution for 5 hour at 4 °C. The CV measurements were made up in 10 mM Hepes buffer at pH 7. Overlapped or poorly resolved peaks were deconvoluted using a homemade program developed on the Origin platform. The formal reduction potentials E°' were taken as the midpoint between the anodic and cathodic peak potentials. All the experiments were repeated at least five times. The reduction potentials were found to be reproducible within ± 2 mV. The rate constant values for the heterogeneous electron transfer (ET) reaction, k_s, obtained from the Laviron's model [32] were found to be reproducible within 6%. The CV experiments at different temperatures were carried out with a cell in a "nonisothermal" setting [30] 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 was under thermostatic control with a water bath. The temperature was varied from 5 to 35°C. With this experimental configuration, the standard entropy change for Fe(III) to Fe(II) cytochrome *c* reduction ($\Delta S°'_{rc}$) is given by [33-35]:

$$\Delta S_{rc}^{\circ'} = S_{red}^{\circ'} - S_{ox}^{\circ'} = nF\left(\frac{dE^{\circ'}}{dT}\right) \qquad (1)$$

Thus, $\Delta S°'_{rc}$ was determined from the slope of the plot of E°' versus T which turns out to be linear under the assumption that $\Delta S°'_{rc}$ is constant over the temperature range investigated. 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 versus 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 [33-35]. The activation enthalpies $\Delta H^{\#}$ were obtained from Arrhenius plots.

3. Results

As reported previously [14], the shape of the cyclic voltammograms (CVs) for the free (unbound) proteins and their CL adducts for bcc, ycc and the triple ycc KtoA variant adsorbed on DT, is dependent of the potential scan rate *v*. Invariably, cathodic peak currents are linearly dependent on *v*, indicating diffusionless electrochemistry. In all cases, at *v* lower than 0.1 V s^{-1} the CVs consist of two cathodic peaks, I_c and II_c and the corresponding anodic peaks, I_a and II_a (Fig. 1). I_c and II_a are intense and well-shaped, while II_c and I_a are much weaker (peak II_c is clearly visible only for bcc) [14]. Under these conditions, for all species the voltammograms are independent of whether the potential scan is started from positive potentials (cathodic scan) or negative potentials (anodic scan). Moreover, from 5 to 35 °C, the responses are reproducible and persist for several cycles, indicating that the protein layer is stable. The CV responses change at higher scan rates. In particular, starting from an oxidizing poise, an increase in *v* does not affect the relative intensity of signals I_c and II_c in the cathodic scan, while in the anodic scan the current of signal I_a increases to the detriment of that of II_a (Fig. 1 and 2). Analogously, starting from a reducing poise, the relative intensity of signals I_a and II_a in the anodic scan is independent of the scan rate (not shown), although it is strongly affected by temperature. In the corresponding cathodic scan, the current of signal II_c increases with increasing scan rate to the detriment of that of the signal I_c (Fig. 2), which at $v > 20$ V s^{-1} has almost disappeared [14]. As shown previously [14], these results indicate the existence, for all immobilized proteins and their adducts with CL, of two redox state-dependent conformers which are mainly stable in the oxidized (signals I) and reduced (signals II) form. These conformers will therefore be termed LP and HP, respectively. At *v* larger than 5 Vs^{-1} the two individual voltammetric features can be recognized and the corresponding individual E°’ values determined (as the average of the cathodic and anodic peak) (Table 1). Consistently, the E°’ value of the HP conformer (signal II) is higher than that of the LP conformer (signal I) by about 150 mV. Notably, the E°’ values obtained for complexed and uncomplexed forms are quite similar, in particular for the HP species. The temperature dependence of E°’ for the adsorbed cytochromes *c*

and the corresponding CL adducts is reported in Fig. 3. The E°' values invariably show a monotonic linear increase with increasing temperature in the range 5-35°C. The corresponding reduction entropy and enthalpy values are listed in Table 1. The rate constants for the electron transfer (ET) process between the adsorbed protein and the electrode, k_s (Table 1), were determined from the scan rate dependence of the anodic and cathodic peak potentials, following the Laviron's model for diffusionless electrochemical systems [32]. The activation enthalpies ($\Delta H^{\#}$) for the ET process calculated by the Arrhenius equation:

$$k_s = A' \exp\left(\frac{-\Delta H^{\#}}{RT}\right) \quad (2)$$

namely from the slope of the plot of ln k_s vs. 1/T (Fig. 4), are listed in Table 1.

3. Discussion

3.1. Oxidation state-dependent heme coordination and thermodynamics of species conversion for the adsorbed proteins

The nature of the species corresponding to signals I and II was determined in a previous study [14]. In particular, for both the unbound proteins and their CL-adducts, signal I (LP) is due to a low-spin six-coordinated (6c) His/His-ligated heme-containing form in which the native axial Met80 heme iron ligand is replaced by a His residue (probably His26 or His33) [4, 14, 36, 37]. Signal II (HP) instead corresponds to a high-spin five-coordinated (5c) state in which Met80 is detached from the iron which thereby features an open coordination site. Thus, in the cathodic potential scan starting from an oxidizing poise, peak I_c corresponding to the stable oxidized low-spin 6c His/His-ligated (LP) form is indeed the main peak, whereas the oxidized high-spin 5c His/- ligated (HP) form, which is stable in the reduced state, yields the minor peak II_c, often absent or barely detectable at best (Fig. 2A and 2B). The ratio of their peak areas (Q_{Ic}/Q_{IIc} hereafter), Q_{Ic} and Q_{IIc}

representing the charge needed to reduce the adsorbed LP and HP protein, respectively, is independent of scan rate, indicating that the surface concentration of the two oxidised forms is constant. The Q_{Ic}/Q_{IIc} ratio does not change appreciably also with temperature, showing us that the populations of the two oxidised forms do not change appreciably with temperature.

The anodic potential scan starting from reducing poise features the reduced high-spin 5c His/- ligated (HP) form (peak II_a) as the main species at all the investigated temperatures (Fig. 2C and 2D). The low-spin reduced 6c His/His-ligated (LP) form (peak I_a) is always present as a minor species. Only for KtoA at T<25°C, peak I_a prevails on II_a. The Q_{Ia}/Q_{IIa} ratio (Q_{Ia} and Q_{IIa} being the charge needed to oxidize the adsorbed LP and HP protein, respectively) is independent of scan rate but is strongly affected by temperature. In particular, peak I_a decreases with increasing temperature and disappears at T>35°C. Thus, interconversion between the ferrous high spin 5c His/- (HP) and low spin 6c His/His (LP) species occurs, which is affected by temperature. The Q_{Ia}/Q_{IIa} ratio can be taken as the ratio between the corresponding surface concentration (Γ_{Ia}/Γ_{IIa}). Under the hypothesis that the activity of the adsorbed species is equal to the corresponding surface concentration ($a_{Ia}= \Gamma_{Ia}$, and $a_{IIa}= \Gamma_{IIa}$), the equilibrium constant, $K_{HP\rightarrow LP}$, for the transition from the ferrous high-spin 5c His/- (HP) form to the ferrous low-spin 6c His/His (LP) form can be calculated as the ratio between the peak currents of signals I_a and II_a at each temperature (Table 2). The van't Hoff plot allows calculation of the standard enthalpy change $\Delta H°_{HP\rightarrow LP}$ and the standard entropy change $\Delta S°_{HP\rightarrow LP}$ associated to the HP to LP transition (Fig. 5, Table 2). The transition enthalpy and entropy are both negative and therefore exert opposite effects on the reaction, which turns out to be favoured by the balance of bonding interactions (enthalpic term) and disfavoured by the change in the number of thermally accessible states of the system (entropic term). The latter contribution is larger at room temperature for all the investigated proteins. This condition is consistent with the fact that the HP→LP transition (ferrous form) involves the formation of a coordination bond ($\Delta H°_{HP\rightarrow LP}<0$), and suggests that this is accompanied by a decrease of the motional freedom in the heme environment likely owing to some structuring effects ($\Delta S°_{HP\rightarrow LP}<0$). The latter contribution increases with

temperature and accounts for the increase in the population of the high spin 5c His/- ligated (HP) form on the electrode with temperature. The $K_{HP \rightarrow LP}$ values increase in the order: bcc, ycc, KtoA for both the unbound proteins and their CL-adducts. Interestingly, adduct formation decreases $K_{HP \rightarrow LP}$. CL therefore stabilizes the 5c HP form, possibly by introducing some structural constraints to the binding of the second axial His to the ferrous iron. The plot of $\Delta H°_{HP \rightarrow LP}$ vs. $T\Delta S°_{HP \rightarrow LP}$ at 293 K (Fig. 6) is approximately linear with a slope of 1.13, indicating a high extent enthalpy/entropy compensation. Enthalpy/entropy compensation phenomena are well known in several (bio)chemical contexts [38-43]. In our case, this behaviour can be ascribed to species dependent transition-induced reorganization of the H-bonding network at protein-solution interfaces [40-43].

3.2. Redox Thermodynamics

3.2.1 Low-spin 6c His/His-ligated (LP) form

The E°' values of the CL-adducts of the 6c His/His (LP) ligated form (signal I) for ycc, KtoA and bcc immobilized on DT are from 15 to 25 mV less negative than those for the corresponding unbound proteins (Table 1), indicating that CL stabilizes ferrous cytochrome *c*. The E°' values of the CL-adducts, particularly for ycc and KtoA, are similar to those for the same cytochromes subjected to urea unfolding immobilized on an anionic surface [33, 44, 45]. Moreover, these E°' values are in agreement with that for cytc covalently bound to an anionic SAM interacting with CL-containing liposomes [46] although other forms of immobilized cytc attributed to adducts with CL with rather different E°' were found for cytc immobilized on cardiolipin/phosphatidylcholine-coated electrodes [47, 48].

As found previously for other His/His-ligated forms of cytochrome *c*, $\Delta S°'_{rc}$ is positive, likely due related to a reduction-induced increased accessibility of the heme center to solvent [33, 44, 45]. Indeed, a decreased heme charge upon reduction (from +1 to 0) would induce a weakening of the electrostatic interaction of the metal center with the surrounding solvent molecules and therefore an increased disorder. However, the reduction thermodynamics, $\Delta S°'_{rc}$ and $\Delta H°'_{rc}$, for the unbound

proteins and their CL-adducts are quite higher than those of the corresponding forms obtained by urea-unfolding. It follows that a compensative effect must exist [38-43]. We may hypothesize that the heme environment of the His/His-ligated forms obtained by urea unfolding immobilized on anionic or hydrophilic surfaces experience a different solvent accessibility with respect to that of the His-His forms immobilized on a hydrophobic surface, independently of the presence of CL [33, 44, 45].

3.2.2. High-spin 5c His/- ligated (HP) form

The E°' values for the 5c His/- ligated (HP) form (signal II) for ycc and KtoA are very similar (Table 1), but are higher than that of bcc. This suggests that the main responsible for E°' for this cytochrome *c* form is not the charge distribution around the native heme crevice (similar for ycc and bcc), but other molecular determinants possibly related to the overall protein structure. As for the 6c His/His-ligated forms, $\Delta S°'_{rc}$ is invariably positive (Tab. 1). Interestingly, unlike the 6c His/His form, the E°' values of the HP species are independent of the presence of CL as a result of marked changes in reduction enthalpy and entropy, which turn out to be exactly compensatory. As shown previously [33, 44], this is typical of an event resulting in a change in the reduction-induced solvent reorganization effects. Therefore the effect of CL binding to this form is limited to a change in the hydrogen bonding network of the water molecules in the hydration sphere of the molecule, without affecting the thermodynamic stability of the heme redox states.

3.3. ET kinetics of the adsorbed species in absence and in the presence of CL

The ET activation enthalpies for the LS 6c His/His-ligated form for all the studied proteins under these conditions (Table 1) are lower than those found for other His/His-ligated cytochromes [33, 44, 45]. This result could be related, at least in part, to a decrease in the accessibility of the heme crevice to solvent and is consistent with the insertion of a hydrophobic chain inside the heme pocket. In particular, for all proteins the ET activation enthalpies of the low spin 6c His/His-ligated (LP) forms are invariably lower than those of the corresponding high spin 5c His/- (HP) forms

(Table 1). Moreover, for both forms, CL binding slows down ET, by increasing the activation enthalpy (Table 1). This suggests that solvent reorganization effects at the protein-SAM interface (which are known to heavily affect the rate of ET [33, 49-51]) are at least partially affected by the presence of CL. Conceivably, the distribution of the water molecules at the interface with the hydrophobic SAM changes remarkably with the characteristics of the protein surface. The activation enthalpy of the low spin 6c His/His-ligated (HP) form of KtoA is higher than those for ycc. Lys 72 and/or 73 and/or 79 could be, therefore, effective in the lowering the solvent reorganization energy by affecting the charge of the protein surface and/or by changing the exposure of the heme center to solvent [22].

4. Conclusions

The equilibrium between the 6c low spin His/His-ligated and the 5c high spin His/- ligated states experienced by cytc immobilized on a hydrophobic SAM is affected by cardiolipin binding. In particular, CL stabilizes the five-coordinated form, which is stable in the reduced state. This effect is paralleled by the CL-induced increase in E°, which is indicative of a stabilization of the ferrous heme. Notably, the immobilized reduced 5c high spin His/- ligated form can electrocatalytically reduce dioxygen at the heme iron, likely to superoxide ion [14]. This effect could possibly be involved in cytc-mediated peroxidation functional to the apoptosis cascade. Therefore, it turns out that CL binding yields a structural modification of cytc at IMM inducing an as yet not fully understood number of consequences, among which, however, we may include a redox effect involving stabilization of the reduced heme which might be important for the biological effect thanks to its affinity for dioxygen. Although the available data do not allow to fully unravel the molecular details of this event, CL binding involves a change in the transition-induced solvent reorganization effects accompanying the ferrous high spin→low spin transition, as demonstrated by the enthalpy-entropy compensation effects. Besides the above thermodynamic effect, CL binding also decreases the rate with which cytochrome *c* exchanges electrons with the electrode. At present,

the biological role of the observed decrease of the ET rate cannot be clearly assessed, although it could be important in the network of the kinetically controlled processes involved in apoptosis.

Acknowledgements

This work has been supported by the Italian Ministry of University and Research (PRIN 2009 prot. N. 20098Z4M5E_002) [M.B.], DM acknowledges the Netherlands Organisation for Scientific Research (NWO) grant 722.011.003. AR acknowledges the LaserLaB Access Program (project number LLAMS001927, LASERLAB-EUROPE grant agreement no. 284464, EC's Seventh Framework Programme).

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Table 1. Thermodynamics of reduction and kinetics of heterogeneous electron transfer for yeast, KtoA and bovine cytochromes *c* and their cardiolipin adducts (+CL) adsorbed on a gold electrode coated with decane-1-thiol.[a]

	yeast wt		KtoA		bovine	
	LP	HP	LP	HP	LP	HP
$E°'$/V [b]	-0.253	-0.106	-0.270	-0.111	-0.227	-0.081
$E°'$/V (+ CL) [b]	-0.226	-0.101	-0.248	-0.106	-0.212	-0.077
$\Delta S°'_{rc}$/J K^{-1} mol^{-1} [c]	106	57	69	58	52	37
$\Delta S°'_{rc}$ /J K^{-1} mol^{-1} (+CL)	90	48	85	54	66	26
$\Delta H°'_{rc}$ /kJ mol^{-1} [d]	55.4	27.2	46.2	27.7	37.1	18.5
$\Delta H°'_{rc}$ /kJ mol^{-1} (+CL)	48.3	23.9	48.8	26.1	40.1	14.9
k_s/s^{-1} [b,e]	339	127	186	97	412	181
k_s/s^{-1} (+CL) [b]	232	119	131	85	298	139
$\Delta H^{\#}$/kJ mol^{-1} [f]	7.9	9.8	8.5	9.1	7.7	9.2
$\Delta H^{\#}$/kJ mol^{-1} (+CL)	8.3	10.1	8.8	9.5	8.1	9.9

[a] Working solution: 10 mM Hepes buffer at pH 7. The average error on $E°'$, $\Delta S°'_{rc}$, $\Delta H°'_{rc}$, k_s and $\Delta H^{\#}$ are ±0.002 V, ±2 J K^{-1} mol^{-1}, 0.8 kJ mol^{-1}, ±6% and 0.2 kJ mol^{-1}, respectively; [b] T = 293 K; [c] $\Delta S°'_{rc}$: standard entropy change for Fe(III) to Fe(II) cytochrome *c* reduction; [d] $\Delta H°'_{rc}$: standard enthalpy change for Fe(III) to Fe(II) cytochrome *c* reduction; [e] k_s: rate constant for the heterogeneous electrode-protein electron transfer; [f] $\Delta H^{\#}$: activation enthalpy for the heterogeneous electrode-protein electron transfer.

Table 2. Thermodynamic parameters for the HP→LP transition for yeast, KtoA and bovine ferrocytochromes *c* and their cardiolipin adducts (+CL) adsorbed on a gold electrode coated with decane-1-thiol.

	yeast wt	KtoA	bovine
$K_{HP \rightarrow LP}$ [a]	0.30±0.02	7.24±0.13	0.23±0.02
$K_{HP \rightarrow LP}$ (+ CL) [a]	0.13±0.01	1.09±0.09	0.008±0.003
$\Delta H°_{HP \rightarrow LP}$ /kJ mol^{-1}	-53.4±0.8	-58.4±0.7	-20.3±0.4
$\Delta H°_{HP \rightarrow LP}$ /kJ mol^{-1} (+ CL)	-56.8±0.9	-66.0±0.8	-21.1±0.4
$\Delta S°_{HP \rightarrow LP}$ /J K^{-1} mol^{-1}	-200±9	-194±9	-84±6
$\Delta S°_{HP \rightarrow LP}$ /J K^{-1} mol^{-1} (+ CL)	-220±11	-236±15	-135±6

[a] Working solution: 10 mM Hepes buffer at pH 7; T= 278 K.

Captions to figures

Fig. 1. Cyclic voltammograms (cathodic scan started after an oxidizing poise at E = +0.2 V followed by the anodic scan) at low scan rate for the triple K72A/K73A/K79A variant of *Saccharomyces cerevisiae* yeast iso-1 cytochrome *c* (KtoA) immobilized on a hydrophobic SAM of decane-1-thiol (solid line) and the corresponding adduct with cardiolipin immobilized under the same conditions (dashed line). Scan rate, 0.1 V s^{-1}, working solution: 10 mM Hepes buffer, pH 7. Analogous CVs were obtained for the other cytochromes.

Fig. 2. Cyclic voltammograms at high scan rate for the triple K72A/K73A/K79A variant of *Saccharomyces cerevisiae* yeast iso-1 cytochrome *c* (KtoA) immobilized on a hydrophobic SAM of decane-1-thiol and the corresponding adducts with cardiolipin immobilized under the same conditions (KtoA-CL). "Cathodic": cathodic scan started after an oxidizing poise at E = +0.2 V followed by the anodic scan; "anodic": anodic scan started after a reducing poise at E = - 0.8 V followed by the cathodic scan. Dotted, dashed and solid lines refer to CV runs at 5, 20 and 30 °C, respectively. Scan rate, 20 V s^{-1}, working solution: 10 mM Hepes buffer, pH 7. Analogous CVs were obtained for the other cytochromes.

Fig. 3. Temperature dependence of the E° values for (A, C) the cytochromes *c* immobilized on a hydrophobic SAM of decane-1-thiol and (B, D) the corresponding adducts with cardiolipin immobilized under the same conditions. A) and B) show the E° values for the LP (6c His/His-ligated heme-containing) forms, while C) and D) refer to the HP (5c His/- ligated heme-containing) forms. (○) ycc, (●) KtoA, (▼) bcc. Scan rate, 20 V s^{-1}. Working solution: 10 mM Hepes buffer, pH 7.

Fig. 4. Arrhenius plots for (A, C) the cytochromes *c* immobilized on a hydrophobic SAM of decane-1-thiol and (B, D) the corresponding adducts with cardiolipin immobilized under the same conditions. A) and B) refer to the LP (6c His/His-ligated heme-containing) forms, while C) and D to

the HP (5c His/- ligated heme-containing) forms. (○) ycc, (●) KtoA, (▼) bcc. Scan rate, 20 V s^{-1}. Working solution: 10 mM Hepes buffer, pH 7.

Fig. 5. van't Hoff plot for the equilibrium constant, $K_{HP \rightarrow LP}$, for the high spin 5c His/- (HP) to low spin 6c His/His (LP) transition for A) the reduced cytochromes *c* immobilized on a hydrophobic SAM of decane-1-thiol and B) the corresponding adducts with cardiolipin immobilized under the same conditions. (○) ycc, (●) KtoA, (▼) bcc. Scan rate, 20 V s^{-1}. Working solution: 10 mM Hepes buffer, pH 7.

Fig. 6. Plot of $\Delta H°_{HP \rightarrow LP}$ *vs.* $T\Delta S°_{HP \rightarrow LP}$ (compensation plot) for the high spin 5c His/- (HP) to low spin 6c His/His (LP) transition for the reduced cytochromes *c* immobilized on a hydrophobic SAM of decane-1-thiol and the corresponding adducts with cardiolipin immobilized under the same conditions. Working solution: 10 mM Hepes buffer, pH 7. T = 293 K.

Figure 1

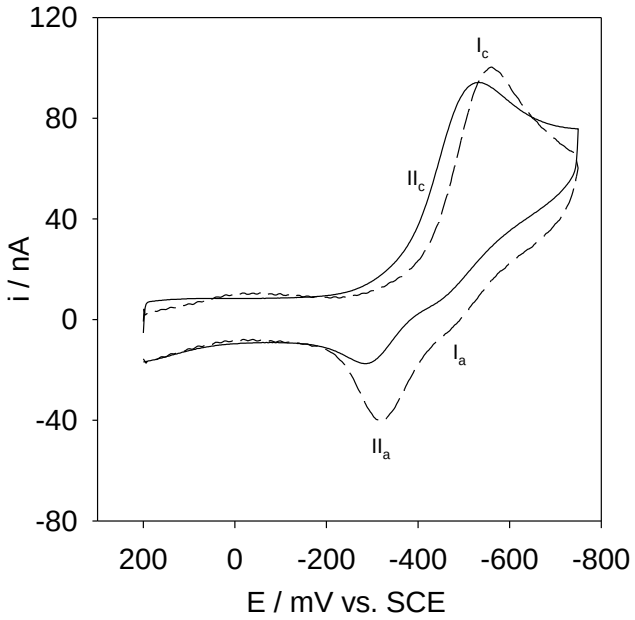

Fig. 1

Figure 2

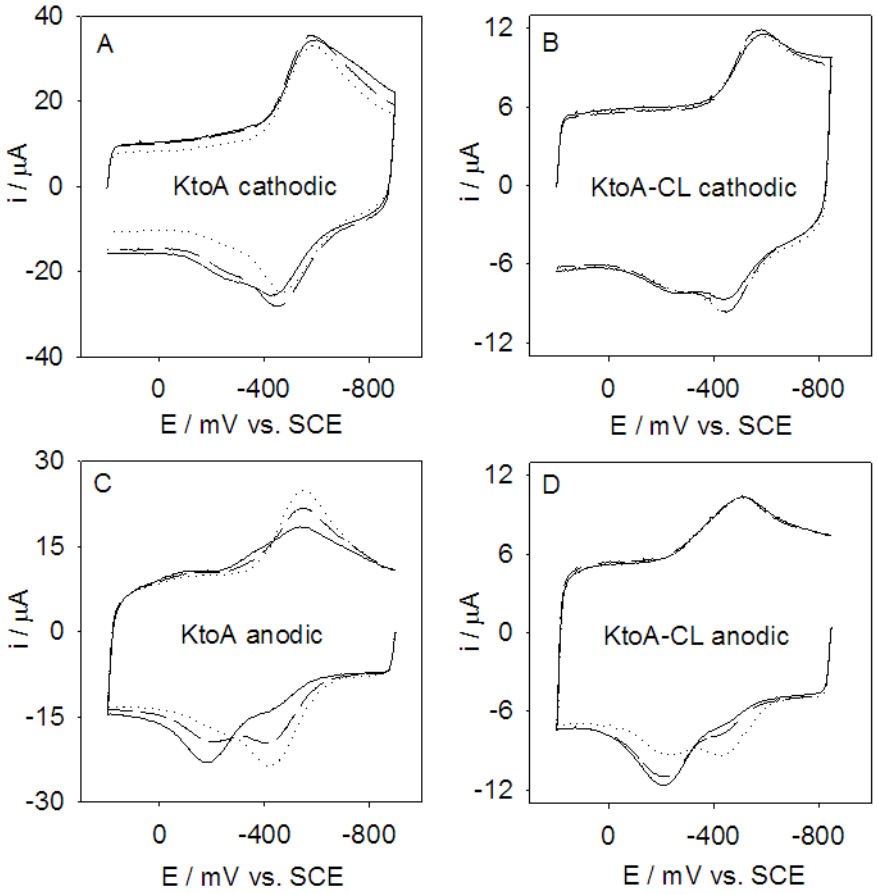

Fig. 2

Figure 3

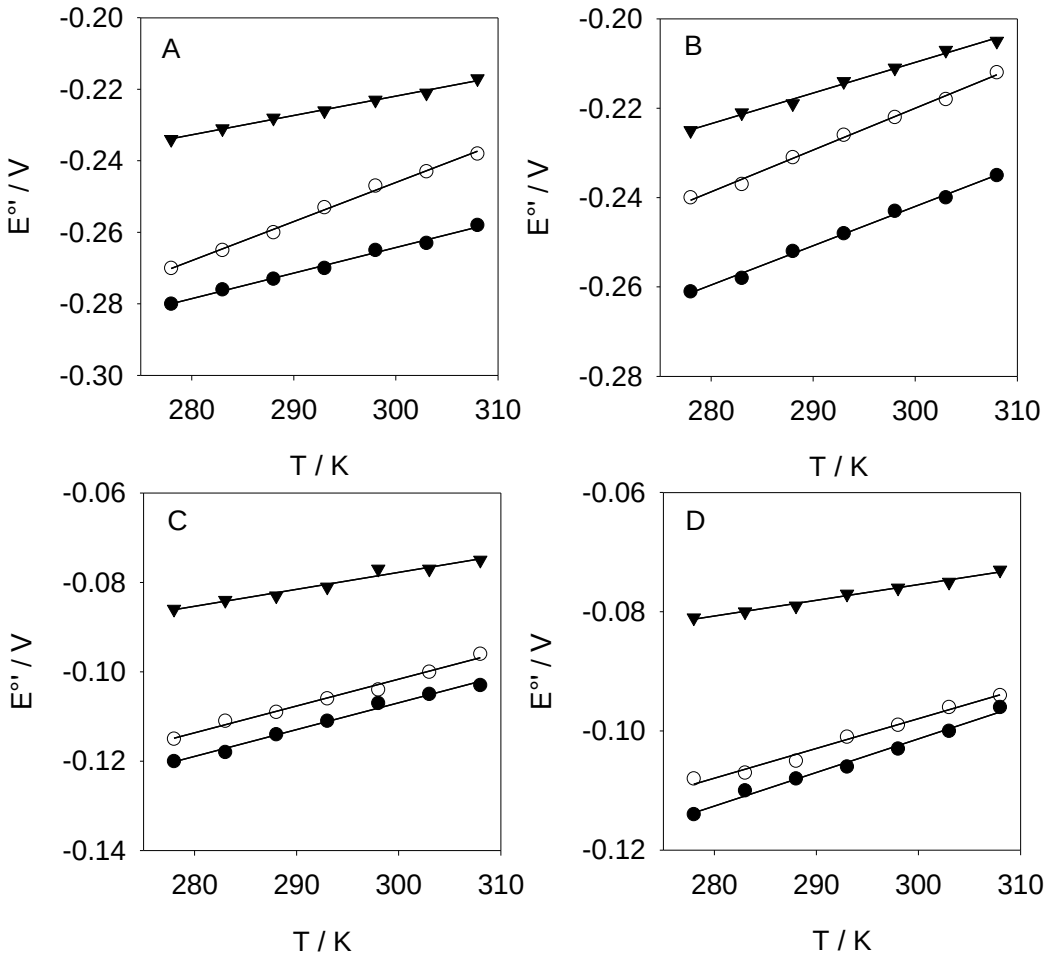

Fig. 3

Figure 4

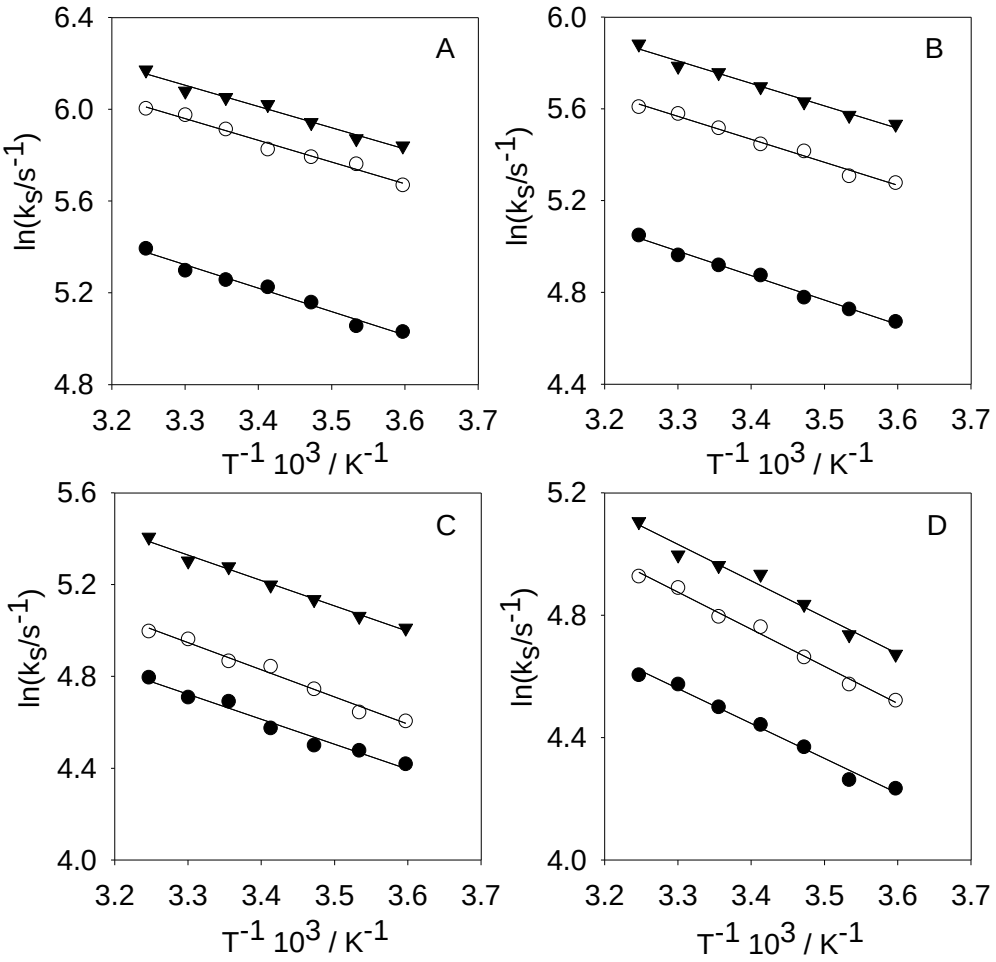

Fig. 4

Figure 5

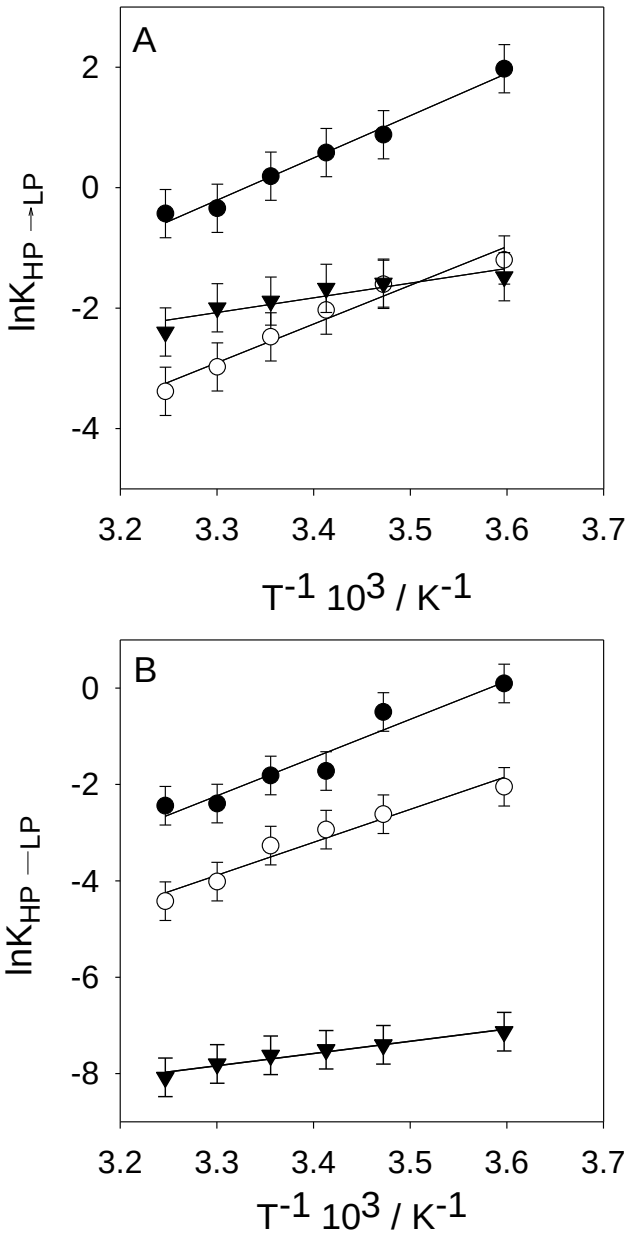

Fig. 5

Figure 6

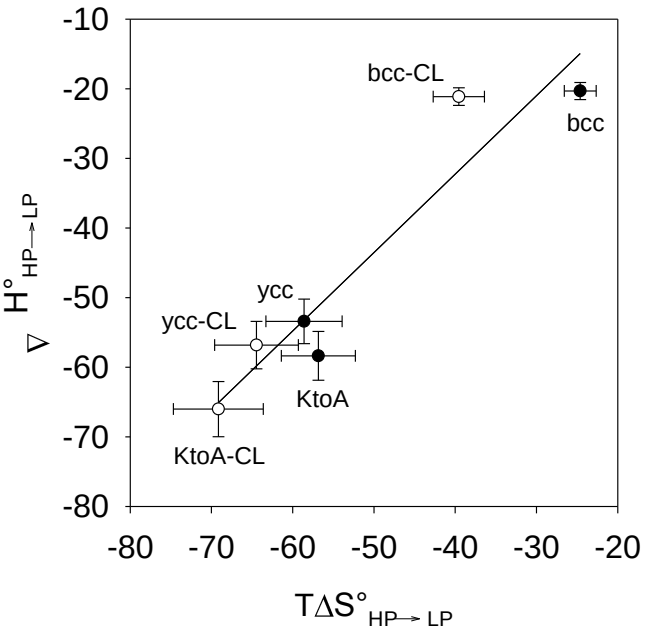

Fig. 6

Highlights

-Cytochrome *c* and its adduct with cardiolipin can be immobilized on a hydrophobic SAM
-Adsorbed cytochrome c and its adduct undergo extensive unfolding and axial ligand substitution
-An equilibrium between a six-coordinated and a five-coordinated form is observed in both cases
-The reduced five-coordinated form is stabilized by cardiolipin binding
-Immobilized cytochrome c exchanges electrons more slowly upon cardiolipin binding