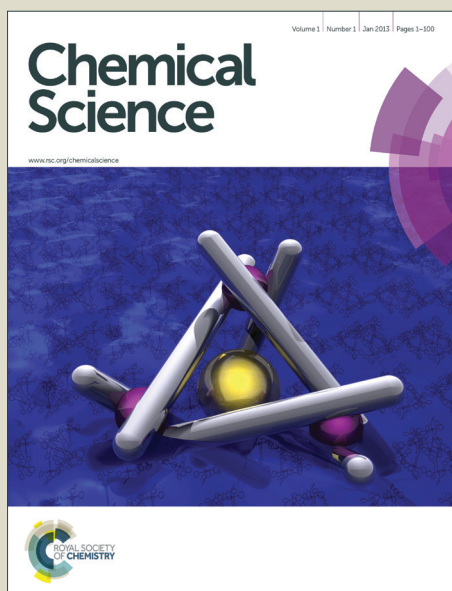


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## ARTICLE

# Specific Methionine Oxidation of Cytochrome *c* in Complexes with Zwitterionic Lipids by Hydrogen Peroxide: Potential Implications for Apoptosis

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Cytochrome *c* (Cyt-*c*) has been previously shown to participate in cardiolipin (CL) oxidation and, therefore, in mitochondrial membrane permeabilization during the early events of apoptosis. The gain of this function has been ascribed to specific CL/Cyt-*c* interactions. Here we report that the cationic protein Cyt-*c* is also able to interact electrostatically with the main lipid components of the mitochondrial membranes, the zwitterionic lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), through mediating phosphate anions that bind specifically to amino groups in the surfaces of the protein and model membranes. In these complexes Cyt-*c* reacts efficiently with H<sub>2</sub>O<sub>2</sub> at submillimolar levels, which oxidizes the sulfur atom of the axial ligand Met80. The modified protein is stable and presents significantly enhanced peroxidatic activity. Based on these results, we postulate that the rise of H<sub>2</sub>O<sub>2</sub> to submillimolar levels registered during the initiation of the apoptotic program may represent one signaling event that triggers the gain of peroxidatic function of Cyt-*c* molecules bound to the abundant PE and PC membrane components. As the activated protein is a chemically stable species, it can possibly bind and oxidize important targets, such as CL.

## 1. Introduction

Cytochrome *c* (Cyt-*c*) is a highly conserved monohemic protein that serves as an electron shuttle between the respiratory complexes III and IV at the level of the inner mitochondrial membrane (IMM), where it is also implicated in the generation, oxidation and trapping of reactive oxygen species (ROs).<sup>1,2</sup> The release of Cyt-*c* from the mitochondrial intermembrane space to the cytosol, on the other hand, plays a pivotal role in apoptosis, thereby highlighting the importance of this small multifunctional protein in sustaining and terminating cellular life.<sup>3</sup> Once in the cytosol Cyt-*c* binds Apaf-1, ATP and pro-caspase-9 to form a multimeric complex called apoptosome that triggers a cascade of events leading to cellular suicide.<sup>2,4-7</sup> The Cyt-*c* mediated caspase activation apoptotic pathway is essential for a large variety of biological events, including brain development, immune system homeostasis, stress-induced and

genotoxic-induced cell death and, therefore, is involved in protecting living organisms from diseases such as cancer. It may also amplify other apoptotic pathways.<sup>2,4-7</sup>

Several mechanisms have been proposed to explain the permeabilization of the outer mitochondrial membrane (OMM) that precedes Cyt-*c* release, as extensively reviewed elsewhere.<sup>2,4-9</sup> In this context, different groups have paid particular attention to the interactions between cationic Cyt-*c* and the anionic phospholipid cardiolipin (CL),<sup>2,10-24</sup> which represents ca. 10-20 % of the total lipid content of the IMM and a much smaller proportion of the OMM<sup>25-28</sup>. In the IMM, CL plays a crucial role in stabilizing the respiratory complexes and in mediating electron transport by Cyt-*c*. Indeed, it has been estimated that about 15 % of the available Cyt-*c* is bound to CL<sup>29-32</sup>. A number of biophysical studies on model systems suggest that, in addition to the unspecific electrostatic interaction, CL forms a specific association with Cyt-*c* that

involves the insertion of one or two hydrocarbon chains into hydrophobic channels of the protein.<sup>11,14–20,24</sup> The interplay of electrostatic and hydrophobic interactions triggers conformational changes that include the detachment of the iron axial ligand Met80 and an augmented accessibility of potential substrates to the metal center. The result is a gain of peroxidase activity of Cyt-c that has been implicated in the selective oxidation of CL observed during the early events of apoptosis.<sup>10,11,23</sup> Good arguments in favor of this proposal are the findings that (a) during apoptosis the amount of CL in the OMM increases significantly and (b) the oxidation of CL appears to be a requisite for the release of proapoptotic factors such as Cyt-c to the cytosol across the OMM.<sup>10,11</sup> Yet, the mechanistic details and, more specifically, the regulation of these processes remain elusive.

In addition to CL, mitochondrial membranes are rich in zwitterionic phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) that comprise more than 70 % of the total lipid content and have a quaternary ammonium and a primary amine bound to the phosphate group, respectively.<sup>25–27</sup> As previously shown, Cyt-c is not expected to present significant electrostatic affinity for such lipids.<sup>14,33,34</sup> Very recent studies, however, have demonstrated high affinity binding of Cyt-c to model systems containing amino and ammonium functional groups that is specifically mediated by inorganic and organic phosphate anions at millimolar levels.<sup>35,36</sup> In the present work we have investigated the chemical reactivity of Cyt-c associated to similar model systems and in complexes with PE- and PC-liposomes. We found that phosphate mediated binding of Cyt-c to these model lipids results in efficient oxidation of the axial ligand Met80 in the presence of H<sub>2</sub>O<sub>2</sub> at submillimolar levels, thus yielding a stable modified protein with high peroxidatic activity. Interestingly, the early stages of the apoptotic program are characterized by a rise of the H<sub>2</sub>O<sub>2</sub> intracellular levels from submicromolar to submillimolar.<sup>37,38</sup> Therefore, we propose that such a mechanism might be involved in the induction of peroxidase activity of Cyt-c that precedes the liberation of proapoptotic molecules from the mitochondria.

## 2. Experimental

### Chemicals.

6-mercapto-1-hexanol, 6-mercaptohexanoic acid, 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, 11-mercaptoundecylphosphoric acid, (11-MercaptoUndecyl)-N,N,N-trimethylammonium bromide and horse heart cytochrome c (Cyt-c) were purchased from Sigma-Aldrich. 11-amino-1-undecanethiol hydrochloride was purchased from Dojindo. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PE) were from Avanti Polar Lipids. Hydrogen peroxide was from Mallinckrodt Baker, Inc. Amplex Red Ultra was from

Invitrogen. The M80A mutant of Cyt-c was prepared according to published procedures.<sup>39</sup>

All chemicals were of the highest available purity and used without further purification. The water used in all experiments was purified by a Millipore system and its resistance was 18.2 MΩ.

### Electrochemistry.

Cyclic voltammetry (CV) experiments were performed using a Gamry REF600 potentiostat. The jacketed three-electrode electrochemical cell was placed inside a Faraday cage (Gamry Vista Shield) and was equipped with a polycrystalline Au bead working electrodes, a Pt wire counter electrode and a Ag/AgCl (3 M KCl) reference electrode to which all potentials in this work are referred. Unless stated otherwise, the electrolyte solution was 10 mM phosphate buffer, pH = 7.0, thoroughly deoxygenated by Ar bubbling. All experiments were carried out at room temperature (22–25 °C).

### Raman Spectroscopy.

Resonance Raman (RR) and surface-enhanced resonance Raman (SERR) spectra were acquired with a confocal Raman microscope (Dilor XY) equipped with a liquid-nitrogen cooled CCD detector. The excitation source was the 413 nm line of a krypton ion laser (Spectra-Physics BeamLok 2060). For SERR determinations the laser beam was focused onto the surface of a home-made rotating Ag electrode by means of a long-working-distance objective (20×, numerical aperture 0.35). The SERR spectroelectrochemical cell has been described in detail elsewhere.<sup>40</sup> RR measurements were performed using a cylindrical quartz cell mounted in a home-made rotating device to avoid laser-induced sample degradation. Typically, experiments were performed with laser powers of 3.5 mW at the sample.

### Electrodes treatment and SAMs preparation.

Au electrodes used for CV were first oxidized in 10% HClO<sub>4</sub> applying a potential of 2.5 V for 2 minutes, then sonicated in HCl 10 % for 15 minutes and rinsed with water. The electrodes were then cleaned with 3:1 v/v H<sub>2</sub>O<sub>2</sub> : H<sub>2</sub>SO<sub>4</sub> mixture at 120 °C. Finally, they were cycled between -0.2 and 1.6 V in 10 % HClO<sub>4</sub> and thoroughly washed with water and ethanol.

The area of these electrodes was determined by CV from the integration of the reduction peak of the surface oxide. For these determinations CVs were recorded at 0.1 Vs<sup>-1</sup> between -0.25 and 1.6 V in 0.5 M H<sub>2</sub>SO<sub>4</sub>, and the conversion factor was 0.44 mC cm<sup>-2</sup> for an oxide monolayer. The average surface area of the electrodes used in this work was calculated to be 0.18 ± 0.08 cm<sup>2</sup> with a roughness factor of 3.4 ± 1.1.

Ag ring electrodes employed for SERRs measurements were mechanically polished and then treated by repetitive electrochemical oxidation/reduction cycles in 0.1 M KCl to create a SER-active nanostructured surface.<sup>40</sup>

After the treatment described above, Au and Ag electrodes were immersed into 2 mM ethanolic solutions of the desired thiols for self-assembly. Unless stated otherwise, the incubation

solutions were 2 mM 1:1 mixtures of 11-mercapto-1-undecanol, which is added as a diluting component of the self-assembled monolayer (SAM), and a second  $\omega$ -functionalized undecanethiol with negatively or positively charged  $\omega$ -groups: carboxylate, phosphate, amino and trimethylammonium. The obtained SAMs are designated as COO-SAM, PO<sub>4</sub>-SAM, NH<sub>2</sub>-SAM and TA-SAM, respectively.

For the specific case NH<sub>2</sub>-SAMs the incubation solvent was a 4:1 v/v mixture of C<sub>2</sub>H<sub>5</sub>OH-HClO<sub>4</sub> 10<sup>-4</sup> M pH = 4 to prevent adsorption via the amino group. In all cases incubations were carried out overnight and the SAM-modified electrodes were kept in the darkness until use.<sup>41</sup> Prior to their use, the electrodes were rinsed thoroughly with ethanol and water and subjected to electrochemical cycling in phosphate buffer as conditioning treatment.<sup>36</sup>

### Preparation of lipid vesicles.

Large unilamellar vesicles were prepared by extrusion of equimolar PC/PE mixtures. Appropriate amounts of lipid stock solutions were mixed in chloroform, evaporated to dryness under a gentle nitrogen stream, and then left under reduced pressure for 1.5 h to remove any residual solvent. The dry lipids were subsequently hydrated with 20 mM HEPES pH 7, at room temperature to yield lipid concentration of 0.5 mM. Afterwards, the samples were passed 20 times through polycarbonate filters with pore size of 100 nm (Nuclepore, Whatman), yielding liposomes of the desired composition. Vesicle size, distribution and stability were checked by dynamic light scattering (SLS 90 Plus/BI-MAS equipped with He-Ne laser operating at 632.8 nm and 15 mW).

### Synthesis and purification of the SO-Cyt protein variant.

Chloramine-T was employed as a selective reagent for oxidizing the Met80 residue of Cyt-c according to previous reports.<sup>42,43</sup> The product obtained is designated as ex-situ generated SO-Cyt. For this purpose, 100  $\mu$ L of 1 mM Cyt-c were mixed with 100  $\mu$ L of 5 mM chloramine-T and the pH was adjusted to 8.4. After 3 hours at room temperature, the mixture was washed 6-times with 10 mL of 10 mM phosphate buffer, pH 7, employing centrifugal filters (Amicon 10 kDa filters). The progress of the reaction was monitored by the disappearance of the 695 nm band from the UV-visible spectrum of Cyt-c (Thermo Scientific Evolution Array spectrophotometer).

The reaction mixture was passed through a cation exchange sulfopropyl-TSK preparative column (21.5 mm x 15.0 cm; Tosoh Biosep) at a flow rate of 3 ml/min. The column was equilibrated with 5 mM ammonium acetate buffer (pH 9.0), kept during 5 min in this buffer and then eluted using a linear gradient from 5 mM to 150 mM ammonium acetate from 5 to 30 min, from 150 mM to 400 mM ammonium acetate from 30 to 75 min and in 500 mM ammonium acetate from 76 to 90 min (see ESI).

The desired product was characterized by mass spectrometry (MS) employing a 4800 MALDI TOF/TOF instrument (Applied Biosystems) in positive ion reflector mode. Mass

spectra were externally calibrated using a mixture of peptide standards (Applied Biosystems). MS/MS spectra were recorded to confirm the site of oxidation in the analyzed peptides (see ESI). Mass spectra were analyzed using DATA explorer and GPMW4 software.

### Peroxidatic activity determinations.

Assessment of the peroxidatic activity was performed employing Amplex UltraRed Reagent (Invitrogen). The fluorescence of the oxidation product, resorufin, was monitored at  $\lambda_{em}$  = 585 nm with  $\lambda_{ex}$  = 570 nm. Briefly, 0.5  $\mu$ M Cyt-c was incubated in HEPES (10 mM plus 100  $\mu$ M DTPA), pH 7. Then, 50  $\mu$ M Amplex Red and 25  $\mu$ M H<sub>2</sub>O<sub>2</sub> were added in HEPES (100 mM plus 100  $\mu$ M DTPA) pH 7 and the fluorescence was registered using a FLUOstar Optima plate fluorimeter (BMG Labtech) or a Variostar Flash Multimode Reader (Thermo). The reaction rate was determined by linear fit of the fluorescence intensity profile.

## 3. Results and discussion

### Reactivity of Cyt-c in biomimetic complexes.

Hydrogen peroxide is a well-documented bleaching agent of Cyt-c that produces oxidative damage of the porphyrin ring.<sup>44</sup> Recent studies, however, suggest that formation of electrostatic complexes with negatively charged surfaces has a protective effect on Cyt-c, thus preventing its degradation in the presence of H<sub>2</sub>O<sub>2</sub> up to concentrations of ca. 20 mM.<sup>45</sup> In this context, we investigated the reactivity of Cyt-c towards H<sub>2</sub>O<sub>2</sub> in electrostatic complexes with different model systems that mimic some essential features of the natural protein/membrane interactions. Specifically, we employed liposomes of different compositions and metal electrodes coated with SAMs of  $\omega$ -functionalized alkanethiols. In the last case the explored  $\omega$  functional groups were carboxylate, phosphate, amino and trimethylammonium (COO-SAM, PO<sub>4</sub>-SAM, NH<sub>2</sub>-SAM and TA-SAM, respectively). The adsorption of Cyt-c on COO- and PO<sub>4</sub>-SAMs involves direct electrostatic interactions through the ring of positively charged lysine residues that surrounds the partially exposed heme edge of the protein.<sup>46</sup> Roughly the same surface residues are implicated in the adsorption onto NH<sub>2</sub>- and TA-SAMs, although in these cases the interactions are mediated by phosphate ions from the buffer solution.<sup>35,36</sup> In all cases the adsorbed protein exhibits quasi reversible cyclic voltammetric (CV) responses (Fig. S1) typical of one-electron redox couples. The formal reduction potentials obtained for Cyt-c on COO-, PO<sub>4</sub>- and NH<sub>2</sub>-SAMs are very close to the value of the native protein in solution while, in excellent agreement with previous observations, the value on TA-SAMs is somewhat more positive.<sup>35,47</sup> Therefore, the detected electrochemical signals can be ascribed to adsorbed Cyt-c which largely preserves the native structure, particularly at the level of the redox site. It is important to point out that the absence of other electrochemical signals does not rule out the

presence of alternative Cyt-c conformations in the adsorbed state that might be kinetically impaired. Indeed, the larger peak separations observed for PO<sub>4</sub>-SAM compared to the other SAMs is indicative of a much slower interfacial electron transfer reaction of Cyt-c in this case, which is compatible with a less favorable and more rigid orientation of the protein in such complexes.<sup>48</sup>

Upon addition of H<sub>2</sub>O<sub>2</sub> to Cyt-c adsorbed on NH<sub>2</sub>-SAMs we observe the appearance of a second mono-electronic and quasi-reversible voltammetric signal with an apparent reduction potential of -119 mV (Fig. 1). The anodic and cathodic peak currents of the new redox active species rise at the expenses of the corresponding signals of native Cyt-c, with a complete transformation at H<sub>2</sub>O<sub>2</sub> concentrations slightly above 1 mM. Interestingly, the new redox couple presents enhanced cathodic currents in the presence of H<sub>2</sub>O<sub>2</sub>, thus indicating the electrocatalytic reduction of H<sub>2</sub>O<sub>2</sub> by the modified protein (pseudoperoxidase activity).

The downshift of the reduction potential of Cyt-c after treatment with H<sub>2</sub>O<sub>2</sub> may in principle indicate a more polar heme environment as a consequence of increased solvent accessibility.<sup>49</sup> However, the addition of several complexing agents such as imidazole, azide, DTPA and methionine has no effect on the electrochemical response (Fig. S2), thus suggesting that the solvent exposure of the heme crevice is not significantly altered.

Replacement of the electrolyte by fresh phosphate buffer once the H<sub>2</sub>O<sub>2</sub>-induced transformation is completed results in stable voltammograms that retain the electrochemical features of the newly generated redox couple (Fig. S3). These results suggest a permanent chemical modification of the adsorbed Cyt-c into a stable species that presents pseudoperoxidase activity and, potentially, peroxidase-like characteristics. Remarkably, this behavior is only observed when Cyt-c is adsorbed on NH<sub>2</sub>- and TA-SAMs in the presence of phosphate buffer (Figs. 1A and S5A). In contrast, when the protein is directly adsorbed on negatively charged COO<sup>-</sup> and PO<sub>4</sub>-SAMs the addition of H<sub>2</sub>O<sub>2</sub> produces a slight decrease of the voltammetric peaks of native Cyt-c, but this partial bleaching is not accompanied by the appearance of new CV signals (Fig. S6). Moreover, pretreatment of Cyt-c solutions with different concentrations of H<sub>2</sub>O<sub>2</sub> (between 1 and 10 mM) previous to incubation with electrodes coated with NH<sub>2</sub>-SAMs leads exclusively to weak voltammetric signals at the potentials expected for native Cyt-c, but there is no indication of other redox active species (Fig. 2B).

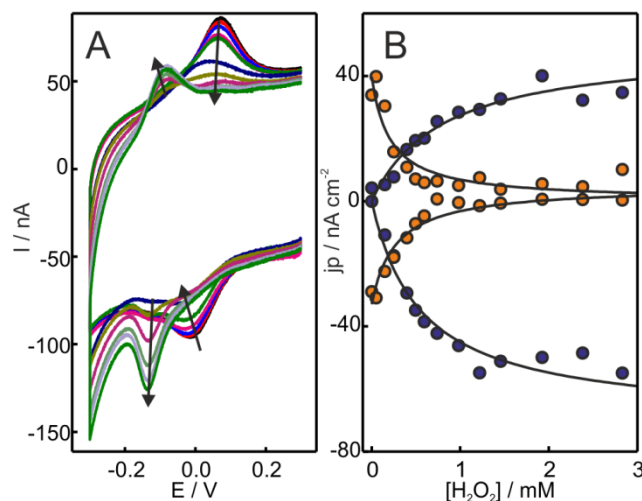


Fig. 1 (A) Voltammetric response of Cyt-c adsorbed on electrodes coated with NH<sub>2</sub>-SAMs. The measurements were performed at 0.05 V s<sup>-1</sup> in 10 mM phosphate buffer, pH = 7.0, in the presence of increasing concentrations of H<sub>2</sub>O<sub>2</sub> from 0 to 500 μM. (B) Background corrected peak current densities for the native Cyt-c redox couple (orange circles) and the newly generated couple (blue circles) as a function of H<sub>2</sub>O<sub>2</sub> concentration. The solid lines are only intended to guide the eye.

Based on these results we investigated the effect of unilamellar vesicles of PC and mixtures of PC/PE. Typically, H<sub>2</sub>O<sub>2</sub> was added up to 1 mM concentrations to solutions containing 200 μM Cyt-c and 0.5 mM PC (or PC/PE). After this treatment the Cyt-c solutions were thoroughly washed using centrifugal filters and the supernatant was incubated with electrodes coated with NH<sub>2</sub>-SAMs in phosphate buffer. Similar to the case of in-situ treatment of Cyt-c adsorbed on NH<sub>2</sub>-SAMs, the modified electrodes exhibit a CV signal with  $E_{1/2} = -119$  mV that also presents enhanced cathodic currents in the presence of H<sub>2</sub>O<sub>2</sub> (Figs. 2C and S7). Therefore, the H<sub>2</sub>O<sub>2</sub>-induced chemical modification that leads to a gain of pseudoperoxidatic (and possibly peroxidatic) activity appears to be a general feature of Cyt-c molecules adsorbed on positively charged and zwitterionic membrane models via mediating phosphate anions.

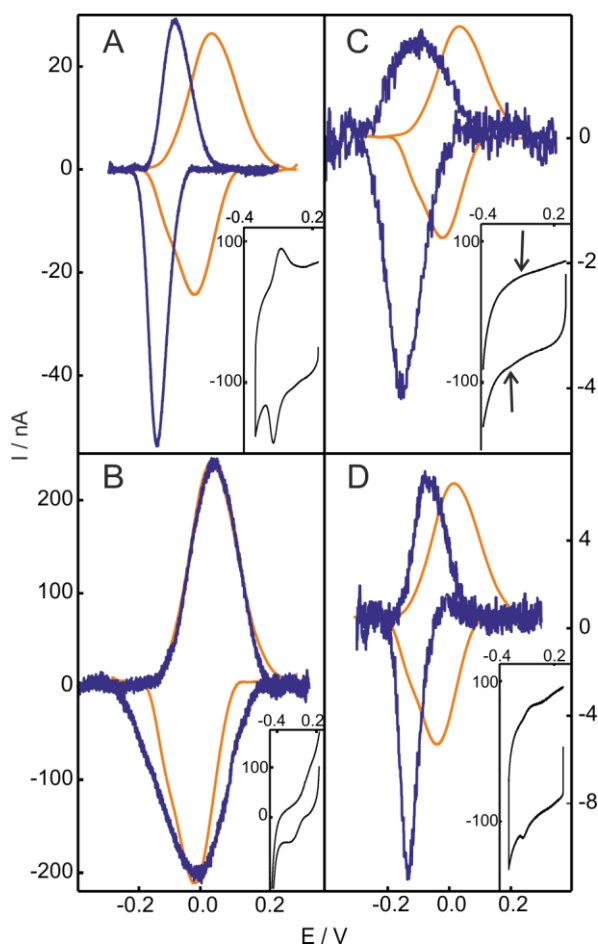


Fig. 2 Baseline subtracted cyclic voltammograms of Cyt-c before (orange) and after (blue) chemical treatment. (A) Cyt-c adsorbed on a  $\text{NH}_2$ -SAM electrode treated with 2 mM  $\text{H}_2\text{O}_2$ . (B) Cyt-c treated pretreated in solution with  $\text{H}_2\text{O}_2$  2 mM and subsequently adsorbed on a  $\text{NH}_2$ -SAM electrode. (C) Cyt-c treated pretreated in solution with  $\text{H}_2\text{O}_2$  2 mM in the presence of PE/PC liposomes, and subsequently adsorbed on a  $\text{NH}_2$ -SAM electrode. (D) Ex-situ generated and purified SO-Cyt adsorbed on a  $\text{NH}_2$ -SAM electrode. All the measurements were performed at 0.1 V s<sup>-1</sup> in 10 mM buffer phosphate pH = 7.0. The insets are the voltammograms signals before background correction.

### Characterization of the catalytically competent species.

The structure of Cyt-c in the different biomimetic complexes was investigated by RR and SERR, as these spectroscopic techniques are able to monitor the redox state, spin and coordination pattern of the heme iron, as well as heme-protein interactions.<sup>40,48,50</sup> In agreement with previous reports, SERR spectra of Cyt-c adsorbed on  $\text{COOH}$ -SAMs are identical to the corresponding RR spectra of the native protein in solution (Fig. S8).<sup>50</sup> Moreover, in agreement with the electrochemical results, the spectra remain unchanged upon addition of moderate amounts of  $\text{H}_2\text{O}_2$  (ca. 4 mM). The SERR spectra of Cyt-c adsorbed on  $\text{PO}_4$ -SAMs are also largely insensitive to the addition of similar amounts of  $\text{H}_2\text{O}_2$  but in this case they reveal, in addition to the native protein, a ca. 30 % spectral contribution of a second species consistent with the replacement of the heme axial ligand Met80 by a histidine residue (Fig. S9A).<sup>50</sup> A similar contribution of *bis*-His species is also observed for Cyt-c adsorbed on  $\text{NH}_2$ -SAMs (Fig. S9B).

The assignment of the *bis*-His species is based on the spectral parameters of the high frequency region shown in Table S2 and in the appearance of a band at 405 cm<sup>-1</sup> assigned to the asymmetric stretching of the heme Fe bound to two imidazolic axial ligands. Upon treatment of the Cyt-c/ $\text{NH}_2$ -SAM complexes with 1 mM  $\text{H}_2\text{O}_2$  we detect the appearance of a new spectroscopic component that resembles the RR spectrum of the ferric M80A mutant and, therefore, is compatible with a  $\text{OH}^-$ /His coordination pattern (Figs. 3 and S10).<sup>51</sup> The rise of the  $\text{OH}^-$ /His spectral component is concomitant with the appearance of the pseudoperoxidatic redox couple and occurs at the expenses of the native spectral component without significant alteration of the *bis*-His contribution (Fig. S10). Nevertheless, within the error of the quantification, we cannot discard that the  $\text{OH}^-$ /His species actually originates from the reaction of  $\text{H}_2\text{O}_2$  with the *bis*-His species, which is in equilibrium with the main native component.

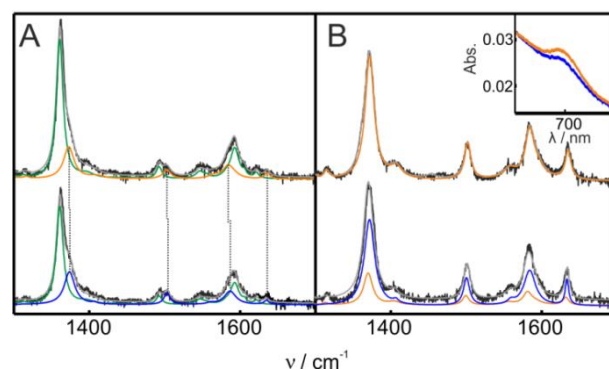


Fig. 3 (A) SERR spectra of Cyt-c adsorbed on an Ag electrode coated with  $\text{NH}_2$ -SAM recorded before (top) and after (bottom) the addition of 1 mM  $\text{H}_2\text{O}_2$  final concentration. The spectra were acquired while cycling the electrode potential between 0.1 V and -0.3 V at 0.05 V s<sup>-1</sup> in 10 mM phosphate buffer, pH 7. The colors indicate the different spectral components. Green: ferrous native Cyt-c. Orange: ferric native Cyt-c. Blue:  $\text{OH}^-$ /His ferric component. (B) RR spectra of 0.2 mM ferric Cyt-c in 10 mM phosphate buffer, pH 7, containing 0.5 mM PC/PE liposomes. The spectra were recorded before (top) and after (bottom) the addition of 1 mM  $\text{H}_2\text{O}_2$  final concentration. The color code of the RR spectral components is the used for SERR in panel (A). The inset shows the UV-Vis charge transfer band before (orange) and after (blue) the addition of  $\text{H}_2\text{O}_2$ .

The fact that the adsorbed Cyt-c is sensitive to  $\text{H}_2\text{O}_2$  only on amino-terminated SAMs suggests the involvement of hydroxyl radical species ( $\cdot\text{OH}$ ) that may be generated by the reaction of the amine groups of the SAMs with  $\text{H}_2\text{O}_2$ .<sup>52</sup> To test this possibility we performed the  $\text{H}_2\text{O}_2$  activation of Cyt-c/ $\text{NH}_2$ -SAM complexes in the presence of DMSO; a very efficient scavenger of  $\cdot\text{OH}$ .<sup>53-55</sup> The results were identical to those obtained in the absence of DMSO, thereby indicating that, most likely,  $\cdot\text{OH}$  is not involved in the formation of the pseudoperoxidatic  $\text{OH}^-$ /His species.

In contrast to native Cyt-c, the reduction potential of the  $\text{OH}^-$ /His species adsorbed on  $\text{NH}_2$ -SAMs exhibits a strong linear pH dependence (Fig. 4A), with a slope of -0.057 V per pH unit that is consistent with the uptake of one proton per incoming electron. For the M80A mutant of Cyt-c cross-linked to electrodes coated with  $\text{COOH}$ -terminated SAMs we obtain an almost identical pH-dependence (Fig. 4B). Moreover, the slope

is also very similar to the one reported for horse radish peroxidase,<sup>56</sup> thus further suggesting close similarities of this species with the “professional” peroxidases.

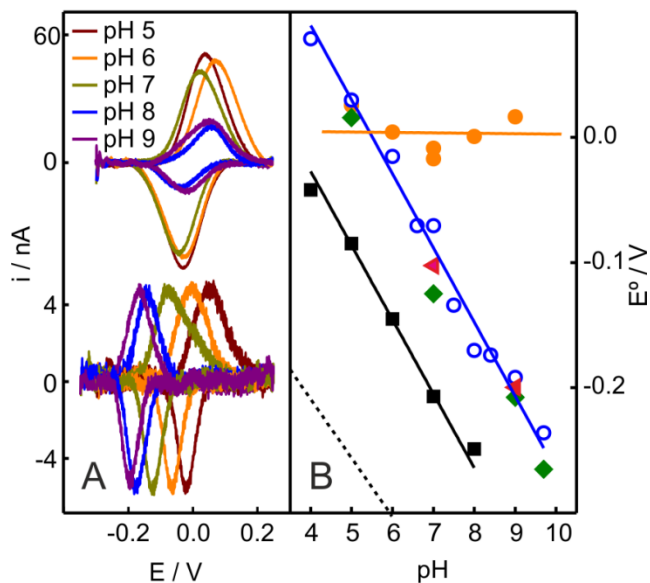


Fig. 4 (A) Voltammetric signals of Cyt-c adsorbed on  $\text{NH}_2\text{-SAM}$  recorded at different pH values before (top) and after (bottom) the addition of 2 mM final concentration of  $\text{H}_2\text{O}_2$ . (B) pH-dependencies of the reduction potentials of different protein species adsorbed on  $\text{NH}_2\text{-SAM}$  electrodes. Orange circles: native Cyt-c. Blue circles: Cyt-c adsorbed on the same electrode after in-situ pretreatment with 1 mM  $\text{H}_2\text{O}_2$ . Green diamonds: Cyt-c pretreated ex-situ with 1 mM  $\text{H}_2\text{O}_2$  in the presence of 5 mM PE liposomes. Red triangles: ex-situ synthesized and purified SO-Cyt. Additionally, the black squares correspond to the M80A Cyt-c mutant cross-linked to a COO-SAM (this work) and the dotted line corresponds to HRP on GCE adopted from reference<sup>56</sup>.

The treatment of Cyt-c/liposome complexes with  $\text{H}_2\text{O}_2$  yields results very similar to the Cyt-c/ $\text{NH}_2\text{-SAM}$  complexes. On the one hand, the RR spectra of the treated Cyt-c/liposome systems are largely dominated by the same spectral component assigned to a ferric OH<sup>-</sup>/His species and, moreover, this spectral change is paralleled by a decrease of the characteristic MetS-Fe charge transfer absorption band at 695 nm (Fig. 3B). On the other, the reduction potentials of the Cyt-c products generated by  $\text{H}_2\text{O}_2$  treatment in both types of complexes exhibit identical values and pH dependencies (Fig. 4B).

Hence, the spectroscopic and electrochemical results point out to the same chemical modification of Cyt-c in the different phosphate-mediated biomimetic complexes that lead to the detachment of the native axial ligand Met80 and the concomitant gain of catalytic activity.

A potential target for the reaction with  $\text{H}_2\text{O}_2$  is the sulfur atom of the Met80 residue, as shown for methionines in general with a variety of oxidant species.<sup>42,57–59</sup> Therefore, we treated Cyt-c with chloramine-T, which has been described as a selective reagent to oxidized Met80 and generate the corresponding sulfoxide species (SO-Cyt).<sup>42,43</sup> The product was purified by high-performance liquid chromatography and characterized by mass spectrometry-based peptide mapping (see ESI for further details).

The purified SO-Cyt exhibits identical RR spectrum to the  $\text{H}_2\text{O}_2$ -generated OH<sup>-</sup>/His species obtained in the biomimetic complexes (Fig. S13). Moreover, as shown in Figs. 2D and 4B, when the ex-situ generated SO-Cyt is adsorbed on electrodes coated with  $\text{NH}_2\text{-SAM}$ s it exhibits nearly identical voltammetric responses and pH dependencies as the in-situ generated OH<sup>-</sup>/His species.

The CV experiments reveal very similar heterogeneous electron transfer rate constants for native Cyt-c and SO-Cyt at all pH values (Table S1), but a large downshift of the reduction potential of SO-Cyt at physiological pH. Thus, the loss of electron shuttling ability of Cyt-c upon Met80 oxidation is essentially a thermodynamic effect.

In summary, the presented results constitute convincing evidence that submillimolar concentrations of  $\text{H}_2\text{O}_2$  are able to efficiently and selectively oxidize the axial M80 ligand of Cyt-c in phosphate-mediated complexes with the main lipid components of the mitochondrial membrane. The oxidized product SO-Cyt is stable and undergoes a coordination and conformational transition that leads to a gain of pseudoperoxidatic activity. The remaining question of whether this transformation also results in a significant gain of truly peroxidatic activity is addressed next.

#### Peroxidatic and pseudo-peroxidatic activity.

The peroxidase-like activity of in-situ generated SO-Cyt was evaluated following different strategies depending on whether it was obtained on  $\text{NH}_2\text{-SAM}$ s or on PC/PE liposomes. Both peroxidase and pseudo-peroxidase activities refer to the ability of catalyzing the reduction of  $\text{H}_2\text{O}_2$  to water. In the first case, electrons are supplied by a molecule (substrate) in solution, which then is oxidized, and homogeneous reaction rates are measured. In contrast, by pseudoperoxidase activity we refer to the electrocatalytic reduction of  $\text{H}_2\text{O}_2$  by the immobilized protein using the heterogeneous electron transfer reaction from the metal electrode to the protein as electron source. The resulting currents measured by CV in the presence of increasing concentrations of  $\text{H}_2\text{O}_2$  are a measure of the reaction rate. As shown in Fig. 5, the addition of  $\text{H}_2\text{O}_2$  results in enhanced cathodic currents, without significant alteration of other parameters such as peak potentials or FWHMs. This type of electrocatalytic behavior has been previously reported for the M80A mutant of yeast iso-cytochrome *c*, and was interpreted in terms of a mechanism that involves  $\text{O}_2$  generation and its subsequent reduction.<sup>60</sup> On the other hand, for the same mutant of the mammalian protein Águila and coworkers showed some evidence of the formation of [Fe(III)OOH] (compound 0) in the reaction with  $\text{H}_2\text{O}_2$ , which then would evolve to ferryl species (compounds I and II).<sup>61</sup> Assuming that this mechanism is correct, and considering that the reduction potential of the Fe(IV)=O intermediate is significantly more positive than for the other species<sup>62</sup>, we can quantitatively analyze the cathodic peak currents in Fig. 5 in terms of a Michaelis-Menten formalism (see ESI for further details).

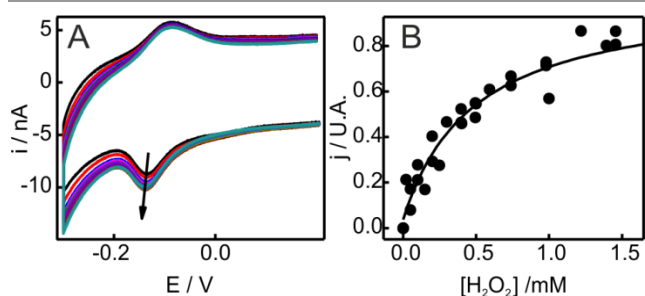


Fig. 5 (A) Voltammetric response as a function of  $\text{H}_2\text{O}_2$  concentration of SO-Cyt generated in-situ upon pretreatment with  $\text{H}_2\text{O}_2$  of Cyt-c adsorbed on an  $\text{NH}_2$ -SAM electrode. All measurements were performed in 10 mM phosphate buffer, pH 7, at  $0.05 \text{ V s}^{-1}$ . The arrow indicates increasing concentrations of  $\text{H}_2\text{O}_2$  from 0 to 2 mM. (B) Normalized cathodic peak current densities obtained from panel (A) as a function of  $\text{H}_2\text{O}_2$  concentration. Line corresponds to fit to Michaelis-Menten-like equation (see ESI).

Table I. Pseudoperoxidase and peroxidase activities of the different Cyt-c variants.

	Activity	$K_m / \text{mM}$	$k_{\text{cat}} / \text{s}^{-1}$	% of control
WT	Pseudo-peroxidase	$144.3^b$	$8^b$	-
	Peroxidase	$65^c$	$5.5^c$	100
SO-Cyt ex-situ	Pseudo-peroxidase <sup>a</sup>	0.24 (0.08)	2.6 (0.8)	-
	Peroxidase	$19^d$	-	719
SO-Cyt in-situ	Pseudo-peroxidase <sup>a</sup>	$0.44^e$ (0.07)	$1.8^e$ (0.6)	-
	Peroxidase	-	-	$644^h$
M80A	Pseudo-peroxidase	$0.00325^f$	-	-
	Peroxidase	$23.2^g$	$72^g$	780

Standard deviations are indicated in brackets. <sup>a</sup>Determined for Cyt-c adsorbed on  $\text{NH}_2$ -SAMs, this work. <sup>b</sup>Cyt-c adsorbed on COO-SAMs, from reference <sup>45</sup>. <sup>c</sup>Cyt-c in solution with ABTS, from reference <sup>63</sup>. <sup>d</sup>SO-Cyt in solution with ABTS, from reference <sup>64</sup>. <sup>e</sup>SO-Cyt obtained in-situ on  $\text{NH}_2$ -SAMs, this work. <sup>f</sup>From reference <sup>60</sup>. <sup>g</sup>Iso-cyt M80A mutant, from reference <sup>65</sup>. <sup>h</sup>SO-Cyt obtained by pretreatment with  $\text{H}_2\text{O}_2$  in the presence of PC/PE liposomes.

The catalytic parameters obtained through this analysis are summarized in Table 1 and Figure 6. While the  $k_{\text{cat}}$  values obtained for in-situ and ex-situ generated SO-Cyt are similar to native Cyt-c, the oxidation of M80 results in a ca. 300-600 increase of affinity towards  $\text{H}_2\text{O}_2$ . As a reference, the value of  $K_m$  reported for horse radish peroxidase on glassy carbon electrodes is ca.  $53 \mu\text{M}$  <sup>56</sup>, while for the M80A mutant of iso-cytochrome *c* it is only  $3.25 \mu\text{M}$ , although this unusually high affinity is accompanied by protein bleaching at  $\text{H}_2\text{O}_2$  concentrations about  $4 \mu\text{M}$ . <sup>60</sup>

The peroxidatic activity of the different species in solution was assessed fluorometrically employing Amplex UltraRed reagent. As summarized in Table 1 and Figure 6, both the oxidation of Met80 (SO-Cyt) as well as its replacement by non-coordinating alanine (M80A) result in increased peroxidatic activity with respect to native Cyt-c by almost 1 order of magnitude, in agreement with previous observations.<sup>39,65</sup> The effect is quantitatively similar for purified SO-Cyt generated ex-situ and for Cyt-c treated with  $\text{H}_2\text{O}_2$  in the presence of PE/PC

liposomes. In spite of the bleaching effect, the treatment of a Cyt-c solution with only  $\text{H}_2\text{O}_2$  has no significant effect on the measured peroxidatic activity (Fig. 6). Similarly, the mere presence of PE/PC liposomes has no effect unless  $\text{H}_2\text{O}_2$  is added, thus confirming that the  $\text{H}_2\text{O}_2$ -induced gain of peroxidatic activity is selective for Cyt-c molecules adsorbed on zwitterionic lipids via mediating phosphate anions.

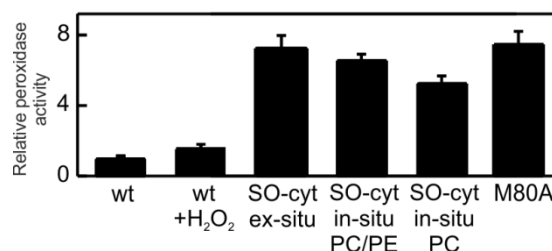


Fig. 6 Peroxidase activities of the different Cyt-c variants relative WT Cyt-c, as determined fluorometrically employing Amplex UltraRed reagent.

## 4. Conclusions

Cyt-c is able to interact electrostatically with the main lipid components of the mitochondrial membranes, the zwitterionic lipids PE and PC, in the presence of biologically relevant amounts of mediating phosphate anions. Under these conditions the adsorbed protein reacts efficiently with  $\text{H}_2\text{O}_2$ . The reaction does not lead to the bleaching of the heme group, but is restricted to sulfur oxidation of the iron axial ligand Met80, thus leading to its detachment and to a concomitant increase of the peroxidatic activity by one order of magnitude.

There is no evidence, though, that PE and PC lipids are peroxidated by Cyt-c to significant amounts.<sup>10,23</sup> Cardiolipin, in contrast, is effectively peroxidated by Cyt-c during the early events of apoptosis, thus leading to membrane permeabilization. The gain of peroxidatic activity has been ascribed to specific Cyt-c/CL interactions. The present findings, on the other hand, suggest a possible additional pathway for membrane permeabilization. Specifically, the rise of  $\text{H}_2\text{O}_2$  concentration to submillimolar levels that characterizes the initiation of apoptosis<sup>37,38</sup> appears to be sufficient to chemically modify a fraction of the Cyt-c molecules that interact (via phosphate anions) with the PE and PC membrane components. The modified protein is a stable peroxidase that could later bind and catalyze the oxidation of membrane components such as CL, thus facilitating the liberation of pro-apoptotic factors, including unmodified Cyt-c.

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## Notes and references



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