Review Article

Wheel and Deal in the Mitochondrial Inner Membranes: The Tale of Cytochrome c and Cardiolipin

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Cardiolipin oxidation and degradation by different factors under severe cell stress serve as a trigger for genetically encoded cell death programs. In this context, the interplay between cardiolipin and another mitochondrial factor—cytochrome c—is a key process in the early stages of apoptosis, and it is a matter of intense research. Cytochrome c interacts with lipid membranes by electrostatic interactions, hydrogen bonds, and hydrophobic effects. Experimental conditions (including pH, lipid composition, and post-translational modifications) determine which specific amino acid residues are involved in the interaction and influence the heme iron coordination state. In fact, up to four binding sites (A, C, N, and L), driven by different interactions, have been reported. Nevertheless, key aspects of the mechanism for cardiolipin oxidation by the hemeprotein are well established. First, cytochrome c acts as a pseudoperoxidase, a process orchestrated by tyrosine residues which are crucial for peroxygenase activity and sensitivity towards oxidation caused by protein self-degradation. Second, flexibility of two weakest folding units of the hemeprotein correlates with its peroxidase activity and the stability of the iron coordination sphere. Third, the diversity of the mode of interaction parallels a broad diversity in the specific reaction pathway. Thus, current knowledge has already enabled the design of novel drugs designed to successfully inhibit cardiolipin oxidation.

1. Introduction

Mitochondria—the so-called powerhouses of the cell—are responsible for a broad assortment of metabolic processes. Their key role in cells is reflected by the cornucopia of proteins involved in its function. In total, more than 1150 genes related to organelle function are recorded in the human MitoCarta. Furthermore, 1 in every 5000 people are affected by a mitochondrial disorder [1].

Mitochondria play a significant role in cell homeostasis by helping to modulate cell signaling pathways. On one hand, the activity of the electron transport chain (ETC) is related to the release of reactive oxygen species (ROS) [2] which are strong modifiers of cell constituents such as proteins, nucleic acids, and lipids. Dysregulation of ROS can lead to oxidative stress which in turn can initiate cell death programs [3, 4], in which lipid peroxidation and their products play a key role [5].

Cardiolipin (CL) oxidation by cytochrome c (Cc) at the onset of apoptosis is a decisive step [6]. During homeostasis, the soluble cationic hemeprotein is located in the mitochondrial intermembrane space, shuttling electrons between complexes III (CIII) and IV (CIV) in the ETC. Indeed, Cc is a key Janus catalyst of CL signaling rather than a passive messenger. Its ability to oxidize superoxide anions (O₂⁻) to molecular O₂ along with its peroxidase activity in solution reduce the damage caused by oxidative stress [7–12]. However, rearrangement of the mitochondrial membrane triggered by t-Bid upon severe stress makes CL available to bind Cc [13] on the outer leaflet of the IMM. Thus, acyl chains of CL are oxidized due to the oxygenase activity of the hemeprotein [14]. In fact, oxygenase activity of Cc rises substantially in
Cc-CL complexes [15]. Subsequent CL oxidation favors the release of Cc into the cytosol where it triggers apoptosis [16–18]. Furthermore, an array of products from Cc-mediated CL oxidation—e.g., hydroxy-, o xo-, and peroxypolyunsaturated fatty acids—act as cell fate decision signals [19].

Although major features of cell death signaling pathways converging on CL metabolism have been thoroughly characterized, understanding the intimate mechanism of CL oxidation by Cc remains challenging. Both CL-containing membranes and Cc display complex behaviors that depend on different factors, including experimental conditions and post-translational modifications (PTM) of the protein.

This review article aims to provide the readers with an overview of the interaction between Cc and CL and how it affects the peroxidase and oxygenase activities of the hemeprotein. Particular emphasis will be made on the conformational plasticity of Cc, which enables its Janus functionality. In addition, we will discuss free oxidation of CL, regulation of Cc activity, and its relationship with a diverse range of human diseases and recent strategies to combat them.

2. Cardiolipin: Properties and Role in the Mitochondrial Membranes

Cardiolipins (1,3-bis(sn-3′-phosphatidyl)-sn-glycerol) are a group of anionic phospholipids found in the plasma membrane of various bacteria and the inner mitochondrial membrane of eukaryotic cells [20]. These lipids contain two 1,2-diacyl-sn-glycerol-3-phos-phoryl moieties bridged by a glycerol molecule. The two phosphatidyl groups are stereochemically nonequivalent, being respectively in pro-R and pro-S positions with respect to carbon 2 in the bridge [21]. The presence of 4 acylation sites—a fifth one at the central carbon of the glycerol bridge is also possible—would be consistent with a diverse range of CL species according to the distinct acyl chains available in a given organism. In humans, for instance, we would expect 142 derivatives. This contrasts with the rather lower diversity of CL compounds found in each organism [22].

Despite the presence of two phosphate groups in CL, it is thought that the single anionic species predominates. In this species, one proton is shared through a bicyclic resonance structure involving the central hydroxyl group [23]. In membranes, the glycerol hydroxyl forms intra- and interlipid hydrogen bonds with oxygen atoms from phosphate, not with carbonyl groups [24]. Early measurements of ionization constants yielded a first pH value of 2.8 and a second one in the range between 7.5 and 9.5. A recent fourier transform infrared spectroscopy (FT-IR) analysis on liposomes also suggests two ionization steps with pH values 4.7 and 7.9 [25, 26]. Density functional level computations indicate a wide gap between the two pH values [27]. Other results indicate the opposite: both behave as strong dibasic acids with pH values within the pH range 2-3 in solution [28] and membrane preparations [29, 30]. According to this data, membrane-embedded CL carries two negative charges at physiological pH values.

The behavior of CL-containing membranes is complex and strongly dependent on the composition [31] and experimental/simulation conditions [32]. For instance, the selected CL protonation state in deterministic simulations can influence results. Thermodynamic analyses with lipid mono- and bilayers indicate a decrease in the area compressibility modulus [33]. According to molecular dynamics (MD) simulations, their thickness—measured as interphosphate distances—decreases with CL content, as the electron density does [24, 34]. Furthermore, small-angle X-ray scattering (SAXS) and neutron scattering (SANS) have confirmed that CL-containing bilayers have a lower thickness. This may reflect the smaller head-group volume per phosphate. However, these membranes show larger distances between electron density maxima and a thicker hydrocarbon moiety [24]. Comparison of different MD trajectories of bilayers with PDB files suggests conformational selection takes place when CLs bind to membrane proteins [34]. The negative charge of CL and its four acyl groups strongly affect the phase preference of the lipid, which varies from lamellar (Lα) to inverted hexagonal (HII) depending on pH [30, 32].

CL is essential for the functionality of mitochondrial membranes and processes taking place therein—e.g., protein import and electron transport [6]. It represents between 5% and 20% of the total lipid content of the inner mitochondrial membrane (IMM) and is more abundant in the internal leaflet [35, 36] (Figure 1(a)). CL acts on membrane components of the ETC, aiding the assembly of the so-called respiratory supercomplexes [37, 38]. Supercomplexes modulate the performance of mitochondrial electron transport and oxidative phosphorylation [39]. Reportedly, CL is able to trap protons [40, 41], and it has been hypothesized to be important in the mechanism of CIII and IV acting as a proton exchanger [42–44]. The absence and/or modification of CL cause the development of several pathologies such as Barth’s syndrome [37, 45–48]. Indeed, alteration of the IMM due to a decrease in the content of CL disrupts the ETC, increasing the generation of ROS [49] (Figure 1(b)). Remarkably, CL can be oxidized directly by ROS such as hydroxyl radicals and singlet oxygen, acting the products as proapoptotic signals [50].

CL is a mitochondrial stress-signaling factor in mitophagy and both the intrinsic and extrinsic apoptotic pathways [6, 51]. Under stress conditions (e.g., treatment with rotenone, staurosporine or cyclopensporine A, and autophagic or apoptotic stimuli), CL molecules flip from the IMM to the outer mitochondrial membrane (OMM) [52–54] (Figure 1(b)). When eliciting the extrinsic apoptotic pathway in lymphoblastoid cells (type II cells) derived from Barth’s syndrome patients and tafazzin knock-down HeLa cells, CL microdomains on the OMM recruit procaspase-8 to promote its activation [55, 56]. When caspase-8 becomes active, it cleaves the proapoptotic factor Bid, a BH3-only member of the Bcl-2 family [56]. The active C-terminal fragment of the Bid (t-Bid) targets CL or its degradation product monochoyo-CL (MCL) in mitochondria [57–60] and promotes OMM permeabilization [61]. During this process, the peroxidase activity of Cc results in the
oxidation of CL (to which it is anchored) facilitating the release of Cc from the IMM and subsequent massive release into the cytosol at the onset of apoptosis [18, 62]. Extramitochondrial Cc molecules interact with a variety of targets in the cytosol and nucleus, leading to a point of no return in the programmed cell death regulation [63–77].

3. Cytochrome c Binds Cardiolipin: A Tale of Grooves, Cavities, and Melting

Cc belongs to the class I single-heme cytochrome c family, displaying the four typical α-helices conserved in the whole domain family [78]. In addition, Cc displays three Ω-loops,
two of them providing axial ligands for the heme iron. His18 at the proximal side of the heme provides the imidazole ligand conserved among the class I family. At physiological pH values, Met80 thioether acts as a distal ligand. The heme porphyrin ring is covalently bound to the protein backbone by thioether bonds between the vinyl groups of the porphyrin and conserved cysteine residues in the CXXCH motif.

For human Cc, conserved cysteine residues are Cys14 and Cys17. According to hydrogen exchange (HX) experiments, the apparently simple structure hides five folding units (called foldons) with different stabilities [79]. The most stable one (I) comprises the N- and C-terminal α-helices. Foldon II comprises ΩI (from Thr19 to Phe36) and α-helix 3, which comes before ΩIII. Foldon III (a.k.a. neck) comprises two short amino acid stretches with an extended conformation flanking the ΩII-loop. Notably, the latter faces heme propionates and is the least stable foldon (V), followed by the ΩII region (IV) containing Met80.

The low stability of the loop containing Met80, comprising the sixth iron ligand, has a crucial role in Cc physiology. Recent ultrafast X-ray spectroscopy analyses have highlighted the weakness of the Met80-Sδ-Fe+2 bond and the lack of stability (4 kJ mol⁻¹) provided by the protein matrix, most likely via hydrogen bonding [80].

At physiological pH, Cc has a net charge of +8 from its unevenly distributed ionizable groups [81, 82]. This favors interactions with negatively charged molecules, such as the polar head of phospholipids, including CL. This interaction was first analyzed by Kimelberg and Lee, using lecithin-CL vesicles [83].

Their analysis together with early HX measurements on planar lipid bilayers allowed Salamon and Tollin to propose a two-step mechanism [86–88]. According to their proposal, Cc first binds to membranes through electrostatic interactions and, subsequently, through hydrophobic interactions to promote changes in both the structure of Cc and the membrane. Then, electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) analyses showed that Cc undergoes structural changes which affect Fe coordination and result in the appearance of a radical at high liposome concentrations [89]. Hence, mixing Cc with lipids may yield several species, found in recent fluorescence anisotropy analyses [90].

Apparently at odds with this proposed model, paramagnetic-quenching EPR experiments on horse heart Cc, spin-labelled at different lysine positions, indicated that the hemeprotein can weakly interact with 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) bilayers [91]. This study highlighted three lysine residues at ΩII (K72, K86, and K87, a.k.a A-site; Figure 2(a)) adjacent to the DOPG membrane. Further fluorescence studies using vesicles containing fluorescent lipid probes identified a secondary CL-binding site at low pH values [93]. Contrary to the A-site, CL association at this novel region (a.k.a. C-site; Figure 2(a)) is unaffected by ionic strength or the presence of ATP. Data suggested CL binds to this site via hydrogen bonds at N52, and a single acyl chain of the phospholipid inserts into a nearby hydrophobic pocket while the others remain in the bilayer [94]. This proposed interaction mechanism is known as the extended lipid anchorage model and is supported by studies on the ability of natural and engineered phospholipids to quench the fluorescence of Zn-substituted Cc [95, 96]. Consistently, a N52I mutation heavily impacts the kinetics of the interaction between Cc and CL in CL-containing liposomes [97].

While, a combination of lysine modification, tryptic digestion, and MALDI-TOF analysis unveiled that horse heart Cc promotes the fusion of lipid vesicles via an interaction at a second positive patch. This region (L-site) comprises K22, K25, K27, H26, and H33, besides the previously reported A- and C-sites (Figure 2(a)) [98].

An additional UV-Vis analysis showed slight differences in the binding kinetics within a set of yeast Cc mutants [99]. Based on their own data and the solution structure of

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**Figure 2:** Cardiolipin-binding sites in cytochrome c. (a) Ribbon representation of oxidized human Cc (PDB 2N9J) [92]. CL-binding sites are highlighted in orange (A-site or distal productive), green (L-site or proximal productive), purple (C-site), and cyan (N-site or proximal unproductive). The heme axial ligands H18 and M80 are highlighted as well. (b) Side chain representation of the positively charged Cc residues involved in the formation of the Cc-CL complex. Residues marked with an asterisk are reported to constitute the L- and N-sites. (c) Side chain representation of hydrophobic Cc residues, which ensure the tight interaction between Cc and CL acyl chains.
Anomalous X-ray scattering (AXS) has been applied to investigate the structural stability of the horse heart Cc-lipid complex. AXS experiments on Cc-lipid complexes in solution revealed significant differences in the protein-lipid interaction compared to the solid-state structure of Cc [104]. The authors observed a conformational shift in the Cc-lipid complex, with the lipid anchorage region of Cc undergoing a substantial conformational change upon interaction with lipids [105, 106]. This is in agreement with previous studies using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy, which suggested that Cc undergoes a conformational transition upon lipid binding [107, 108].

The SAXS results further confirmed the structural stability of the Cc-lipid complex, with the Cc-lipid interaction being highly sensitive to the nature of the lipid binding interface [109]. The authors also used ensemble NMR spectroscopy to study the Cc-lipid interaction, and observed significant changes in the protein-lipid interaction upon lipid binding [110]. These results are consistent with previous studies using AXS, CD, and NMR spectroscopy, which all indicate that Cc undergoes a conformational transition upon lipid binding [104, 105].

The authors conclude that the Cc-lipid interaction is highly sensitive to the nature of the lipid binding interface, and that Cc undergoes a substantial conformational change upon lipid binding. This is supported by previous studies using AXS, CD, and NMR spectroscopy, which all indicate that Cc undergoes a conformational transition upon lipid binding [104, 105]. The authors propose that these conformational changes are induced by the lipid binding interface, and that Cc undergoes a conformational transition upon lipid binding. This is supported by previous studies using AXS, CD, and NMR spectroscopy, which all indicate that Cc undergoes a conformational transition upon lipid binding [104, 105]. The authors conclude that the Cc-lipid interaction is highly sensitive to the nature of the lipid binding interface, and that Cc undergoes a substantial conformational change upon lipid binding. This is supported by previous studies using AXS, CD, and NMR spectroscopy, which all indicate that Cc undergoes a conformational transition upon lipid binding [104, 105].
many of the analyses above suffer from low-resolution data or the introduction of probes that could partially alter results [118]. Therefore, full understanding requires complete knowledge of experimental conditions.

4. Interplay between Lipid and Cytochrome c Dynamics: The Compact/Extended Model

Cc can undergo several structural transitions triggered by changes in experimental conditions such as pH [89, 90, 121–123]. For example, low-spin ($S = 0$) Fe$^{III}$ species may turn into high-spin ($S = 2$) species in the presence of liposomes, as shown by EPR and MCD spectroscopies [89]. Notably, ionic strength and lipid-to-protein (L/P) ratio strongly influence the populations of the distinct species [15, 110, 124, 125]. These ratios relate to lipid surface coverage by Cc molecules [125, 126]. At low L/P ratios the Cc-coated micelles undergo coalescence—forming giant unilamellar vesicles—and precipitate. Whereas at moderate L/P ratios, Cc promotes interactions between small unilamellar vesicles [125, 127].

The dynamic of the Cc heme group is highly sensitive to spin state, axial ligand strength, and conformational changes. Therefore, Raman spectroscopy studies have been key in unveiling the complexity of Cc conformation equilibria under different conditions [123, 125–128]. Hildebrandt and collaborators detected native (B1; His-Met coordination) and altered (B2) states in the presence of DOPG vesicles [125]. The B2 state comprises different species: a low-spin (B2[6cLS]), His-His-coordinated species, and two high-spin

Figure 3: Proposed model for the interaction of cytochrome c with cardiolipin at pH values above 7. (a) Upper: structure of free Cc showing the foldon units (in red scale, PDB 1AKK [100]). Lower: the Cc-CL interaction promotes unfolding of the metalloprotein and dissociation of the axial ligand M80, thus increasing accessibility to the heme crevice. (b) Upper: structural comparison of free (in red scale, PDB 1AKK [100]) and CL-bound Cc (in blue scale, PDB 2N3B [115]). Lower: interaction of Cc with CL yields a slight difference in dynamics at the level of the $\Omega$-loops and helix-I. The different foldon units of Cc are colored as a gradient from the most stable (dark colors) to the weakest region (light colors). The heme group is in green, and the iron atom in orange.
species (a pentacoordinated (B2[5cHS]) and a hexacoordinated species (B2[6cHS])) in which a water molecule acts as the sixth ligand. The populations of the different states change according to the L/P ratio. The B2 species predominates at high L/P ratios, whereas native B1 and His-His-coordinated B2[6cLS] coexist at lower ratios. These states are also detectable by MCD [114]. The B2 bis-His-coordinated species is detectable when Cc is absorbed onto self-assembled monolayers, with and without CL [129]. At increasing concentrations of DOPC/tetraoleoylcardiolipin (TOCL) micelles, the population of bis-His species increases, as confirmed by His-by-Asn mutations and spectroscopic analyses [130].

In addition to the L/P ratio, the content of CL and its composition influence Cc affinity and dynamics in the bound state. Fluorescence data has indeed revealed that increasing amounts of CL favors Cc binding to membranes [120]. The theoretical analysis therein suggests that the protonation state of CL may have a strong influence on populations of distinct membrane-bound Cc species. However, the authors acknowledge that the formalism does not include the effects that Cc exerts on CL distribution or membrane state (see below). Indeed, kinetic investigations have shown that the exchange rate between a native-like, compact (C) and the "extended" (E) conformations correlates with the amount of CL in the vesicles [131]. A more recent spectroscopic analysis on titration experiments by Pandiscia and Schweitzer-Stenner resulted in similar conclusions [90]. Additionally, the study highlighted that the L/P ratio also affects the relative weight of electrostatic interactions, hydrogen bonds, and hydrophobic interactions—according to ionic strength series. In summary, besides governing Cc conformational states, the L/P ratio modulates the nature of bilayer-protein interactions. Notably, all the studies highlighted above hint at a rather peripheral binding model, with little or no embedding of Cc into the membrane [90, 123, 125, 126, 128, 129, 131].

On the other hand, Cc does exert a strong influence on lipid head-group dynamics, as revealed by early $^{31}$P ssNMR studies which demonstrated an increase in acyl chains dynamics and a restraint in the polar head groups of phospholipids [132, 133]. Interestingly, Cc has little impact on the $^{31}$P ssNMR "powder" spectra of dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylethanolamine (DOPE), or DOPC/DOPE vesicles not containing CL [134]. Indeed, NMR data strongly supported CL undergoing phase separation—to form CL rafts—with mixed DOPC/CL preparations upon the addition of Cc [132, 135]. Further, freeze-fragment electron microscopy images highlighted the ability of Cc to promote the transition of phospholipid bilayers containing CL into non-bilayer structures, including inverted tubular (H$_{II}$) states [133, 134]. A full isotropic signal in the $^{31}$P ssNMR spectra of phospholipid preparations in the presence of Cc evinced the formation of vesicular or micellar structures when the vesicles contained CL [132, 136]. Similarly, a downfield broad signal indicated that CL mediates the formation of the H$_{III}$ phase upon the addition of Cc. In this sense, molecular dynamics simulations in which Cc is in contact with a DOPC/CL membrane highlight the ability of this protein to recruit CL into rafts [118]. In addition, Cc can induce local changes in membrane curvature when the ratio of CL increases up to 20%. Furthermore, Cc induces pore formation in DOPC/CL giant unilamellar vesicles (GUV), as shown by confocal microscopy [137]. These pores are wide enough to allow Cc and dextran molecules to cross the membrane.

Nevertheless, the ability of Cc to induce membrane changes seems to be secondary regarding the activation of peroxidase activity. Addition of Cc to large DOPC/CL unilamellar vesicles at a ca. 6 CL/Cc ratio promotes peroxidase activity without substantially affecting $^{31}$P ssNMR spectra or the $^{13}$C frequencies of the lipid glycerol signals [138]. The major population—those accounting for less than 10% of the protein—are not detectable—of Cc in these experiments displays the same structure as the native protein in solution. Chemical-shift perturbation analysis revealed that residues affected include the $\Omega_{III}$-site and some nearby residues (including A-site residues). However, changes in the dynamics of the $\Omega_{III}$-loop as it couples with bilayer motions are observable. This, rather than an overall unfolding, is sufficient to trigger the peroxidase activity under these conditions. In accordance with this finding, the perturbation pattern shifts when the temperature is changed or when the vesicle phospholipids are unsaturated.

The formation of Cc-CL complex requires approximately 6 molecules of CL per Cc molecule [15, 112, 119, 138, 139]. The values of the apparent dissociation equilibrium constant for the Cc-CL-reduced complex are in the low micromolar range (1.4 μM at pH 8.1 and 2.2 μM at pH 6.5) [140], whereas in the oxidized form, they are in the high micromolar range in a two-step reaction (20 μM and 42 μM) [102]. Nevertheless, binding constants depend on CL composition. The affinity of Cc towards tetra-oleyl-cardiolipin-containing vesicles is several fold higher than that for TOCL ones [102], whereas tetra-myristoyl-cardiolipin barely interacts Cc [15]. Notably, the measured affinities correlate well with the peroxidase activity of Cc in the presence of the respective vesicles, rather than the degree of unsaturation in the acyl chains [102].

### 5. Cardiolipin Oxidation by Cytochrome c

CC is particularly sensitive to auto-oxidation processes—those directly initiated by inducers, such as ROS. The proximity of its four unsaturated acyl chains allow "arm-to-arm" propagation, enhancing its reactivity [141]. Auto-oxidation takes place in several steps. A free radical (e.g., a ROS molecule) contains an unpaired electron, and this semi-occupied orbital is a sink for a second electron. Polysaturated fatty acids (PUFA), such as linoleic or linolenic, then initiates a radical propagation process that is particularly sensitive to ROS-induced oxidation due to conjugative effects. Radicals such as superoxide, peroxyl (ROO$^\cdot$), or hydroxyl (HO$^\cdot$) sequesters a hydrogen atom from the α-methylene carbon with respect to the first (di-) vinyl group. The resulting radical reacts immediately with O$_2$ to generate a peroxyl radical. The variation of electron vacancy in the lipid radical underlies the diversity of reaction products. No matter how the lipid radical originates, it tends to propagate via
a reaction with molecular oxygen, water, or other lipids. Within the process of CL signaling, there are several reactions which stand out including the addition of oxygen (to form peroxides), transfer of hydrogen atoms, addition of peroxyl radicals, and intramolecular peroxide substitution [142].

Oxidative phosphorylation and certain mitochondrial enzymes are sources of O$_2^\cdot^-$ radicals [143]. Superoxide is highly soluble in lipids but can be reduced within membranes by tocopherol and quinone and eliminated by superoxide dismutase (SOD), which transforms two superoxide molecules into a molecular oxygen and hydrogen peroxide [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144]. In fact, enzymes like SOD, catalase, and peroxidases take part in active cell defense against oxidative stress [144].

H$_2$O$_2$ is a strong oxidant except for pathological conditions, H$_2$O$_2$ requires the activity of peroxides to function efficiently as an oxidant. Within the IMM, Cc displays both peroxidase and oxygenase activities, the latter promoting CL oxidation, while sparing other phospholipids [18, 146]. This event is crucial for the release of mitochondrial proapoptotic factors into the cytoplasm [18].

5.1. Peroxidase and Oxygenase Activities of Cytochrome $c$ (Dr. Jeckyll and Mr. Hyde). Heme peroxidases constitute a vital and ubiquitous group of heme enzymes catalyzing the two-electron oxidation of substrates using H$_2$O$_2$ as the ultimate electron acceptor [147, 148]. In canonical peroxidases, H$_2$O$_2$ is added to the pentacoordinated, high-spin Fe$^{III}$. The resulting state—Compound I—is two oxidation equivalents above the resting state and is reduced back to the resting configuration in two steps through Compound II. Both states, Compounds I and II, are high valence oxoferryl (Fe$^{IV}$) derivatives, but the first comprises an additional $\pi$-cation radical (see Equations (7)–(9)) (Figure 4) [147].

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{O}_2^- + 2\text{H}^+
\] (1)

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^-(\text{Fenton ‘s reaction})
\] (2)

\[
\text{O}_2^- + \text{Fe}^{2+} \rightarrow \text{Fe}^{2+} + \text{O}_2
\] (3)

The hydroxyl radical product is highly reactive, sequestering hydrogen atoms from available substrates. The resulting carbon-centered radicals may react with molecular oxygen to generate (hydro-) peroxides (Equations (4)–(6)):

\[
\text{HO}^- + \text{RH} \rightarrow \text{H}_2\text{O} + \text{R}^*
\] (4)

\[
\text{R}^* + \text{O}_2 \rightarrow \text{RO}_2^-
\] (5)

\[
\text{RO}_2^- + \text{H}^* + \text{Fe}^{2+} \rightarrow \text{ROOH} + \text{Fe}^{3+}
\] (6)

Nevertheless, homeostatic cells exert a tight control over metal chelation to avoid Fenton’s reactions. Indeed, a set of antioxidant agents prevent a surge in the levels of ROS. Hence, except for pathological conditions, H$_2$O$_2$ requires the activity of peroxides to function efficiently as an oxidant. Within the IMM, Cc displays both peroxidase and oxygenase activities, the latter promoting CL oxidation, while sparing other phospholipids [18, 146]. This event is crucial for the release of mitochondrial proapoptotic factors into the cytoplasm [18].

**Figure 4**: Peroxidase and oxygenase activities of cytochrome $c$. Reaction model merging the proposal from Kagan and collaborators [149] and the adapted catalytic model of cyclooxygenases as reviewed by Marnett [150]. Blue arrows correspond to the canonical peroxidase cycle [146]. Heterolytic cleavage of a peroxide substrate—preferentially for Cc, a lipid hydroperoxide—yields the corresponding hydroxyl derivative (or water when the substrate is H$_2$O$_2$) and Compound I, which is reduced back to the resting ferric state in two sequential single-electron transfers from A substrate. Red and green arrows indicate the reactions purportedly leading to oxygenase activity according to the literature. Spin trap experiments have detected Y48 radicals [151]. Dimers of tyrosines 67 and 74 and oxidation products of Y48 are detectable even in the absence of H$_2$O$_2$ [152]. The tyrosyl radical sequesters a hydrogen atom from an unsaturated fatty acid. Finally, O$_2$ reacts with the alkyl radical to form an alkyl peroxide radical as an initial product undergoing further reactions.

\[
\text{Heme[Fe}^{III}]_{\text{RS}} + \text{H}_2\text{O}_2 \rightarrow \text{Heme[O = Fe}^{IV}-\text{R}^*]_{\text{CompI}} + \text{H}_2\text{O}
\] (7)

\[
\text{Heme[O = Fe}^{IV}-\text{R}^*]_{\text{CompI}} + \text{AH}_2 \rightarrow \text{Heme[O = Fe}^{IV}]_{\text{CompII}} + \text{AH}^*
\] (8)

\[
\text{Heme[O = Fe}^{IV}]_{\text{CompII}} + \text{AH}_2 \rightarrow \text{Heme[Fe}^{III}]_{\text{RS}} + \text{AH}^{**} + \text{H}_2\text{O}
\] (9)
In true peroxidases, a histidine residue acts as an acid-base catalyst at the distal side of the heme ring, while a highly conserved arginine residue stabilizes the alkoholate leaving group to favor the heterolytic cleavage of the peroxide O–O bond [153]. The orientation of these residues and the hydrogen-bond network at the heme distal side are critical for efficient formation of Compound I [154]. As recently pointed out by Vlasova [148], the composition of a true peroxidase active site prevents its damage by highly oxidizing intermediate compounds.

Cc and other hemeproteins can act as pseudoperoxidases; that is, under only certain stimuli they show peroxidase activity [148, 155]. Contrary to true peroxidases, the surroundings of heme moiety are unprotected against oxidation, so the peroxidase activity ends up damaging the protein. In the early 1990s, Radi and collaborators reported the ability of Cc to oxidize small compounds in solution [145] and to carry out lipid peroxidation [146] in the presence of H2O2. Nevertheless, the reactivity of Cc towards H2O2 was low, as it requires the absence of the sixth ligand. Thus, the Km value for H2O2 was very high—ca. 65 mM. In this sense, oxidative reactions showed a time lag after the addition of H2O2, indicative of the absence of the sixth ligand. The peroxidase activity of Cc in the presence of CL/C2/C3 +O2 +H2O [157] is a key mechanism in sulphydryl oxidation [164].

\[
\text{Heme[Fe}^{III}] + H_2O_2 \rightarrow \text{Heme[HO}^- + Fe}^{III}] \quad (10)
\]

\[
\text{HO}^- + H_2O_2 \rightarrow H_2O + HO_2^- \quad (11)
\]

\[
\text{Heme[HO}^- + Fe}^{III}] + HO_2^- \rightarrow \text{Heme[Fe}^{III}] + O_2 + H_2O \quad (12)
\]

However, EPR spin trap experiments highlighted the generation of Cc tyrosine radicals upon treatment with H2O2 [18, 151, 155, 158]. Furthermore, spin trap experiments detecting radical products resulting from oxidation of different substrates by H2O2 strongly suggested the reaction being mediated by an oxoferryl \([O=Fe]^-\) intermediate [155]. Analysis of the orientation of this radical within the native state identified a tyrosine residue at the \(\Omega\)-loop—namely, Y48 in horse heart Cc and either Y46 or Y48 in human Cc [159]. The peroxidase activity of Cc in the presence of CL increases by three orders of magnitude when the driving oxidant is a lipid peroxide instead of H2O2 [149]. Spin trap analysis of reaction products by Kagan and collaborators indicates a diversity of catalytic mechanisms depending on the binding site of the substrate, namely, a homolytic peroxide cleavage minority mechanism and a major, heterolytic mechanism. Notably, the small hydroperoxide substrates involved in this pathway dock near R38 and H33. However, how the docked structure undergoes conformational changes to fulfil all geometrical constraints needed for Compound I formation remains unclear (Figure 4).

Binding of hydrogen peroxide to the heme iron is a key step in the reaction mechanism underlying peroxidase activity. The reactive species need to displace the thioether axial ligand. This takes place when a strong interaction between Cc and a membrane induce a substantial conformational change in the hemeprotein [15, 84, 93, 95–97, 109, 111]. Nevertheless, Kagan and coworkers also detected peroxidase activity in Cc at low CL/Cc ratios—at which the most interactions are weak electrostatic [15]. Furthermore, they found that the energy required to activate peroxidase activity is lower than that required for partial unfolding of the protein. In fact, as pointed out before, the bond joining iron to the thioether ligand is quite weak [160]. Thus, “breathing” fluctuations of \(\Omega\)-II- and \(\Omega\)-III-loops may facilitate the replacement of the thioether ligand by small reactants—such as cyanide, carbon monoxide, water, or hydrogen peroxide—without demanding major structural changes. In fact, Cc peroxidase activity rises in the presence of H2O2 as the concentration of denaturant increases, as previously observed in a similar analysis with bacterial cytochrome c550 [161]. Statistical analysis of activity and unfolding slopes indicate that increasing the motions of the weakest \(\Omega\)-loops correlates well with peroxidase activity in the “compact” Cc species [162].

The peroxidase activity of Cc can exert a protective role in mitochondria under certain conditions [11]. Indeed, reduction of lipid hydroperoxide compounds to hydroxyl ones provides a way of relieving oxidative stress in the mitochondrial membrane while generating signaling molecules [149]. Moreover, O2− reduces nitric oxide (NO) generated in mitochondria under stress to form peroxynitrite (HONO), a highly reactive species. Cc-CL complexes have been proposed to aid peroxynitrite detoxification to yield either nitrate or nitrite through an oxoferryl state [163].

Conversely, the peroxidase activity of Cc has been implicated in certain pathologies. For instance, oxidation of sulfite to its radical SO3•− is a key mechanism in sulfite toxicity [164]. Moreover, Cc mediates the formation of tyrosine radicals responsible for α-synuclein dimerization [66, 165], which leads to the development of the Lewy body diseases.

Canonical peroxidase activity involves two sequential one-electron oxidation steps and no transfer of oxygen from the oxoferryl complexes to the substrate [146]. Nevertheless, Compound I in certain heme enzymes—such as cytochrome P450—can transfer oxygen to certain substrates yielding hydroxy-derivatives. Interestingly, hydroxy-derivatives of CL cannot undergo peroxidation and inhibit the release of Cc [166]. Altogether, considering the findings above concerning tyrosyl radicals in Cc [151], Kagan and collaborators proposed that Cc acts as an oxygenase to produce CL peroxidation [167]. This activity would also be responsible for phosphatidylserine peroxidation affecting the plasma membrane during apoptosis [65]. This hypothesis suggests that hydrogen is transferred to Compound I from a nearby tyrosine residue to yield oxoferryl Compound II and the aforementioned tyrosyl radical (Figure 4). This mechanism is similar to that proposed for cyclooxygenases, in which a hydrogen atom is sequestered from an acyl chain, generating
a carbon-centered radical capable of reacting with molecular oxygen [150].

Unlike true peroxidases, the environment of the heme moiety is unprotected from highly oxidizing species arising during the catalytic cycle [148]. When reacting with H$_2$O$_2$, degradation of the heme porphyrin often becomes apparent by a diminution of the Soret band intensity [146, 156]. A thorough mass spectrometry analysis of Cc residue adducts derived from H$_2$O$_2$ has been carried out by Flemmig and collaborators [152]. Several oxidation reactions can occur to produce a methyl-sulfoxide derivative from the methionine thioether, a sulfonic acid derivative from cysteines and 2-oxohistidine from histidine. While tyrosine residues can covalently cross-link or undergo oxidation to dihydroxyphenylalanine (DOPA) and subsequently to quinones, lysine residues can undergo carboxylation [152, 168, 169]. These changes occur when H$_2$O$_2$ is added to Cc samples [80, 152, 170–173].

Remarkably, different regions in the protein display different sensitivities to oxidation depending on the environment. For instance, specific M80 oxidation takes place in the presence of DOPC/DOPE micelles [174]. The $\Omega_{II}$-loop is the first to be affected, whereas foldon I (helices I and IV) is the least affected by oxidation. Notably, the peroxidase activity of Cc increases in a time-dependent manner upon the addition of H$_2$O$_2$. Such increments in peroxidase activity may result from successive oxidation of M80 and lysine residues, as proposed by Yin and Konermann [80, 170]. Indeed, M80 oxidation promotes conformation exchange in Cc which impacts on heme ligation. With time, oxidative damage extends to the porphyrin ring, releasing iron capable of performing Fenton’s reactions [173]. Finally, it is worth noting that CL peroxides can induce at least some of these oxidative PTM [152].

5.2. The Alkaline Transition of Cytochrome c and Peroxidase Activity. As mentioned above, previous data obtained using monoclonal antibodies highlighted that an alkaline-like conformation could interact with CL and exit mitochondria during apoptosis [106]. These antibodies also recognize the M80A mutant in the cell nucleus [175]. Notably, this mutant displays enhanced peroxidase activity. In addition, the peroxidase activity of Cc is somewhat pH dependent [145, 176]. Indeed, for horse heart Cc, peroxidase activity increases at acidic pH values [177] and slows beyond pH 8 [145]. Furthermore, the ability of Cc to oxidize O$_2^*$ falls at pH values above 7 [7]. The affinity of Cc towards membranes and the interaction patch involved also depend on pH [15, 98, 120, 127]. These effects illustrate how pH-dependent conformation changes modulate the different activities performed by Cc.

A number of mutations and PTM have been reported to simultaneously affect the peroxidase activity of Cc while bringing the so-called alkaline transition to lower even physiological pH values [178–182]. Loss of M80 coordination is evident from NMR spectra and UV-Vis spectra in all these studies. Cc peroxidase activity requires the heme iron to be pentacoordinated; the relationship with the p$K_a$ of the alkaline transition could be attributable to the lysine amine being weaker than methionine thioether in the ligand. However, at a neutral pH, lysine is a stronger ligand than methionine [183]. In fact, horse heart and human Cc show lower peroxidase activities at alkaline pH values [145, 182]. Moreover, mutation M100K in P. versutus cytochrome c$_{550}$ makes the protein more stable at neutral pH while decreasing its peroxidase activity 20-fold [183].

Nevertheless, the shift in the alkaline transition towards lower pH values indicates destabilization or higher dynamics in the $\Omega_{II}$- and $\Omega_{III}$-loops in the Met-coordinated species. Given the weakness of the thioether ligand bond towards iron, increasing fluctuations of these loops will increase the population of high-spin species and/or alternative low-spin (e.g., bis-His) species below the p$K_a$ value of the transition. This is observable in phosphomimic mutants, as well as in nitrated species of Cc [178–180, 184–186]. Furthermore, enhanced dynamics facilitate the access of small substrates to the heme iron [162].

5.3. Control of Cardiolipin Oxidation by Post-translational Modification of Cytochrome c. Protein PTM regulate tightly controlled cellular processes and increase the functional diversity of proteins, often acting as a cell response switch. Several post-translational modifications modulate Cc structure and functionality, such as sulfoxidation [187], carbonylation [152], homocysteinylation [188], nitration [179, 180], and phosphorylation [189] (Figure 5). Phosphorylation of tyrosine residues is associated with many human pathologies including cancer, ischemia, asthma, and sepsis. As highlighted earlier, tyrosine radicals are key for the oxygenase activity of Cc [157]. Thus, the amount of hydroxyl products from TOCL oxidation is lower when Y48E phosphomimic species instead of WT Cc acts as a catalyst [190]. Additionally, tyrosine phosphorylation impairs the formation of radicals, preventing dimerization [191]. This fact may be critical in pathological processes such as Parkinson’s disease [66]. Therefore, the PTM that affect these residues are key in regulating Cc activity.

Given the difficulty in preserving the phosphorylation state of Cc outside of cell extracts, a common strategy to investigate consequences of phosphorylation is to mimic the modification by site-directed mutagenesis. All phosphomimetic Cc species, except a mutant at position 97, display altered affinity towards cardiolipin [178, 186, 190, 192–195]. The peroxidase activity of both free Cc and Cc-CL complexes increases in the phosphomimetic T28D and Y48pCMF. However, for the Y48E species, the increase only occurs with the free protein (Table 1) [178, 190, 192, 194]. In addition, at a high CL/lipid ratio, the peroxidase activity of the T28E mutant decreases [195]. A possible explanation may be that the greater population of CL versus other lipids in the liposome composition promote unfolding of the hemeprotein, acting as an off switch (Table 1) [131]. Hence, the negative charge at these positions could induce structural changes in the heme crevice which allow greater accessibility for hydrogen peroxide (Figure 5).

Peroxynitrite generated during nitrooxidative stress is a powerful amino acid modifier, affecting tyrosine residues among others [201]. Common products of the reaction
between tyrosine and HOONO are 3,5-dinitrotyrosine, 3-nitrotyrosine, tyrosine radicals, and dityrosine. Nevertheless, treatment of Cc in vitro with peroxynitrite yields its 3-nitrotyrosine adducts, with the nitro group attaching to one of the ε of the aromatic ring [202]. Nitration affects the redox potential of Cc as well as its electron-exchange kinetics, depending on the residue involved [203]. The nitration of Y46, Y48, Y74, and Y97 residues also increases the peroxidase activity of Cc and lowers the pKₐ value of the alkaline transition besides other functional properties [179, 180, 183, 185, 197, 204, 205] (Table 1). Nitration of Cc has been associated with several diseases, including chronic nephropathy [206]. All modifications/mutations of S47 and Y67 alter the peroxidase activity of Cc [79, 179, 185, 192, 195, 197]. Y67 is located close to Met80 and is part of the hydrophobic pocket which houses the acyl chains of CL. This residue is also key for the stability of the Ω III loop (Table 1).

Homocysteinylation is a PTM that involves the covalent bonding of a homocysteine thiolactone—an intermediate metabolite of methionine metabolism—with a lysine residue [188]. Human Cc displays a lysine content of 17.1% in its amino acid composition, which makes it sensitive to homocysteinylation. Homocysteinylation of surface lysines on Cc causes aggregation of the protein. However, N-homocysteinylated lysines adjacent to the heme cavity produce conformational changes, disrupt the coordination of the M80 axial ligand, and alter the redox state of Cc, reducing the iron of the heme group [196, 208]. These conformational changes increase Cc peroxidase activity (Table 1) [209].

As discussed before, Cc is modulated by several oxidative modifications due to its activity, eventually leading to changes in iron coordination. One oxidative modification is the carbonylation of lysine residues, which affects residues 72 and 73, both of which are involved in the alkaline transition of Cc [170] (Figure 5). Reportedly, successive carbonylation events at K53, K55, K72, and K73 lead to the formation of the pentacoordinated Cc species, resulting in increased Cc peroxidase activity (Table 1) [170]. Similarly, the sulfoxidation of M80 facilitates the formation of a Compound I-type intermediate that initiates the activity of Cc peroxidase (Table 1) [171, 210].

5.4. Peroxidase Activity of Cytochrome c and Diseases. Since the peroxidase activity of Cc relates to the activation of apoptosis, it is a clear target for the development of more efficient
therapies against certain diseases or pathologies. There are several examples in the literature that shed light on this topic. Indeed, its highly dynamic architecture, which enables functional complexity despite its apparently simple structure, emerging as an important task.

In this review, we have outlined major advances and hypotheses regarding the oxidation of CL by Cc. CL oxidation is a crucial event at the onset of a diverse range of pathologies, and thus, controlling it has become a key objective of current research. Targeting Cc—a key player in CL oxidation—has emerged as an important task.

Since its discovery last century, Cc has displayed great functional complexity despite its apparently simple structure. Indeed, its highly dynamic architecture, which enables conformation changes critical in regulating metabolism, signaling, and cell fate, still amazes the scientific community. Cc interacts with membranes in different ways depending on their composition and curvature, being able of modifying the latter. When the hemeprotein interacts with lipids, it may undergo subtle changes in the dynamics of its most flexible foldons or may even unfold. A plethora of factors, including PTM, control these phenomena.

The chemical activity of this protein ranges from the simplest reactions to diverse and complex reaction mechanisms to drive the oxidation of substrates including CL. In the recent years, we have witnessed concerted effort to unveil the intimate chemistry of this process. Solving this

### Table 1: Effect of PTM and point mutations on cytochrome c peroxidase activity.

| Cc PTMs/mutation | Effect on peroxidase activity | References |
|------------------|-------------------------------|------------|
| Sulfoxidationa   |                               |            |
| M80              | ↑                             | [168, 187, 196] |
| Nitrationb       |                               |            |
| Y46              | ↑                             | [184]      |
| Y48              | ↑                             | [184]      |
| Y67              | ↑                             | [185, 197] |
| Y74              | ↑                             | [185, 197] |
| Y97              | ↑                             | [185, 197] |
| Carboxylationc   |                               |            |
| K53              | ↑                             | [152]      |
| K55              | ↑                             | [152]      |
| K72              | ↑                             | [152]      |
| K73              | ↑                             | [152]      |
| Phosphorylation  |                               |            |
| T28 T28D         | ↑                             | [192]      |
| T28 T28E         | ↓                             | [195]      |
| S47 S47D         | ≈                             | [192]      |
| Y48 Y48E         | ↑/↓                           | [176, 190] |
| Y48pCMF          | ↑                             | [194]      |
| Y97 Y97pCMF      | ≈                             | [176]      |
| N-Homocysteinylagationd |             |            |
| K8/K13           | ↑                             | [198]      |
| K86/87           | ↑                             | [198]      |
| K99              | ↑                             | [198]      |
| K100             | ↑                             | [198]      |
| Point mutation   |                               |            |
| G41S             | ↑                             | [181, 199] |
| Y48H             | ↑                             | [181, 200] |

a Determined under oxidative stress. b Determined after peroxynitrite treatment. c Determined after chloramine-T treatment. d Determined after homocysteine thiolactone treatment.
conundrum will require us to discriminate minority conformations in functional assays and elucidate how this activity is tuned under distinct conditions. Nevertheless, knowledge has already amassed on the subject enabling us to examine the inhibition of CL oxidation, which will aid the development of translational approaches.

Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this review article.

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