

Covalent Binding and Anchoring of Cytochrome *c* to Mitochondrial Mimetic Membranes Promoted by Cholesterol Carboxyaldehyde

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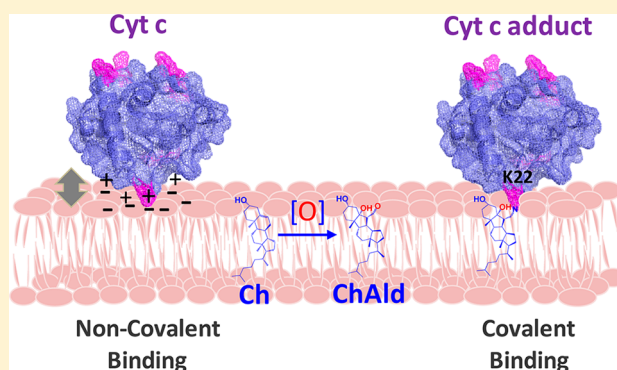
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S Supporting Information

ABSTRACT: Mitochondrial cholesterol has been reported to be increased under specific pathological conditions associated with enhanced oxidative stress parameters. In this scenario, cholesterol oxidation would be increased, leading to the production of reactive aldehydes, including cholesterol carboxyaldehyde (ChAld). By using SDS micelles as a mitochondrial mimetic model, we have demonstrated that ChAld covalently modifies cytochrome *c* (cytc), a protein known to participate in electron transport and apoptosis signaling. This mimetic model induces changes in cytc structure in the same way as mitochondrial membranes do. Tryptic digestion of the cytc-ChAld adduct followed by MALDI-TOF/TOF analyses revealed that modifications occur at Lys residues (K22) localized at cytc site L, a site involved in protein–protein and protein–membrane interactions. Interestingly, ChAld ligation prevented cytc detachment from liposomes even under high ionic strength conditions. Overall, it can be concluded that ChAld ligation to Lys residues at site L creates a hydrophobic tail at cytc, which promotes cytc anchoring to the membrane. Although not investigated in detail in this study, cytc adduction to cholesterol derived aldehydes could have implications in cytc release from mitochondria under apoptotic stimuli.



INTRODUCTION

Cytochrome *c* (cytc) is a small heme protein attached to the external side of the inner mitochondrial membrane where it is responsible for the electron transport between cytochrome *c* reductase and cytochrome *c* oxidase.¹ The electron transport mediated by cytc occurs through a lateral diffusion of this protein on the external side of the membrane, where it interacts with phospholipids.² The interaction between cytc and the mitochondrial membrane is mostly mediated by electrostatic interactions between lysine residues and the phosphate groups of phospholipids, especially cardiolipin.^{2–4} It has also been proposed that an acyl chain from cardiolipin penetrates in a hydrophobic core of the protein and contributes for the protein anchorage to the membrane.^{5,6} In specific situations and under the proper stimuli, cytc is released to the cytosol where it is involved in apoptosis signaling.^{1,7,8}

Cholesterol is a neutral amphiphilic lipid widely found in the cell membrane of eukaryotes where it plays an important role by maintaining lipid organization and fluidity.^{9–11} It is enriched in specific membrane regions known as lipid rafts.^{12–14} These regions are recognized to act as scaffolds to membrane-bound proteins and to have an influence in signaling transduction.^{12,14,15} Unlike the cell plasma membrane that is rich

in cholesterol, the mitochondrial inner membrane has a very small pool of this lipid, corresponding to 0.5 to 3% of the amount found in the cell membrane.^{11,16} However, several studies reported elevated cholesterol levels (2–10-folds higher concentration) in the mitochondria of cancerous cells^{17–19} and in nervous tissues of patients with Alzheimer's disease,^{9,20} which makes cholesterol a major target for lipid peroxidation. In addition, the increased cholesterol levels in the mitochondria are described to deplete mitochondrial glutathione (mGSH), probably by impairing the cytosolic mGSH influx through the mitochondrial membranes.^{20,21} Since mitochondria are primary sources of reactive oxygen species (ROS), this depletion in mGSH could increase the oxidative stress and, consequently, lipid peroxidation inside the mitochondria.

Cholesterol oxidation promoted by reactive oxygen species generates several products including hydroperoxides and aldehydes.^{22–26} In the last 10 years, studies have identified the formation of cholesterol-derived aldehydes, namely, the 3 β -hydroxy-5-oxo-5,6-secholestan-6-al (CSeC) and its aldolization product 3 β -hydroxy-5 β -hydroxy-B-norcholestan-6 β -car-

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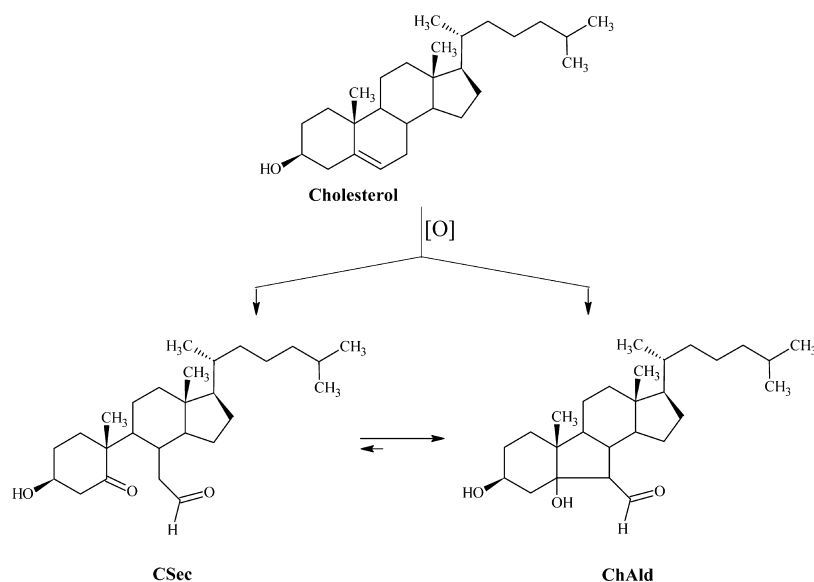


Figure 1. Chemical structures of 3 β -hydroxy-5-oxo-5,6-secholestan-6-al (CSec) and its aldolization product 3 β -hydroxy-5 β -hydroxy-B-norcholestan-6 β -carboxaldehyde (ChAld). Both compounds have a molecular weight of 418.65 g/mol.

boxaldehyde (ChAld) during cholesterol oxidation mediated by ozone (O_3)^{25,26} and/or singlet molecular oxygen (1O_2)^{23,24} (see Figure 1 for the aldehydes chemical structures). Wentworth Jr. and co-workers reported the formation of cholesterol aldehydes in atherosclerotic plaques *in vivo* and associated their presence with the formation of ozone in human arteries.²⁵ However, later studies have demonstrated that singlet molecular oxygen can also generate cholesterol aldehydes, in particular ChAld.^{23,24} Indeed, this aldehyde was detected as the major aldehyde product both *in vitro* and *in vivo*.²⁷ In this sense, singlet molecular oxygen can be generated by a number of biologically relevant chemical pathways involving hydrogen peroxide and lipid hydroperoxides.^{28–30} Among these pathways, we have recently demonstrated that the interaction of cytc with cardiolipin (CL) generates singlet oxygen,³¹ indicating that this excited species can be generated in mitochondria where it can contribute to the oxidation of mitochondrial lipids.

ChAld exerted cytotoxic effects in different cell types present within atheromatous arteries. These aldehydes were also able to trigger a loss of secondary structure of apolipoprotein B-100 upon binding to LDL.²⁵ Moreover, ChAld was implicated in proatherogenic processes and was also reported to induce amyloidogenesis in several biological relevant proteins.³² Since ChAld is believed to be the major cholesterol aldehyde found *in vivo*,²⁵ in the present study we investigated only the effect of this cholesterol derivative.

Several studies on cytc modification have been published demonstrating the ability of aldehydes derived from lipid peroxidation to react with basic amino acids of the protein.^{33–35} For instance, the covalent addition of both 4-hydroxynonenal (HNE)³³ and 2,4-decadienal (DDE)³⁴ to cytc was reported. The addition mechanisms were either a condensation reaction, formation of a Schiff base adduct, or a Michael addition. From a biological point of view, several critiques can be made to studies using cytc adduction by lipid electrophiles in solution. Cytc is a membrane-bound protein that displays different conformation and physicochemical properties when bound to membranes when compared to the “free” protein in solution. Hence, depending on how the protein anchors to the membrane, the

adduction may occur at different portions on the protein (e.g., to different amino acid residues) and by different mechanisms. In this sense, the use of membrane models to mimic the interaction between cytc and the membrane will induce the expected conformation and physicochemical alterations to the protein, inducing the adduction at a more biologically relevant site on the protein. In addition, this approach may also help understand the biological implications of these adductions.

In the present study, we used micelles of sodium dodecyl sulfate (SDS) to mimic the inner mitochondrial membrane by providing the anchor base to cytc. The interactions between cytc and SDS micelles have been extensively characterized by Mugnol and co-workers.³⁶ The authors showed that, upon interaction with the micelles, cytc changes its tertiary structure and assumes an alternative low spin state, in which it holds the same structure and physicochemical properties as the cytc bound to the mitochondrial membrane.³⁶ In our study, the use of this particular model comes in handy since in addition to the alterations induced on the protein, it also behaves as a membrane model, preventing protein aggregation and sterol precipitation. Therefore, by using this model we were able to bring together ChAld and cytc in a manner similar to that presumed to occur in the mitochondrial membrane. Another convenience of using this model is that, unlike polyunsaturated fatty acids, SDS cannot be oxidized, which makes it a simpler model for elucidating the mechanisms proposed in this work. By doing that, we showed that cytc is covalently modified by ChAld in either the presence or the absence of the mimetic membrane. Relevantly, in the presence of mimetic membranes cytc modification was residue-specific and occurred primarily at Lys22, a residue situated in site L, a pH-dependent binding site of the protein. Interestingly, release of cytc from ChAld-containing liposomes is impaired when compared to cholesterol-containing ones, suggesting that covalent adduction could possibly influence cellular events involving cytc mobility. To our best knowledge, this is the first study to use this approach to elucidate the mechanisms of protein adduction induced by cholesterol electrophiles. Possible implications of these modifications to electron transport and apoptosis signaling are also discussed.

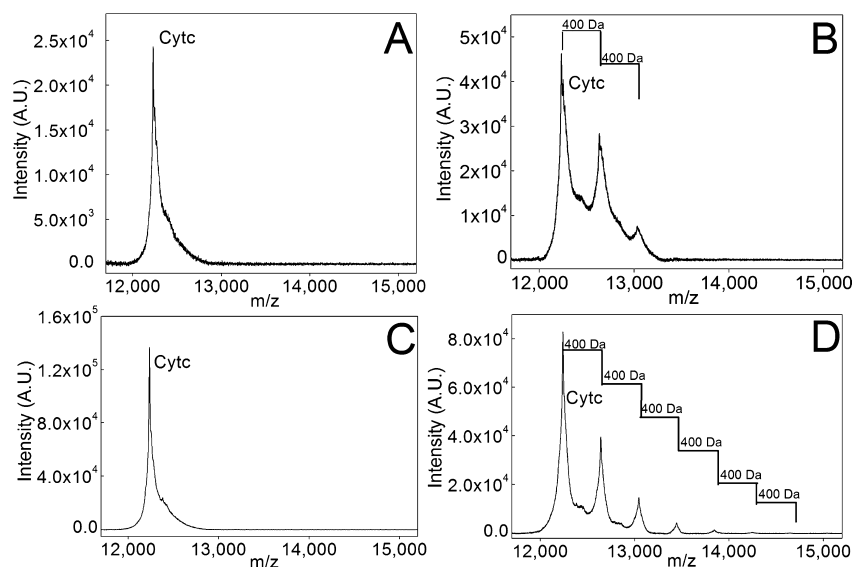


Figure 2. MALDI-TOF MS of cytc before and after 24 h of reaction with ChAld in the presence (panels A and B) and absence (panels C and D) of SDS micelles. Reactions were conducted in bicarbonate buffered media (10 mM, pH 7.4) containing 8 mM SDS (when present), 100 μ M cytc, and 1 mM ChAld. Panels A and C show the control conditions performed in the absence of ChAld. Panels B and D represent the incubations conducted in the presence of 1 mM ChAld. The addition of ChAld leads to the appearance of peaks separated by 400 Da. Two and six ChAld additions can be observed at panels B and D, respectively.

EXPERIMENTAL PROCEDURES

Chemicals. Bovine heart cytochrome *c* (Fe(III)), ammonium bicarbonate (NH_4HCO_3), cholesterol (cholest-5-en-3-ol), sodium dodecyl sulfate (SDS), silica gel 60 (230–400 mesh), methylene blue, potassium monobasic phosphate (KH_2PO_4), potassium dibasic phosphate (K_2HPO_4), potassium chloride (KCl), and deuterated chloroform (CDCl_3) were purchased from Sigma (St. Louis, MO). Tetraoleoylcardiolipin (TOCL) and dipalmitoylphosphatidylcholine (DPPC) were from Avanti Polar Lipids, Inc. Isopropanol and acetonitrile were purchased from JTBaker. Trypsin Gold (mass spectrometry grade) was purchased from Promega. All other reagents were of analytical grade. The K22A cytc mutant was prepared exactly as described in Kawai et al.³⁷ Stock solutions of ammonium bicarbonate buffer (pH 7.4) were freshly prepared in Milli-Q water and the pH adjusted to 7.4 prior to use.

Synthesis of 3-Hydroxy-5-hydroxy- β -norcholestane-6-carboxaldehyde (ChAld) by the Photooxidation of Cholesterol. ChAld was synthesized as described by Uemi and co-workers.²³ Briefly, cholesterol (200 mg) was dissolved in 20 mL of chloroform in a 100 mL round-bottomed flask, and 250 μ L of methylene blue solution (10 mM in methanol) was added. The solution was ice-cooled and irradiated using two tungsten lamps (500 and 300 W) for 2.5 h under continuous stirring and in an oxygen-saturated atmosphere. ChAld was purified by flash column chromatography using silica gel 60 (230–400 mesh). The column was equilibrated with hexane, and a gradient of hexane and ethyl ether was used. After purification, the solvent was evaporated, and the aldehyde was resuspended in isopropanol and stored at -80°C for further use. ChAld's identity was confirmed by NMR spectroscopy, and its concentration was determined using propionaldehyde as an internal standard.

Cytc Incubation with SDS Micelles. The reaction between cytc and ChAld in the presence of SDS micelles was done in the presence of 10 mM ammonium bicarbonate buffer (pH 7.4). SDS (8 mM final concentration) was added prior to ChAld and cytc. After the addition of SDS, ChAld was added, and the mixture was agitated. After 5 min, cytc was added to the solution, and the reaction was carried out at 37°C under constant agitation. The reaction in the absence of SDS was conducted exactly in the same way without adding SDS.

Tryptic Digestion of Cytochrome *c*. Cytc samples incubated in the absence and presence of ChAld were digested for 18 h with proteomic grade trypsin (Promega) in a 1:50 (w/w) ratio at 37°C .

The reaction medium contained 50 mM ammonium bicarbonate (pH 8.0) and 1 mM CaCl_2 .

MALDI-TOF MS. Cytc samples incubated in the absence and presence of ChAld were analyzed by MALDI-TOF mass spectrometry. Samples were mixed in a 1:4 (v/v) ratio with a saturated solution of α -cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/0.1% aqueous trifluoroacetic acid (1:1, v/v). Approximately 0.5 μ L of the resulting mixture was spotted onto a MALDI target and analyzed by MALDI-TOF MS. The analyses were performed in the linear, positive ion mode in an UltrafleXtreme spectrometer (Bruker Daltonics, Germany) using an acceleration voltage of 25 kV. The resulting spectra were analyzed by flexAnalysis software (Bruker Daltonics, Germany). The instrument was calibrated using an external calibration mixture containing cytochrome *c* and myoglobin as standards (Protein Standard I from Bruker Daltonics). For MALDI-TOF analysis of digested protein, the samples were passed through a C18 ZipTip column (Millipore) and then prepared as described above. Approximately 0.5 μ L of the resulting mixture was spotted onto a MALDI target and analyzed by MALDI-TOF MS. Analyses of the digest were performed in reflector mode in an UltrafleXtreme spectrometer. The spectra were analyzed using the flexAnalysis software (Bruker, Germany) aided by the Biotools and Sequence editor software.

Circular Dichroism (CD). CD measurements were carried out in a Jasco J-720 spectropolarimeter (Easton, MD) using quartz cuvettes of 0.1 cm optical path. The instrument parameters were bandwidth, 1.0 nm; scanning speed, 200 nm/min; response time, 0.25 s; and accumulations, 6. Protein concentration was determined spectrophotometrically at 410 nm ($\epsilon_{410\text{ nm}} 106.1\text{ mM}^{-1}\text{cm}^{-1}$) prior to the spectral acquisition.

Spectroscopic Determinations. Spectrophotometric analyses were carried out in a Varian model Cary 50 Bio spectrophotometer in a 10 mm path length cuvette. Incubations were carried out in the presence of 10 mM bicarbonate buffer (pH 7.4) at 37°C under constant stirring. Incubation times are given in the figure legends. Cytochrome *c* concentrations were determined at 410 nm.³⁸ Spectra of blanks were subtracted from those of samples.

Vesicle Preparation and Binding Experiments. Dry films of a mixture containing dipalmitoylphosphatidylcholine (DPPC), tetraoleoylcardiolipin (TOCL), and either cholesterol (Ch) or ChAld were prepared from stock solutions in methanol. The solvent was evaporated under nitrogen gas (N_2), and the resulting mixture was

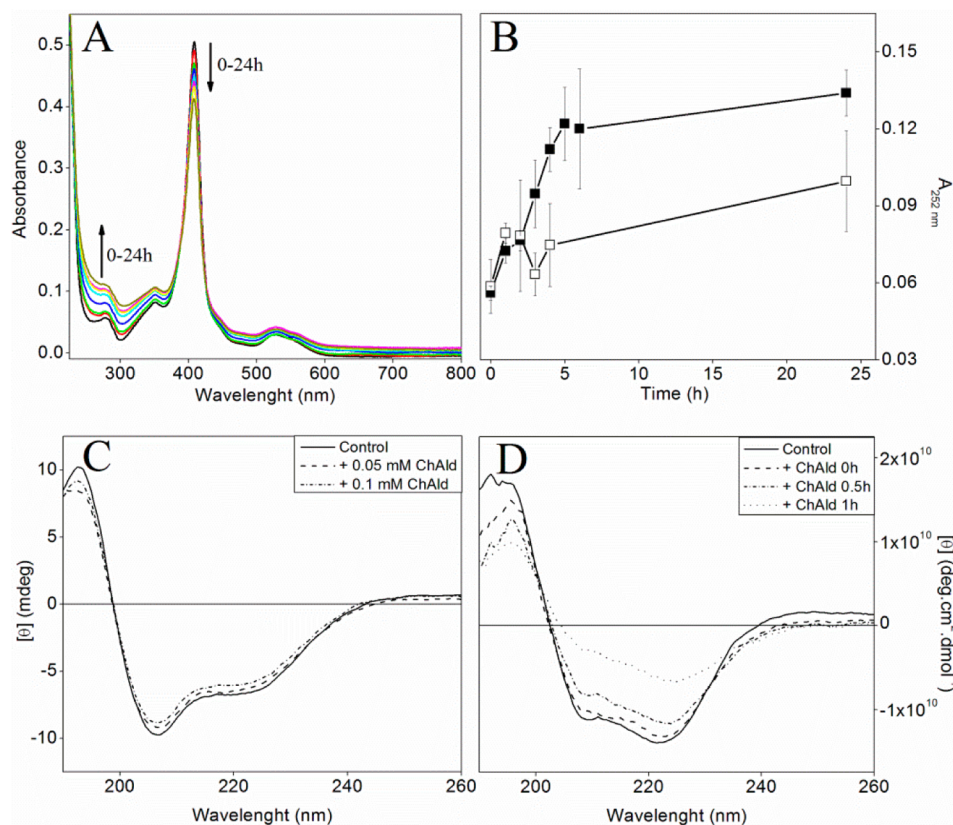


Figure 3. Spectroscopic determinations of cytochrome after the reaction with ChAld. Analyses were conducted in a SDS micelles-containing medium (except for panel D, which was conducted in the absence of SDS micelles) in the presence and absence of ChAld. Incubations were conducted in bicarbonate buffered media (10 mM, pH 7.4) containing 8 mM SDS (except for panel D). Panel A shows scans (190 to 800 nm) of cytc in the presence of ChAld. Experimental conditions: 5 μ M cytc and 50 μ M ChAld (when present). Incubations were conducted for 24 h with the absorbance values recorded hourly in the first 6 h. Panel B depicts the absorbance values recorded at 252 nm along the incubation time in the presence (■) and absence (□) of ChAld. Panels C and D show far-UV CD analyses of cytc before (control) and after incubation with ChAld. In panel C, incubations were conducted for 24 h at 37 °C, under constant agitation. No differences from the time-zero reaction were observed in all experimental conditions. Panel D depicts an initial period of the reaction (up to 1 h) in which major alterations in the protein's secondary structure are observed. The reaction media also contained 10 μ M cytc and 100 μ M ChAld. The spectrum marked cytc control corresponds to the incubation conducted in the absence of ChAld, which remained unaltered through the entire incubation period. Spectrum marked + ChAld 0 h was recorded immediately after the addition of cytc to the medium. All spectra are representative of 3 different analyses.

left under vacuum for 1 h to remove traces of the organic solvents. The lipid films were resuspended in phosphate buffer (50 mM) and agitated for a few minutes. The final lipid concentration was 1 mM (0.7 mM DPPC, 0.1 mM TOCL, and 0.2 mM Ch or ChAld). Unilamellar vesicles with a diameter of about 100 nm were prepared by extrusion through polycarbonate membranes (Avestin). Samples were passed through the membrane 21 times.

Cytc binding to liposomes was measured as described by Oellerich and co-workers.³⁸ Briefly, cytc was incubated with either Ch or ChAld-containing liposomes for 24 h. The samples were then submitted to ultracentrifugation at 104,000g for 4 h at 4 °C using a Beckman Optima TLX ultracentrifuge. Binding was evaluated by the residual content of cytc in the supernatant. Cytc concentration was determined spectrophotometrically at 410 nm.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The procedure of SDS–PAGE was adapted from Laemmli.³⁹ Briefly, cytc incubated in the presence and absence of ChAld was subjected to electrophoresis in 15% acrylamide gel under nonreducing conditions. Gels were prepared with 15% acrylamide, 0.4 M TRIS buffer (pH 8.8), 0.1% ammonium persulfate, and 0.1% SDS. Samples were mixed with the sample buffer (62 mM Tris-HCl buffer (pH 6.8), 10% v/v glycerol, 2% w/v SDS, and 0.01% w/v bromophenol blue) and placed at 95 °C for 5 min for heat denaturation. After the electrophoretic run, the samples were silver stained.

RESULTS

ChAld Covalently Modifies Cytochrome c. Cytc was incubated with ChAld in buffered media (pH 7.4) and then analyzed by MALDI-TOF mass spectrometry. The incubation was conducted in the presence of SDS in order to mimic the interaction between cytc and the mitochondrial membrane. ChAld was incorporated in the micelle according to the protocol described by Rawat and Chattopadhyay,⁴⁰ and then cytochrome c was added to the suspension of ChAld-containing SDS micelles. This incubation led to the formation of two modified cytc species differing by 400 Da, as evidenced by the appearance of two additional peaks in the MS spectrum (Figure 2B). This result is consistent with the addition of either one or two ChAld molecules to cytc. The same experiment conducted in the absence of SDS micelles revealed a much more aggressive modification pattern, in which cytc molecules presenting up to six covalent additions of ChAld were identified (Figure 2D). In the absence of ChAld, no addition was observed either in the presence or in the absence of SDS (Figures 2A and C, respectively). Importantly, ChAld has a molecular weight of 418 Da, and each addition corresponds to 400 Da, indicating a loss of one water molecule. This type of addition is consistent with the formation of Schiff base adducts between the aldehyde

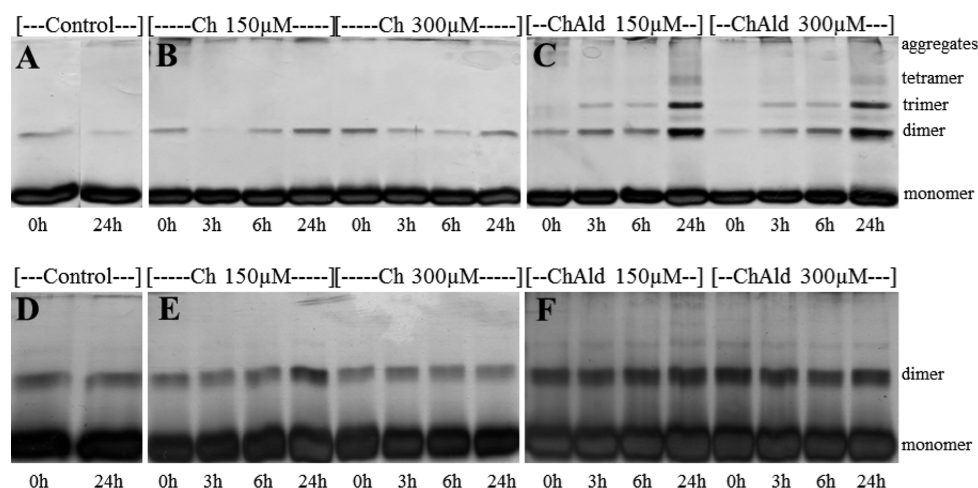


Figure 4. SDS–PAGE of cytc in the absence and presence of SDS. All experimental conditions contain 10 mM bicarbonate buffer (pH 7.4) and 30 μ M cytc. Panels A to C represent the incubations conducted in the absence of SDS micelles. Panels D to F represent the incubations conducted in the presence of SDS micelles (8 mM SDS). Panels A and D are control conditions containing only cytc. Panels B and E represent the conditions in the presence of cholesterol. Panels C and F represent the conditions containing ChAld. Cholesterol was used as a control to show that the aggregation is not associated with the presence of a hydrophobic molecule in solution. Cholesterol and ChAld concentrations are depicted above each gel. Incubation times are shown below each lane. The results are representative of, at least, 3 repetitions.

group and the side chain of basic amino acid residues such as lysine and/or arginine.³³

Schiff Base Adducts and Cytc Conformational Changes. Schiff base adducts (imine bond) are known to absorb light when irradiated at 252 nm.⁴¹ In order to attest the formation of Schiff base adducts between ChAld and cytc, we monitored the reaction for 24 h. Simultaneously, we observed the cytc Soret band. The Soret band had a 2 nm blue shift upon incubation with the SDS micelles, being now centered at 408 nm.³⁶ In the presence of ChAld, a slight bleaching of the Soret band was observed (Figure 3A). The bleaching was accompanied by the increase of the absorbance at 252 nm, consistent with the formation of Schiff base adducts (Figure 3B). An isosbestic point can be observed at 385 nm, suggesting that the bleaching is a consequence of the formation of the Schiff base adducts. Unlike the cytochrome *c* samples incubated with ChAld (closed squares) that presented the progressive increase of absorbance at 252 nm, the incubation of cytochrome *c* in the control conditions (open squares) led to no significant difference within 24 h. Altogether, these observations strongly suggest that ChAld covalently modifies cytc through a Schiff base mechanism. It is worth mentioning that we were unable to use this spectroscopic approach to study the reaction in the absence of SDS because cytc precipitated upon reaction with ChAld. This is an indication that cytc undergoes aggregation upon reaction with ChAld (discussed in more detail below).

Chemical modifications of amino acids are known to induce conformational changes in both secondary and tertiary structures of proteins. We assessed these features by employing CD and fluorescence spectroscopies. CD spectra in the far-UV region showed that the secondary structure of cytc remained unaltered upon incubation with two different concentrations of ChAld for 24 h in the presence of SDS (Figure 3C). Furthermore, intrinsic tryptophan fluorescence experiments revealed that the chemical environment of the fluorophore did not change upon incubation with ChAld and that the amino acid fluorescence remained quenched due to the proximity to the heme iron (Figure S1, Supporting Information).³⁶

However, CD analyses revealed that, in the absence of SDS micelles, cytc had its secondary structure severely altered upon reaction with ChAld (Figure 3D). The major alterations include a loss of α -helix and a gain in β -sheet structures, which may suggest the formation of high molecular weight species. Besides the modifications induced by ChAld, the secondary structure of cytc changes upon incubation with SDS (control conditions at Figures 3C and D), a behavior already observed and discussed by Mugnol and co-workers.³⁶

Cytc Aggregates in the Absence of SDS Micelles. The incubation of cytc with ChAld for 1 h in the absence of SDS micelles leads to the formation of a red precipitate. In order to characterize the aggregation pattern, we performed SDS–PAGE electrophoresis. The experiments were done with two different cytc/ChAld ratios (1:5 and 1:10), and aliquots were taken along the incubation time and analyzed. Cytc itself appears in the gel as two bands, one corresponding to the monomer and the other to the dimer (see reactant information brochure at www.sigmaldrich.com). This electrophoretic pattern remained unchanged along the 24 h of incubation in the control conditions (Figure 4A). In order to test the hypothesis that cholesterol itself could induce cytc aggregation, we incubated cytc with the same amounts of either cholesterol (Ch) or ChAld. The presence of cholesterol did not change cytc electrophoretic pattern under all conditions tested (Figure 4B). However, bands corresponding to species with higher molecular weight appeared when cytc was incubated with ChAld (Figure 4C), correlating aggregation with the reaction with the aldehyde. Cytc aggregation was further confirmed via dynamic light scattering (DLS). This technique revealed that aggregates are formed when cytc is mixed with ChAld (Figure S2, Supporting Information). In addition, making use of FESEM, we observed that amorphous cytc aggregates are formed under these conditions (Figures S3A–D, Supporting Information).

In contrast to the above-described scenario, when cytc is incubated with ChAld in the presence of SDS micelles, no aggregation was observed (Figures 4D to F). The cytc electrophoretic pattern remained unaltered in all experimental

conditions in the presence of SDS micelles, suggesting that aggregation would only occur in conditions in which the modified cytc molecules are released from the mimetic membrane and move randomly in solution.

Cytc Is Modified in Specific Lysine Residues. In order to further characterize these modifications, we attempted to identify the specific amino acid residues that are modified by ChAld. Cytc samples incubated in the presence of SDS micelles, either in the presence or absence of ChAld, were digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry (Figure 5, upper panel).

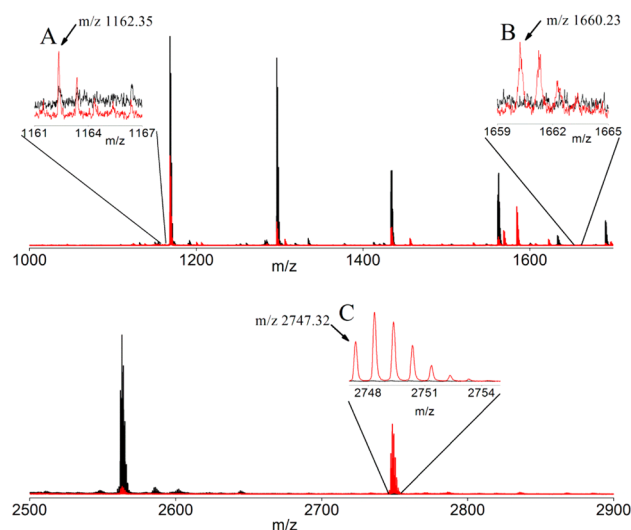


Figure 5. Sections of MALDI-TOF-MS of the peptides resulting from the tryptic digestion of cytc before (black spectra) and after (red spectra) incubation with ChAld. Typical reactions contained bicarbonate buffer (10 mM, pH 7.4), 8 mM SDS, 50 μ M cytc and 500 μ M ChAld. Spectra are representative of, at least, 3 different experiments. The upper panel shows the tryptic digestion of the wild type cytc. The lower panel shows the tryptic digestion of the K22A mutant. Panels A and B show mass peaks (m/z 1162.35 and 1660.36) found only after the incubation of WT cytc with ChAld. Panel C shows a mass peak with m/z 2747.32 found only after the incubation of ChAld with K22A cytc. Note that in the control conditions (black spectra in panels A–C) no peaks were detected. See Table 1 for more details concerning the tryptic digestion of cytc.

The peptides from each sample were analyzed using the peptide mass fingerprinting (PMF) approach. Briefly, PMF consists of matching the experimental peptides to theoretical peptides generated from protein digestion, usually aided by computer software (in this case, MASCOT, Matrix Science Inc., Boston, MA, USA).⁴² PMF analyses revealed that two peptides in the native protein sequence were covalently modified by ChAld

(see Table 1 for more details). The unmodified peptides have m/z ratios of 762.49 and 1260.58, and the corresponding peaks were identified in the mass spectrum (see Table 1). Incubation with ChAld leads to the appearance of a new peak with m/z 1162.35 corresponding to the modified peptide K*IFVQK (residues 8–13) and another peak with m/z 1660.36 corresponding to the modified peptide CAQCHTVEK*GGK (residues 14–25) (Figure 5A and B, respectively). An MS/MS analysis of the peptide containing Lys22 revealed that this residue is covalently modified by ChAld (Figure S4, Supporting Information). In order to confirm that Lys22 is indeed the modified residue (see Table 1 for more details) in this sequence, we performed the same experiments with a K22A cytc mutant, in which Lys22 was replaced by Ala (Figure 5, lower panel, and Figure S6, Supporting Information). These results show that the peptide mentioned above (residues 14–25) is no longer modified at any of its residues. However, a new covalent addition of ChAld was observed at Lys13 of the K22A cytc mutant, which replaced the modification at Lys22 (Figure 5C).

In addition to the modification observed in Lys13, a modification in Lys8 was also observed at incubations of ChAld with K22A cytc. The mass peak observed in Figure 5C corresponds to the sequence KIFVQKCAQCHTVEAGGK (residues 8 to 25 in the K22A cytc mutant), in which the lysine residues at positions 8 and 13 are covalently modified by ChAld (see Figure S5, Supporting Information, for the theoretical simulation of the observed mass peak in Figure 5C). Another evidence that the residues mentioned above (Lys 8, 13, and 22) are indeed modified by ChAld is that they were not recognized by trypsin, characterizing missing cleavage sites. Since trypsin cleaves after K and R residues, a modification would render them unrecognizable by the enzyme.

ChAld Impairs Cytc Release from Liposomes. In order to study the effect of these covalent additions on cytc binding to membranes, we performed experiments using a liposome model and making use of ultracentrifugation, as described by Oellerich and co-workers.³⁸ Briefly, liposomes containing 10% mol/mol TOCL, 70% mol/mol DPPC, and either ChAld (20% mol/mol) or cholesterol (as a control condition 20% mol/mol) were incubated with cytc for 24 h. After the incubation time, samples were centrifuged at 104,000g for 4 h, and the absorbance of the supernatant was recorded at 410 nm. The results presented in Figure 6 show that cytc binds to the liposome containing TOCL in the presence of either cholesterol or ChAld, as revealed by the decrease in cytc concentration in the supernatant after centrifugation (in both conditions, 22% of cytc was released from the liposome). An increase in the ionic strength before centrifugation (by adding 250 mM KCl) released cytc from cholesterol-containing

Table 1. Modified Peptides Identified by MALDI-TOF MS after Tryptic Digestion^a

sequence ^b	residues	unmodified ^c m/z	expected ^d m/z	observed ^e m/z
K*IFVQK	8–13	762.49	1162.82	1162.35
CAQCHTVEK*GGK	14–25	1260.58	1660.91	1660.36
K*IFVQK*CAQCHTVEAGGK	8–25	1946.99	2747.66	2747.34

^aAll m/z depicted at the table correspond to the $[M + H]^+$ values. It is worth noticing that another suggestion that the residues marked with * are indeed modified by ChAld is that they were not recognized by trypsin. Note that trypsin cleaves the peptide bond after K and/or R residues.

^bSequence of the modified peptide. Residues marked with * were covalently modified by ChAld. The alanine residue underlined in the third sequence corresponds to the K22A mutation. ^cValues correspond to the theoretical m/z for the unmodified peptide. ^dValues correspond to the expected m/z for the ChAld-modified peptide. ^eValues correspond to the observed m/z for the ChAld-modified peptide.

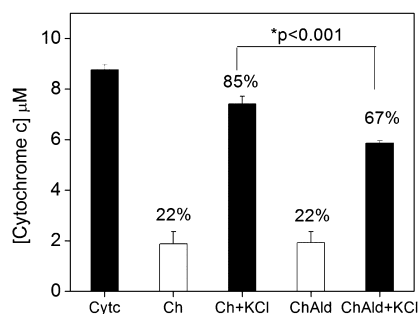


Figure 6. Binding of cytc to ChAld-containing liposomes. Incubations were conducted in phosphate buffered media (10 mM, pH 7.4) at 37 °C. After 24 h of incubation, samples were centrifuged at 104,000g for 4 h at 4 °C. The final lipid content was 1 mM (700 μM of DPPC, 100 μM of TOCL, and 200 μM of either ChAld or cholesterol (Ch)), 10 μM cytc, and 250 mM KCl (when present). The first condition (Cytc) refers to the incubation conducted in absence of the liposomes. The second condition (Ch) refers to the incubation conducted in the presence of cholesterol-containing liposomes. The third condition (Ch + KCl) is the same as Ch with the addition of KCl after 24 h of incubation and prior to centrifugation. The fourth (ChAld) and fifth (ChAld + KCl) conditions were equal to Ch and Ch + KCl, respectively, where cholesterol was replaced by ChAld.

liposomes (85% of total cytc was released upon treatment with KCl), showing that the interaction between the protein and the vesicle is mostly electrostatic. However, the increase in ionic strength in liposomes containing ChAld had a smaller effect in releasing cytc from the membrane (only 67% of total cytc was released upon treatment with KCl), suggesting that the covalent modification is preventing its release from liposomes.

DISCUSSION

Oxidative stress is known to be associated with pathological conditions such as cancer and Alzheimer's and Parkinson's diseases, situations recognized to produce increased amounts of lipid peroxidation products, including reactive aldehydes.^{20,21,25} Proteins are excellent targets for modification by lipid aldehydes, mainly by covalent addition to nucleophilic amino acid residues.^{33–35} Among these aldehydes, ChAld is of major interest since it is reported to be increased *in vivo* in cells under pathological conditions.⁴³ ChAld can react with several proteins, including those attached to the inner mitochondrial membrane, such as cytc. Since cytc integrity is vital for many physiological processes, its modification upon reaction with ChAld could have implications to the cell, including impairment of both electron transport and apoptosis signaling.

The production of ChAld *in vivo* can occur by three different mechanisms, in which cholesterol is oxidized by ozone and/or singlet molecular oxygen.^{23,25} Briefly, ChAld can be formed by (i) Hock cleavage of cholesterol 5 α -hydroperoxide;²⁴ (ii) ozonolysis;²⁵ and (iii) [2 + 2] cycloaddition to cholesterol followed by dioxetane thermolysis, originating CSec and, ultimately, ChAld.⁴⁴ In this sense, quantitative analysis of human arterial plaque extracts revealed the presence of approximately 32 \pm 15 pmol/mg tissue.²⁷ Moreover, these aldehydes have also been detected at nanomolar levels in stimulated neutrophils.⁴⁵

Cytc modification has been previously studied using other lipid-derived aldehydes, such as HNE,³³ DDE,³⁴ and trans-2-hexenal.⁴⁶ In the study with HNE-induced cytc modification, Isom and co-workers, making use of mass spectrometry,

identified three covalent additions of HNE, where the modified residues were His33, Arg38, and Lys87. In the study regarding DDE-induced cytc modification, Sigolo and co-workers reported up to seven time-dependent covalent additions of DDE to cytc (H18, K22, K25, K73, K86, K87, and K99 were the supposed modified sites), a behavior similar to that observed in this study in the absence of SDS micelles. Moreover, the authors also identified Lys22 as a possible modified residue, which is the same residue found modified by ChAld using the SDS mimetic model, a residue known to participate in protein–protein and protein–membrane interactions. It is worth mentioning, however, that all studies mentioned above characterized cytc modifications promoted by lipid aldehydes in experiments conducted in the absence of mimetic membranes. In this situation, cytc is moving randomly in the solution, and therefore, the modification sites are spread along the entire sequence of the protein.⁴⁶ In our conditions the aldehyde was inserted in the SDS micelles which, in turn, bind cytc at specific sites on its surface. In this situation, cytc is approximated to the aldehyde, which guides the reaction to specific residues, particularly residues situated at the binding sites of cytc.

Since cytc is positively charged (isoelectric point 10.2), the interaction between this protein and lipid membranes carrying negatively charged phospholipids is initially guided by electrostatic interactions.^{2,47,48} These interactions are assumed to occur through three sites in the protein surface, named A, C, and L sites, corresponding to 30% of the protein surface.^{6,47,48} Site A comprises a basic portion on the protein containing the positively charged Lys72 and Lys73 residues and is characterized as contributing to electrostatic and lipid-extended interactions between cytc and phospholipid membranes.^{49,50} Site C, in turn, is assumed to interact through a hydrogen bond between the invariant Asn52 and protonated phospholipids in the membrane exposed to acidic media.^{6,48} Site L has its interaction with acidic phospholipids modulated by the pH of the medium, in which lysine residues 22 and 27 play a pivotal role in the attachment of cytc to membranes.⁴ These two lysine residues have their side chain in a convergent position toward the membrane, which induces a lowering of their ϵ -amino pK_a values (around 6.0).⁴ The pK_a values of lysine residues of cytc site L indicate that these residues are predominantly protonated at the inner mitochondrial membrane interface when the medium is acidified by proton pumping to the intermembrane space. However, the increase of pH in the intermembrane space promoted by the loss of pH gradient changes the acid–base equilibrium of these ϵ -amino groups to the deprotonated base conjugated form.⁴ In this sense, the identification of Lys22 as a modified residue can be partially explained by the low pK_a value of Lys22 since the reaction between the amino group and the aldehyde can only occur when the amino group is deprotonated.

Cholesterol overload to the mitochondria is suggested to decrease mGSH levels, increasing oxidative stress inside the organelle and favoring, for instance, lipid peroxidation processes.^{9,21} In this situation, cholesterol aldehyde production could be enhanced, and the reaction with proteins, including cytc, would be more likely to occur. The result presented in Figure 6 reveals that the release of cytc from cardiolipin-containing liposomes is impaired when ChAld is added to the liposome. The increase in ionic strength (i.e., by adding 250 mM KCl) is known to disrupt the electrostatic interactions between the protein and the membrane, releasing cytc to the

bulk solution. In the presence of ChAld, however, less cytc is released into solution, suggesting that adduction could interfere with the release of cytc by keeping it covalently attached to the aldehyde under oxidative stress conditions. Other types of aldehydes, such as aldehydes formed from phospholipids (e.g., cardiolipin) could also contribute to covalently anchor cytc to the membrane. This hypothesis is under investigation in the laboratory. Although not further investigated in this study, cytc adduction to membrane lipids could have an impact on apoptosis signaling, contributing, for instance, for the resistance of cancerous cells to undergo apoptosis.

In conclusion, our data indicate that cytc can be a potential target for cholesterol-aldehyde-induced protein modification. When cytc is bound to mimetic biological membranes, the modifications are more restricted and occur at specific lysine residues in the protein, namely, Lys22 located at a pH-sensitive binding site of cytc. Although protein adduction seems to occur through a Schiff base formation (formation of an imine bond), further characterization is needed in order to confirm this mechanism. We were also able to demonstrate that these modifications impair cytc detachment under high ionic strength conditions by keeping this protein covalently bound to the aldehyde present in the membrane. The extension of the biological implications of these modifications is still unknown. However, several hypotheses can be made based on the results reported herein, including impairment of the electron transport and cytc release under an apoptosis stimulus. Further experiments are underway in order to find out the implications of these adductions to the cell.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental procedures for FESEM, DLS, and fluorescence spectroscopy and figures of fluorescence spectroscopy, DLS, FESEM, MS/MS spectrum of the modified peptide, simulation of the MS peak corresponding to the modified peptide in the K22A cytc mutant, and an expanded version of the MS spectrum shown in Figure 5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ChAld, 3 β -hydroxy-5 β -hydroxy-B-norcholestane-6 β -carboxaldehyde; cytc, cytochrome c; SDS, sodium dodecyl sulfate; mGSH, mitochondrial reduced glutathione; ROS, reactive oxygen species; CSec, 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al; O₃, ozone; ¹O₂, singlet molecular oxygen; CL, cardiolipin; LDL, low density lipoprotein; HNE, 4-hydroxynonenal; DDE, 2,4-decadienal; MALDI, matrix assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; HCCA, alpha-cyano-4-hydroxycinnamic acid; CD, circular dichroism; DPPC, dipalmitoylphosphatidylcholine; TOCL, tetraoleoylcardiolipin; Ch, cholesterol; FESEM, field emission scanning electron microscopy; DLS, dynamic light scattering; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PMF, peptide mass fingerprinting

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