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PAPER

Cytochrome c-promoted cardiolipin oxidation generates singlet molecular oxygen†

Sayuri Miyamoto,*[a] Iseli L. Nantes,[b] Priscila A. Faria,[b] Daniela Cunha,[a] Graziella E. Ronsein,[a] Marisa H. G. Medeiros[a] and Paolo Di Mascio*[a]

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The interaction of cytochrome c (cyt c) with cardiolipin (CL) induces protein conformational changes that favor peroxidase activity. This process has been correlated with CL oxidation and the induction of cell death. Here we report evidence demonstrating the generation of singlet molecular oxygen [$O_2(^1\Delta_g)$] by a cyt c–CL complex in a model membrane containing CL. The formation of singlet oxygen was directly evidenced by luminescence measurements at 1270 nm and by chemical trapping experiments. Singlet oxygen generation required cyt c–CL binding and occurred at pH values higher than 6, consistent with lipid–protein interactions involving fully deprotonated CL species and positively charged residues in the protein. Moreover, singlet oxygen formation was specifically observed for tetralinoleoyl CL species and was not observed with monounsaturated and saturated CL species. Our results show that there are at least two mechanisms leading to singlet oxygen formation: one with fast kinetics involving the generation of singlet oxygen directly from CL hydroperoxide decomposition and the other involving CL oxidation. The contribution of the first mechanism was clearly evidenced by the detection of labeled singlet oxygen [$^{18}O_2(^1\Delta_g)$] from liposomes supplemented with 18-oxygen-labeled CL hydroperoxides. However quantitative analysis showed that singlet oxygen yield from CL hydroperoxides was minor (<5%) and that most of the singlet oxygen is formed from the second mechanism. Based on these data and previous findings we propose a mechanism of singlet oxygen generation through reactions involving peroxyl radicals (Russell mechanism) and excited triplet carbonyl intermediates (energy transfer mechanism).

Introduction

Cytochrome c (cyt c) is a small globular heme-protein (13 kDa) present in the mitochondrial intermembrane space. Besides its well established role as an electron carrier between complexes III and IV of the mitochondrial respiratory chain, studies conducted over the last twenty years established new functions for this protein. Cyt c participates in programmed cell death,[1,2] induces fatty acid amidation,[3,4] catalyzes *S*-nitrosothiol formation,[5] and displays peroxidase/oxygenase activity especially when bound to anionic phospholipids.[6] Recently attention has been focused on cyt c peroxidase activity, which has been associated with selective cardiolipin (CL) oxidation and induction of cyt c detachment from the inner mitochondrial membrane.[6–9]

Within mitochondria, a significant proportion of cyt c is bound to the inner membrane.[9–15] Cyt c has a net positive charge at neutral pH and strongly interacts with membranes containing acidic phospholipids, such as CL. Cardiolipin is a unique anionic dimeric phospholipid found almost exclusively in the inner mitochondrial membrane. In most mammalian tissues CL is comprised of four unsaturated fatty acids, namely linoleoyl acyl chains.[16,17] Importantly, binding to CL plays an essential role in determining the conformational and catalytic properties of cyt c.

It is well known that native cyt c has a compact globular structure with a hexacoordinated heme iron, which does not allow the interaction of heme iron with external ligands. However, cyt c interaction with negatively charged CL induces protein conformational changes leading to the opening of the heme crevice, disruption of the heme sixth ligand (Fe–Met80 bond),[18] and alterations in the heme iron spin state,[19] dramatically altering cyt c redox properties and reactivity. For example, the loss of the Met80 ligand enables exogenous ligands, such as H_2O_2 and other hydroperoxides, to access the heme catalytic site and trigger peroxidase activity on the protein.[6] Notably, the rate constants for amplex red oxidation by the cyt c–CL complex were

[a]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP26077, CEP 05513-970 São Paulo, SP, Brazil. E-mail: miyamoto@iq.usp.br, pdmascio@iq.usp.br; Fax: +55 11 3815 5579; Tel: +55 11 3091 8497*
[b]*Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Rua Santa Adélia 166, Bairro Bangu, CEP 09210-170 Santo André, SP, Brazil*

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about three orders of magnitude higher in the presence of free fatty acid hydroperoxides ($\sim 10^4$ M^{-1} s^{-1}) than with hydrogen peroxide ($\sim 10^1$ M^{-1} s^{-1}).[20]

Singlet oxygen [$O_2(^1\Delta_g)$] is an excited state of molecular oxygen that plays an important role in photodynamic therapy[21,22] and in the destruction of invading microorganisms during phagocytosis.[23–25] Several photochemical and non-photochemical reactions have been shown to produce singlet oxygen in biological systems.[22,24,26,27] In previous studies, we have demonstrated that biologically relevant hydroperoxides, such as lipid hydroperoxides, generate singlet oxygen in the presence of metal ions, peroxynitrite and hypochlorous acid.[27–30]

In the present study, we have investigated whether cyt c–CL interaction could induce the generation of singlet oxygen. Chemical trapping and luminescence measurements at 1270 nm clearly showed the generation of singlet oxygen in cyt c incubation with CL liposomes. Quantitative analysis revealed the existence of at least two sources of singlet oxygen in our model, one directly related to CL hydroperoxide decomposition and another to CL oxidation. Several experiments were conducted to elucidate the main mechanism responsible for the singlet oxygen generation, including experiments using 18-oxygen labeled CL hydroperoxides.

Materials and methods

Materials

Cytochrome c (cyt c; from bovine heart), dimyristoyl phosphatidylcholine (DMPC), bovine heart cardiolipin (CL), egg yolk phosphatidylcholine (EYPC), diethylenetriaminepentaacetic acid (DTPA) and sodium azide were obtained from Sigma (St. Louis, MO). Deuterium oxide (D_2O, 99.9%) was purchased from Aldrich (Steinheim, Germany). Tetramyristoyl (TMCL) and tetraoleoyl cardiolipin (TOCL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The disodium salt of anthracene-9,10-diyldiethane-2,1-diyl disulfate (EAS) and 3,3′-(1,4-epidioxynaphthalene)-1,4-diylbis-[*N*-(2,3-dihydroxypropyl)propanamide] ($DHPNO_2$) were synthesized as described by Di Mascio and Sies[31] and Martinez *et al.*[32] respectively. Standard EAS endoperoxide ($EASO_2$) was prepared by photooxidation in D_2O and purified by HPLC using the conditions described for $EASO_2$ analysis and quantified spectrophotometrically (ε_{212} = 40 738 M^{-1} cm^{-1}).[32] Deuterated phosphate buffer at specified pD values (pD=pH + 0.4) was prepared by mixing stock solutions of K_2HPO_4 and KH_2PO_4 prepared in D_2O. All solvents were of HPLC grade and were acquired from Mallinckrodt Baker (Phillipsburg, NJ).

Purification of CL and determination of hydroperoxide content in CL stock solutions

CL purification was done on a reversed phase C8 column (150 × 6 mm, 5 μm particle size; Tosoh, Japan) at a flow rate of 1 mL min^{-1} using a gradient of methanol (solvent B) in 10 mM ammonium formate (solvent A) as follows: 90% B for 5 min, 90% to 100% B in 20 min, 100% B for 5 min and 100% to 90% B in 5 min. The eluent was monitored at 205 nm and 235 nm for CL and CL hydroperoxide (CLOOH) detection (Fig. S1, ESI†). Peaks corresponding to CL and CL mono- [$CL(OOH)_1$], di- [$CL(OOH)_2$], and tri-hydroperoxides [$CL(OOH)_3$] appeared at 35, 13, 21 and 29 min, respectively. Purified CL was obtained by collecting the peak at 35 min. Collected fractions were concentrated using a rotary evaporator and the final CL concentration was determined by a phosphorus assay using the modified micro-procedure of Bartlett.[33] To determine CLOOH concentration in CL stock solutions, hydroperoxide standard samples were prepared by photooxidation, purified by HPLC and quantified by the phosphorus assay. Using these standards, a calibration curve was constructed for $CL(OOH)_1$, $CL(OOH)_2$ and $CL(OOH)_3$.

Synthesis of 18-oxygen-labeled CL hydroperoxides

Labeled CL hydroperoxides ($CL^{18}O^{18}OH$) were prepared by the photosensitized oxidation of CL under an 18-oxygen ($^{18}O_2$) atmosphere by a similar procedure to that described for fatty acid hydroperoxides.[28] Briefly, CL solution was completely degassed and irradiated in the presence of methylene blue in a closed system saturated with $^{18}O_2$. The 18-oxygen-labeled CL mono-, di-, and tri-hydroperoxides [$CL(^{18}O^{18}OH)_1$, $CL(^{18}O^{18}OH)_2$, $CL(^{18}O^{18}OH)_3$] were purified by HPLC and the incorporation of 18-oxygen into the hydroperoxides was confirmed by MS analysis (Fig. S2, ESI†).

Liposome preparation

Large unilamellar vesicle liposomes were prepared by an extrusion technique[34] using a mixture of DMPC and CL (1 : 1, mol/mol; to a final concentration of 5 mM each). Briefly, vesicles were prepared by mixing individual phospholipids in methanol or ethanol followed by complete solvent evaporation under nitrogen gas and vacuum for 1 h. The thin lipid film was hydrated in phosphate buffer by vortexing for 1 min and extruded 21 times through a polycarbonate membrane to obtain large unilamellar vesicles of a defined size (100 nm).[35] All buffers contained 100 μM of DTPA to avoid reactions involving free metal ions. For the preparation of liposomes containing labeled hydroperoxides, 1 μmol of DMPC was mixed with 0.6 μmol of purified CL and 0.4 μmol of $CL(^{18}O^{18}OH)_1$, $CL(^{18}O^{18}OH)_2$, or $CL(^{18}O^{18}OH)_3$. This lipid mixture was dried and hydrated in 200 μL of 10 mM deuterated phosphate buffer at pD 8.4 to obtain final liposome preparations containing 5 mM DMPC, 3 mM CL and 2 mM labeled CL hydroperoxides. All liposomes were prepared just before the experiment and diluted to get a final concentration of 1 mM CL.

Detection of singlet oxygen monomol emission

Monomolecular photoemission of singlet oxygen at 1270 nm was recorded using a photocounting apparatus described previously.[28,29] All reactions were conducted in a quartz cuvette under continuous stirring at room temperature. Typically, cuvettes were filled with a 1.35 mL DMPC–CL liposome solution (1 : 1; to a final concentration of 2 mM each) in 10 mM phosphate buffer at pD 7.8 containing 100 μM DTPA. After recording the baseline, 150 μL of a 1 mM cyt c solution in D_2O

was manually injected through a small polyethylene tube. The area under the emission signal was integrated and singlet oxygen yield was estimated using $DHPNO_2$ as a standard clean source of singlet oxygen (Fig. S3, ESI†).[30,36]

Detection of singlet oxygen by chemical trapping

Chemical trapping experiments were conducted in the presence of 8 mM EAS, a concentration at which EAS traps 50% of available singlet oxygen.[31] Incubations containing liposomes, cyt c and EAS were continuously mixed in a Thermomixer® (Eppendorf AG, Hamburg, Germany) at 37 °C for up to 2 h of incubation. Before analysis, aliquots of the samples were filtered through Ultrafree MC-5000 NMWL filter units (Millipore Corporation, Bedford, MA) at 7900*g* for 30 min. For the HPLC analysis, 10 μL of the filtered sample was injected into the Shimadzu Prominence HPLC system composed of two HPLC pumps (LC-20AT), an autoinjector (SIL-20A) and a system controller (CBM-20A). Endoperoxides were separated on a reversed-phase C18 column (Gemini, 250 × 4.6 mm, 5 μm particle size; Phenomenex, CA), using a gradient of acetonitrile (solvent B) and 25 mM ammonium formate (solvent A). Elution was started with solvent B at 19% for 20 min, then increased to 60% in 2 min, maintained at 60% for 4 min and finally restored to 19% in 2 min. The flow rate was set to 1 mL min^{-1} and the eluent was monitored at 215 nm. For MS/MS analysis, the eluent from the column was split to send 10% of the flow rate into the mass spectrometer (Quattro II triple quadrupole, Micromass, Manchester, UK). Analysis was done with electrospray ionization in the negative ion mode. The source temperature of the mass spectrometer was held at 150 °C, the cone voltage was 15 V, the collision energy was set to 10 eV, and the capillary and the electrode potentials were set to 3.5 and 0.5 kV, respectively. Unlabeled and labeled endoperoxides were specifically detected in the multiple reaction monitoring (MRM) mode by detecting the loss of an oxygen molecule from the doubly negatively charged endoperoxide according to the following: $EAS^{16}O^{16}O$, *m*/*z* 228 → 212, $EAS^{16}O^{18}O$, *m*/*z* 229 → 212, $EAS^{18}O^{18}O$, *m*/*z* 230 → 212.

Quantification of CL and CL hydroperoxides by HPLC-MS/MS

Liposomes (DMPC–CL 1 mM : 1 mM final concentration) were incubated with cyt c (50 μM) in phosphate buffer (10 mM, pD 7.8 containing 100 μM of DTPA) at 37 °C for up to 2 h. At specified times, 80 μL of the sample were mixed with 20 μL of TMCL (0.5 mM) and extracted with 300 μL of chloroform–methanol (2 : 1, vol/vol) containing 1 mM BHT. After vigorous vortexing, the organic phase was collected, evaporated under a stream of nitrogen and dissolved in 400 μL of methanol. For the analysis an aliquot of 30 μL was injected into the HPLC-MS/MS. HPLC separation was carried out on a reversed phase C8 column (150 × 6 mm, 5 μm particle size; Tosoh, Japan) using the gradient described for CL purification. For MS analysis, the eluent from the column was monitored at 235 nm and then split to send 10% of the flow rate into the mass spectrometer. Analysis in the mass spectrometer was done using electrospray ionization in the negative ion mode. The source temperature of the mass spectrometer was held at 100 °C, the cone voltage was 30 V, the collision energy was set to 30 eV, and the capillary and the electrode potentials were set to 3.0 and 0.5 kV, respectively. Full scan data were acquired over a mass range of 200–1600 *m*/*z*. MRM analysis of CL and its hydroperoxides was done by monitoring the following transitions: *m*/*z* 724 to 279 for TLCL, *m*/*z* 620 to 227 for TMCL, *m*/*z* 740 to 279 for CL monohydroperoxide [$CL(OOH)_1$], *m*/*z* 756 to 279 for CL dihydroperoxide [$CL(OOH)_2$] and *m*/*z* 772 to 279 for CL trihydroperoxide [$CL(OOH)_3$]. For the calibration curve a dilution series of TLCL and CL hydroperoxide standards containing 26.5 μM of TMCL were prepared.

Results

Singlet molecular oxygen detection by chemical trapping

The generation of singlet oxygen by the cyt c–CL system was first investigated using the water-soluble chemical trap, EAS. The reaction of EAS with singlet oxygen yields a stable endoperoxide ($EASO_2$), which was detected and quantified by reversed-phase HPLC. Fig. 1A shows a typical chromatogram of EAS and $EASO_2$ analysis for the reaction system consisting of cyt c and CL-liposomes. A peak corresponding to $EASO_2$ was detected at approximately 11 min (Fig. 1A, line a). This peak was not detected in control incubations containing only liposomes, cyt c or EAS (Fig. 1A, lines b, c and d). Moreover, the identity of the peak assigned as $EASO_2$ was confirmed by mass spectrometry, which exhibited two major ions, one at *m*/*z* 228 and another at 212, corresponding to the doubly charged molecular ions of the $EASO_2$ molecule ($[M - 2H]^{2-}$) and its fragment ($[M - 2H - 2O]^{2-}$) formed by the loss of two oxygen atoms from the endoperoxide (Fig. 1B).

To evaluate the time-dependent formation of singlet oxygen by the cyt c–CL system we measured the cumulative formation of $EASO_2$ up to 2 h incubation. The plot of $EASO_2$ *versus* time showed a hyperbolic curve, with a *plateau* being attained after 1 h incubation (Fig. 1C). The yield of $EASO_2$ determined at the *plateau* was 71 ± 6 μM. Considering that EAS traps only 50% of the singlet oxygen generated,[31] the estimated yield of singlet oxygen for the incubation containing 50 μM cyt c and 1 mM CL was 142 ± 12 μM. This value corresponds to 14% of the CL content and almost three times the amount of cyt c present in the reaction system.

Previous studies showed that the lipid to protein ratio affects cyt c conformational changes, heme coordination state and reactivity.[19] To investigate the influence of the CL/cyt c ratio on singlet oxygen yield, increasing concentrations of cyt c were added to a fixed concentration of CL liposomes (1 mM). The plot of singlet oxygen yield *versus* the CL/cyt c ratio exhibited a quadratic hyperbolic profile. Singlet oxygen yield increased at higher lipid to protein ratios, reaching a saturation level at a CL/cyt c ratio greater than 100 : 1 (Fig. 1D). Zucchi *et al.* observed that high lipid/protein ratios induce alterations in the cyt c spin state from the native low spin state to the "alternative low-spin form". This state has a more open heme crevice and thus is able to react more efficiently with peroxides.[15,19] As a result, the increased generation of singlet oxygen at high lipid/

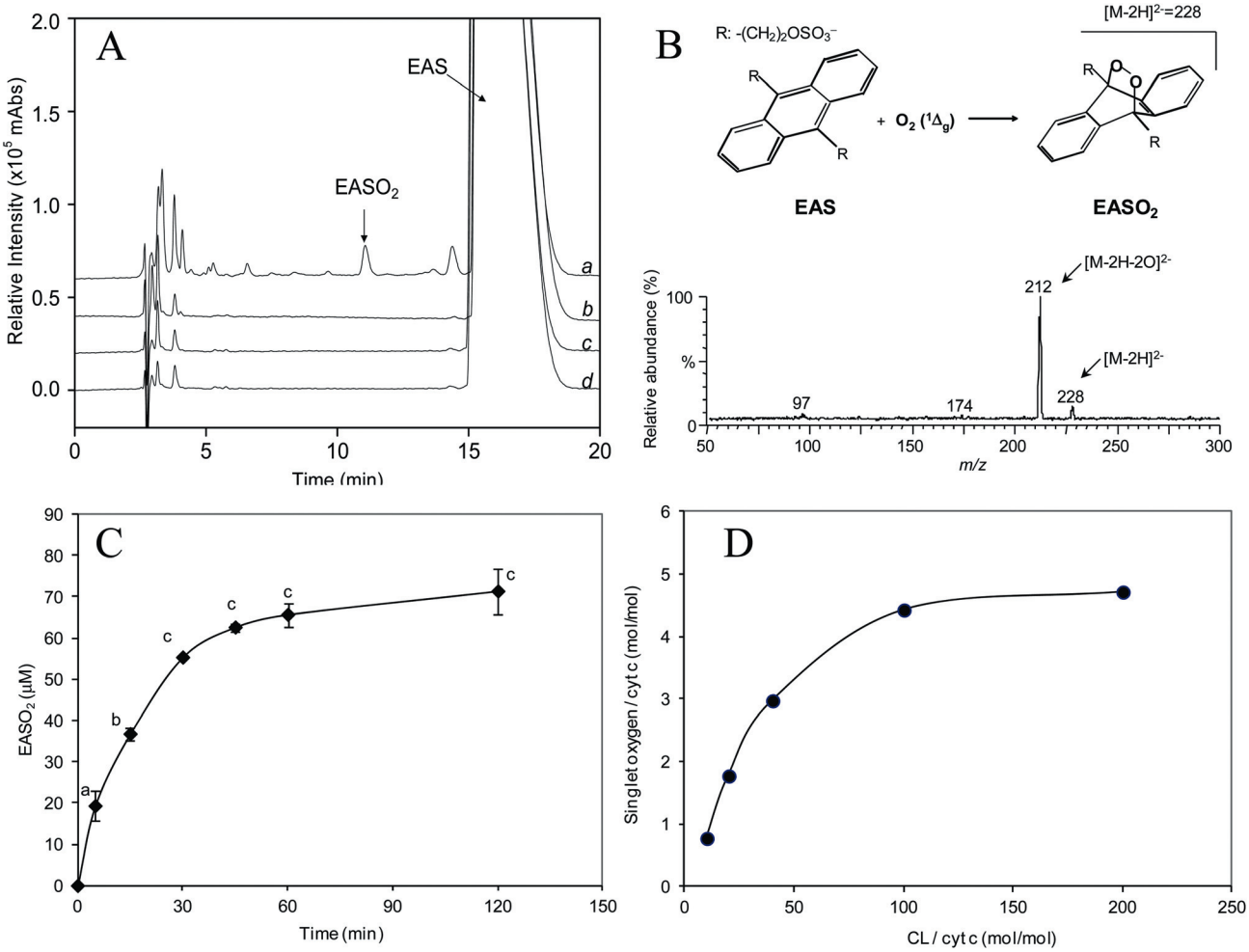


Fig. 1 Singlet oxygen detection in cyt c–CL incubations by chemical trapping. All experiments were carried out in the presence of 8 mM of the chemical trap EAS in 10 mM deuterated phosphate buffer, pD 7.8 at 37 °C. The CL liposomes used for the experiments consisted of DMPC–CL (1 mM : 1 mM, final concentration). A. Typical chromatogram of $EASO_2$ detection for the complete reaction system consisting of CL liposomes and 50 μM cyt c (a); and for incubations containing CL liposomes (b); cyt c (c); and EAS only (d). B. Scheme of the reaction of EAS with singlet oxygen generating $EASO_2$ and mass spectrum of the peak detected at 11 min. C. Time-course analysis of $EASO_2$ formed in the incubation of 1 mM CL liposomes with 50 μM cyt c. Results are expressed as mean ± SD ($n = 3$, values with different letters are significantly different, $p < 0.05$, t-test). D. Effect of the lipid/protein ratio on singlet oxygen formation. Increasing concentrations of cyt c were incubated with a fixed concentration of 1 mM CL liposomes. Representative data from a duplicate experiment.

protein ratios is probably related to the more efficient conversion (activation) of cyt c to the "alternative low-spin form".

Effect of phospholipid and fatty acyl type on singlet oxygen generation

Cyt c–CL binding involves both electrostatic and hydrophobic interactions. To investigate the significance of protein binding for singlet oxygen generation, CL was replaced by egg yolk PC, a zwitterionic phospholipid. As shown in Fig. 2, endoperoxides were not detected in incubations containing only PC, indicating that cyt c electrostatic binding to the negatively charged CL is critical for singlet oxygen generation. This hypothesis was also confirmed by experiments using high ionic strength buffers. Singlet oxygen formation was reduced by more than 70 and 90% in the presence of 100 and 250 mM of KCl, respectively. These data show that salt-induced dissociation of the cyt c–CL complex inhibits the generation of singlet oxygen, pointing to the essential role of cyt c–CL electrostatic interactions in this reaction.

It has been shown that the approximation of cyt c to CL through electrostatic interactions is followed by partial or complete insertion of the protein into the phospholipid membrane.[13,37] While still not completely understood, cyt c–CL hydrophobic interactions involve the insertion of one or two fatty acyl chains of CL into a hydrophobic channel in the protein.[37] As a result, cyt c reactivity is also directly affected by the type and nature of the lipid acyl chain.[19,38] To test the influence of fatty acid type we compared the formation of singlet oxygen in liposomes containing saturated (TMCL), monounsaturated CL (TOCL), or polyunsaturated CL species (TLCL). Interestingly, singlet oxygen was only observed for the cyt c incubations with polyunsaturated CL species and a link between

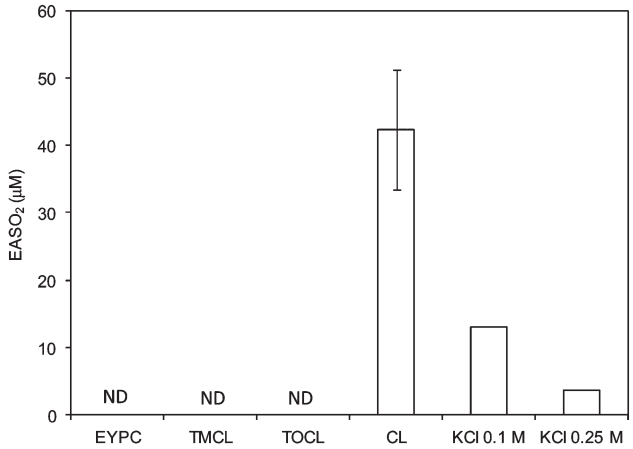


Fig. 2 Effect of phospholipid type and increasing ionic strength on singlet oxygen generation. Cyt c (50 μM) was incubated with liposomes prepared with DMPC and different phospholipids (EYPC, TMCL, TOCL or bovine heart CL) at a 1 : 1 molar ratio and a final concentration of 1 mM of each phospholipid. The effect of increasing ionic strength was tested by incubating DMPC–CL liposomes in the presence of 100 and 250 mM KCl. All reactions were conducted in the presence of 8 mM EAS in 10 mM deuterated phosphate buffer, pD 7.8 at 37 °C for 1 h. After incubation samples were filtered to remove protein and an aliquot of 10 μL was injected into the HPLC. ND: not detected.

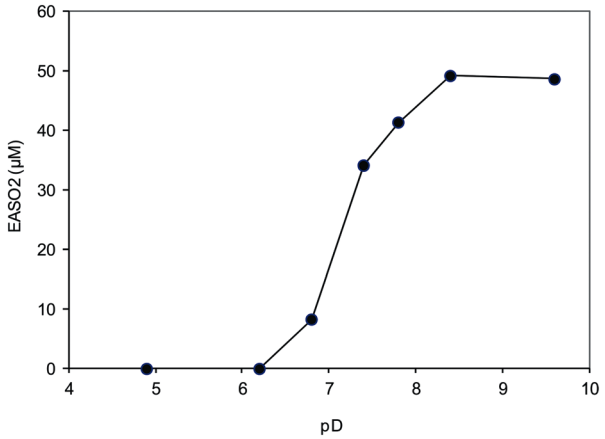


Fig. 3 Effect of pH on singlet oxygen formation. Liposomes consisting of DMPC–CL (1 mM : 1 mM) were incubated with cyt c (50 μM) in the presence of 8 mM EAS at 37 °C for 1 h in 10 mM deuterated phosphate buffer at different pH values. After incubation samples were filtered to remove protein and an aliquot of 10 μL was injected into the HPLC. Values are means of two independent experiments.

singlet oxygen generation and CL fatty acyl oxidation can be hypothesized since linoleoyl side chains are much more prone to oxidation than oleoyl chains.

pH effect

To gain further details on the type of cyt c–CL interaction involved in singlet oxygen generation, we evaluated the pH dependence of the process. Cyt c incubations with CL liposomes were conducted at different pH values in the presence of EAS. All experiments were conducted in deuterated phosphate buffer to ensure maximum detection of singlet oxygen. The plot of $EASO_2$ formation *versus* pD showed a sigmoidal curve with a pK_a at around pD 7.2 (equivalent to pH 6.8) with singlet oxygen being observed only at pD > 6.2 (pH >5 .8) (Fig. 3). This value is closely related to the pK_a of the electrostatic binding of cyt c to deprotonated CL species.[10,11,39] Cardiolipin has two pK values, one at 2.8 and another around 6.[14] Thus above pH 6, CL becomes fully deprotonated, a condition that favors electrostatic interactions with basic residues in the protein. Cyt c has two clusters of positively charged residues: site A containing lysine residues with high pK_a (Lys-72 and Lys-73) and site L containing ionizable groups with pK_a values around 7.0 (Lys-22, Lys-25, Lys-27, His-26 and His-33).[11] It has been suggested that at physiological pH, cyt c is bound to membranes containing CL mainly through site A,[11] whereas at more acidic conditions (pH < 7) cyt c–CL interaction involves an additional binding site named as site L.[39] The observation of singlet oxygen only at pH higher than 6 indicates that this excited species is formed at a condition where cyt c binds to CL most probably through site A.

Singlet molecular oxygen detection by luminescence measurements at 1270 nm

To confirm the generation of singlet oxygen, we measured the light emission corresponding to the monomolecular decay of singlet oxygen at 1270 nm.[28] These experiments were done using a photomultiplier apparatus previously described.[28] After monitoring the baseline, cyt c was infused into the liposome solution (Fig. 4). Purified CL yielded a very small signal that lasted less than 10 s (Fig. 4, line a). On the other hand, cyt c infusion into auto-oxidized CL solutions containing 5 and 30% of CL hydroperoxides triggered higher emission signals. Integration of the area under the emission signal showed that singlet oxygen yield increased linearly with CLOOH content (Fig. 4 inset). However, the yield of singlet oxygen obtained by this assay was much lower compared to the yield determined by chemical trapping experiments.

For example, CL liposomes used in the chemical trapping experiment contained approximately 5% of CLOOH and the yield of singlet oxygen determined by chemical trapping was 142 ± 12 μM. However, singlet oxygen yield determined by the light emission measurement was 3 μM, which corresponds to only approximately 2% of the total amount determined with EAS. This discrepancy can be explained by the existence of at least two pathways leading to singlet oxygen generation. One with fast kinetics that was responsible for the flash of light observed just after cyt c addition and was directly dependent on hydroperoxide reaction with cyt c; and the other displaying a slower kinetics, which was responsible for the major fraction (>90%) of singlet oxygen detected by chemical trapping.

It is important to note that in comparison to chemical trapping experiments the luminescence assay is less sensitive. While chemical traps allow the cumulative detection of singlet oxygen by the formation of stable endoperoxides, the luminescence assay measures it directly by recording the light derived from its monomolecular decay. Detection limits for singlet oxygen in our photomultiplier were in the range of 1–2 μM of singlet oxygen

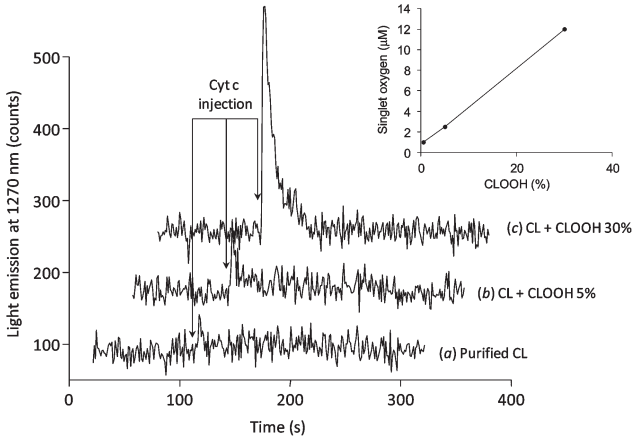


Fig. 4 Detection of singlet oxygen monomol light emission at 1270 nm. Reactions were carried out in a cuvette containing 1.45 mL of DMPC–CL liposome (1 : 1, final concentration 2 mM) in deuterated phosphate buffer (10 mM) containing 100 μM DTPA at pD 7.8. After recording the baseline an aliquot of 0.15 mL of 1 mM cyt c (final concentration 100 μM) was infused through a small tube directly into the solution under continuous stirring. Liposomes were prepared with purified CL, and CL stock solutions containing 5 and 30% CLOOH. The amount of singlet oxygen was estimated by the integration of the area under the emission signal using $DHPNO_2$ as a standard. The inset shows the plot of singlet oxygen yield *versus* CLOOH content (%) in CL liposomes. Data are from a single experiment done in duplicate.

which explains why we could not observe the continuous generation of singlet oxygen in cyt c–CL incubation by the luminescence assay. In fact, chemical trapping data (Fig. 1C) show that singlet oxygen generation rate ($\Delta EASO_2/\Delta t$) decreases with incubation time. The highest rate was observed in the first 5 min of incubation where singlet oxygen was generated at an average rate of approximately 0.14 μM per second, a value below the detection limit of our photomultiplier system. Taken together the luminescence and chemical trapping data clearly point to the existence of at least two mechanisms leading to singlet oxygen generation.

Analysis of CL and CL hydroperoxides

To get more insights into the mechanism behind singlet oxygen generation, we did a time-course analysis of CL consumption and CLOOH formation. Quantitative analysis of CL and its hydroperoxides was performed by HPLC-MS/MS using TMCL as an internal standard (Fig. 5). Cyt c incubation with CL liposomes showed an exponential decrease of CL (specifically TLCL) content, particularly at the first 10 min of reaction, with a nearly total CL consumption after 30 min of incubation (Fig. 5B). Comparative analysis of Fig. 1C and 5 indicates that CL consumption is closely related to singlet oxygen. Additionally, the rapid decrease in CL content was paralleled by a 5-fold increase in $CL(OOH)_1$ content (from approx. 5 μM to 25 μM), especially at the first 10 min of the reaction. This is probably related to the higher rates of singlet oxygen formed at the initial stages of the cyt–CL reaction. After 10 min, $CL(OOH)_1$ was gradually consumed returning to the basal level after 1 h incubation. $CL(OOH)_2$ and $CL(OOH)_3$ were only slightly increased, indicating that further oxygenation of the CL monohydroperoxides is not favoured.

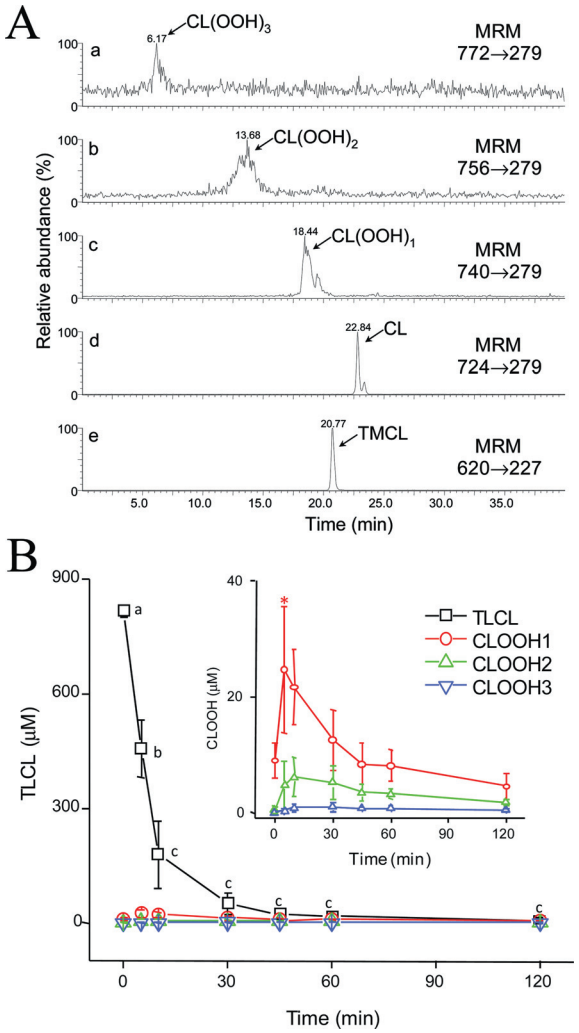


Fig. 5 Time dependent consumption of CL and formation of CL hydroperoxides in cyt c–CL incubation. For the experiment, cyt c (50 μM) was incubated with CL liposome (DMPC–CL, 1 mM : 1 mM) in deuterated phosphate buffer at pD 7.8 at 37 °C for 5, 10, 30, 45, 60 and 120 min. After incubation TMCL was added and lipids were extracted and then analyzed by HPLC-MS/MS. A. Typical mass chromatograms obtained for CL and CL hydroperoxide analysis in the MRM mode. TLCL (*m/z* 724), CL(OOH)1 (*m/z* 740), CL(OOH)2 (*m/z* 755), and CL(OOH)3 (*m/z* 771) were detected by monitoring the fragmentation of the doubly charged molecular ions ($[M - 2H]^{2-}$) yielding a fragment at *m/z* 279 corresponding to linoleic acid. TMCL (*m/z* 620) was detected by monitoring the fragmentation yielding myristic acid at *m/z* 227. B. The concentrations of CL and CL hydroperoxides were determined by the integration of the peak area, which was normalized by the area of the internal standard and converted to concentration using a standard curve prepared for each compound. Results are expressed as mean ± SD ($n = 3$). Values with different letters or * are significantly different ($p < 0.05$).

Effect of CL hydroperoxides on singlet oxygen generation. Studies using 18-oxygen labeled cardiolipin hydroperoxides

Previous studies conducted in our laboratory showed that singlet oxygen can be directly generated from hydroperoxides. To clarify the generation of singlet oxygen from CL hydroperoxides

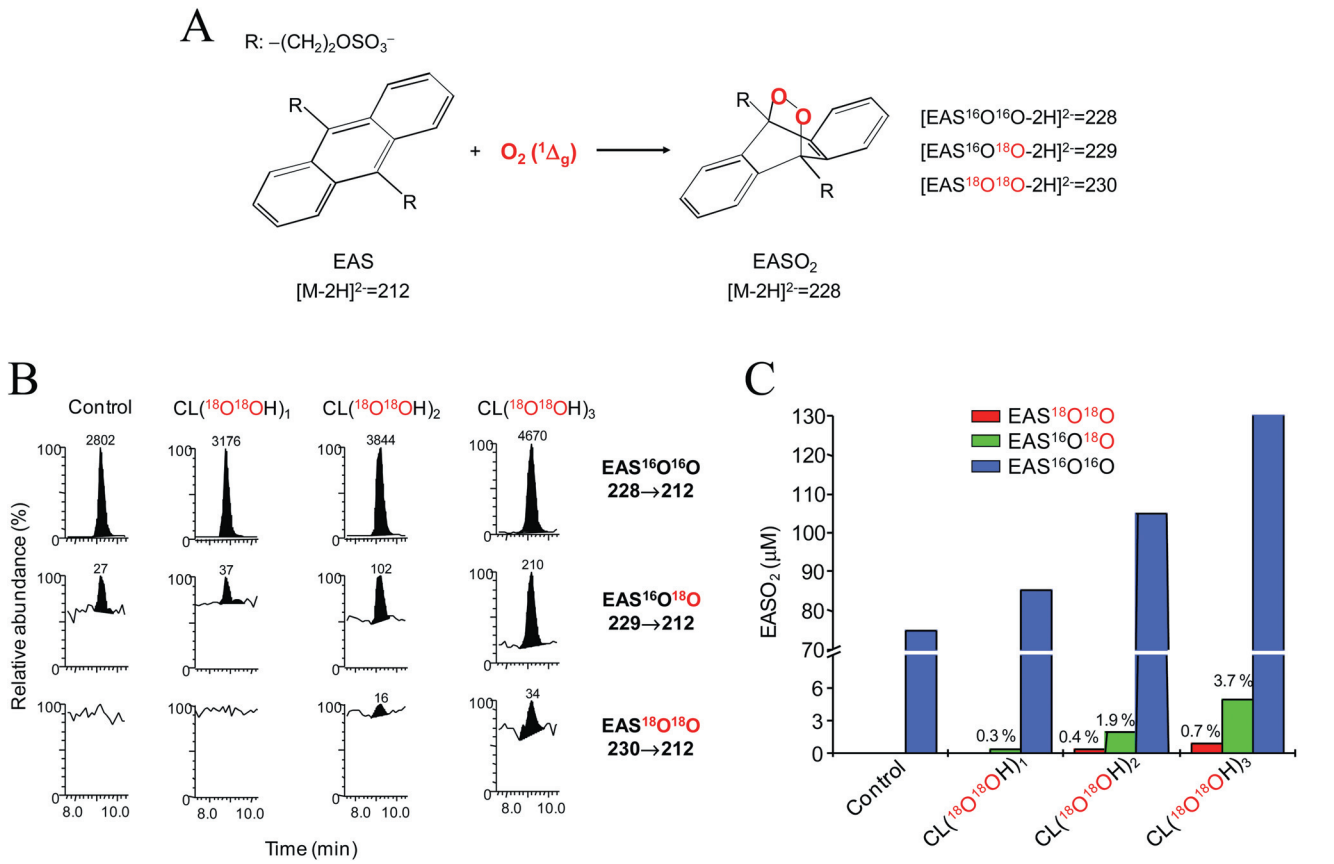


Fig. 6 Detection of 18-oxygen-labeled singlet oxygen in cyt c incubations with liposomes containing labeled CL hydroperoxides. Liposomes containing DMPC–CL (1 mM : 1 mM) or DMPC–CL–$CL^{18}O^{18}OH$ (1 mM : 0.6 mM : 0.4 mM) were incubated with 200 μM cyt c in the presence of 8 mM EAS in 10 mM deuterated phosphate buffer (pD 8.4, containing 100 μM of DTPA) at 37 °C for 1 h. A. Scheme showing the formation of labeled endoperoxides (EAS^xO^xO, x = 16 or 18). B. Mass chromatograms showing the specific detection of $EAS^{16}O^{16}O$, $EAS^{16}O^{18}O$ and $EAS^{18}O^{18}O$ by MRM analysis. Peak area is shown at the top of each peak. C. Concentrations of unlabeled and labeled endoperoxides detected in the incubations. The relative percentages of labeled endoperoxides are shown.

we used liposomes supplemented with 18-oxygen-labeled CL hydroperoxides. Labeled hydroperoxides were synthesized and purified as described in detail in the Methods section. Liposomes containing DMPC–CL–$CL(^{18}O^{18}OH)_n$ at a 1 : 0.6 : 0.4 molar ratio (final 1 mM concentration of CL) were prepared and incubated with cyt c (200 μM) in deuterated phosphate buffer (10 mM) in the presence of EAS. To ensure maximum formation of singlet oxygen, reactions were conducted in deuterated buffer at pD 8.4. After 1 h incubation, EAS endoperoxides were analyzed by HPLC-MS/MS (Fig. 6). Unlabeled, partially labeled and fully labeled endoperoxides were detected using the MRM mode analysis (Fig. 6B). It is important to mention that labeled CL hydroperoxides used for the experiments were almost 100% pure (Fig. S2, ESI†). Moreover, care was also taken to use CL stock solutions containing less than 1% of pre-formed hydroperoxides and also to avoid hydroperoxide formation during liposome preparation.

Fig. 6C shows the concentration of endoperoxides detected for a control reaction containing only CL and for the incubations containing CL plus $CL(^{18}O^{18}OH)_1$, $CL(^{18}O^{18}OH)_2$, or CL $(^{18}O^{18}OH)_3$, respectively. The amount of singlet oxygen detected was higher in liposomes supplemented with hydroperoxides. However, most of the detected endoperoxides were unlabeled, indicating that singlet oxygen generation directly from CL hydroperoxide decomposition is low. Importantly, although at low yields, partially ($EAS^{16}O^{18}O$) and fully labeled ($EAS^{18}O^{18}O$) endoperoxides were detected, and this was particularly evident in incubations containing $CL(^{18}O^{18}OH)_2$ or CL $(^{18}O^{18}OH)_3$. Based on our previous studies,[28] partially and fully labeled singlet oxygen species can be formed by one labeled and unlabeled peroxyl radicals, or by reactions involving two labeled peroxyl radicals ($CL^{18}O^{18}O^{\cdot}$), respectively. Thus, the detection of labeled endoperoxides serves as evidence for the formation of peroxyl radicals directly from the CL hydroperoxide reaction with cyt c.

Discussion

Cyt c–CL complex formation has been related to selective CL oxygenation and induction of apoptosis.[40] Here we present evidence demonstrating the generation of singlet oxygen during cyt c–CL interaction using a model membrane containing CL. Singlet oxygen formation was evidenced by chemical trapping experiments (Fig. 1), and confirmed by the direct measurement of singlet oxygen monomol light emission at 1270 nm (Fig. 4).

Importantly, the generation of singlet oxygen occurred specifically for membranes containing polyunsaturated CL species (TLCL) and was inhibited by increasing the ionic strength of the buffer indicating that cyt c–CL binding is required for singlet oxygen generation (Fig. 2).

Cyt c–CL binding and singlet oxygen generation

Cyt c–CL binding involves a combination of electrostatic and hydrophobic interactions as well as hydrogen bonding.[10,11,13,19,39] Three sites of interaction have been described for cyt c.[11,39] Site A is comprised of basic amino acid residues (Lys-72 and -73) that interact with the deprotonated phosphate group, and site C is comprised of the invariant Asn-52 which is involved in the hydrogen bonding with the protonated phosphate group of the phospholipid. More recently, an additional binding site named site L has been identified, which contains positively charged residues (Lys-22, -25, -27, and His-26 and -33) with lower pK_a (around 7.0).[39]

Although still not completely understood, it has been proposed that at neutral pH, cyt c binds to CL both *via* sites A and C. Binding at this condition involves hydrogen bonding of one protonated phosphate moiety to Asn-52 with simultaneous electrostatic binding of deprotonated phosphate to the Lys residues and accommodation of one acyl chain within the hydrophobic channel (extended lipid anchorage)[12,13] of the protein. On the other hand, under acidic conditions (pH < 7), the protonation of basic residues at site L creates an additional binding site that has been shown to mediate membrane fusion in a model of mitochondrial membrane.[39]

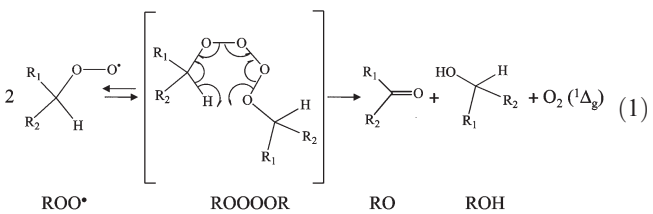
Interestingly, the pH dependent profile of singlet oxygen generation showed a sigmoid curve with a pK_a around 6.8 (Fig. 3). A similar turning point was observed for cyt c binding to site L. However, singlet oxygen generation displayed an inverse profile compared to site L binding. Singlet oxygen was only detected at pH values higher than 6 and was generated at highest yields in the pH range of 8–9. Based on ionization behavior of basic groups at sites A and L,[39,41] these data indicate that singlet oxygen is generated at a condition favouring electrostatic interactions with site A. Further studies are necessary to corroborate this hypothesis.

In addition to cyt c–CL electrostatic interactions, the formation of singlet oxygen was affected by CL fatty acyl chain type. Singlet oxygen was only observed with polyunsaturated CL species and was not detected for saturated or monounsaturated CL species. It has been suggested that both saturated and unsaturated acyl chains can be accommodated in the cyt c hydrophobic channel but produce different effects in heme iron crevice geometry.[19,42,43] In experiments measuring binding strength, TLCL displayed a 6-fold higher binding efficiency compared to TOCL.[43] Moreover, unsaturated CL species have been shown to induce the formation of an "alternative" low spin form of cyt c. This alternative form displays a more open heme crevice with increased accessibility of the heme for reaction with peroxides and carbonyl compounds.[19] Thus singlet oxygen formation induced in the presence of TLCL is possibly related to linoleoyl conformation which allows higher binding efficiency and formation of activated states of cyt c. Moreover, considering that a major fraction of singlet oxygen is formed by a pathway involving CL oxidation, the formation of singlet oxygen with TLCL and not with TOCL can also be ascribed to the higher oxidizability of linoleoyl acyl chains compared to oleoyl chains.[44]

Possible routes leading to singlet oxygen generation

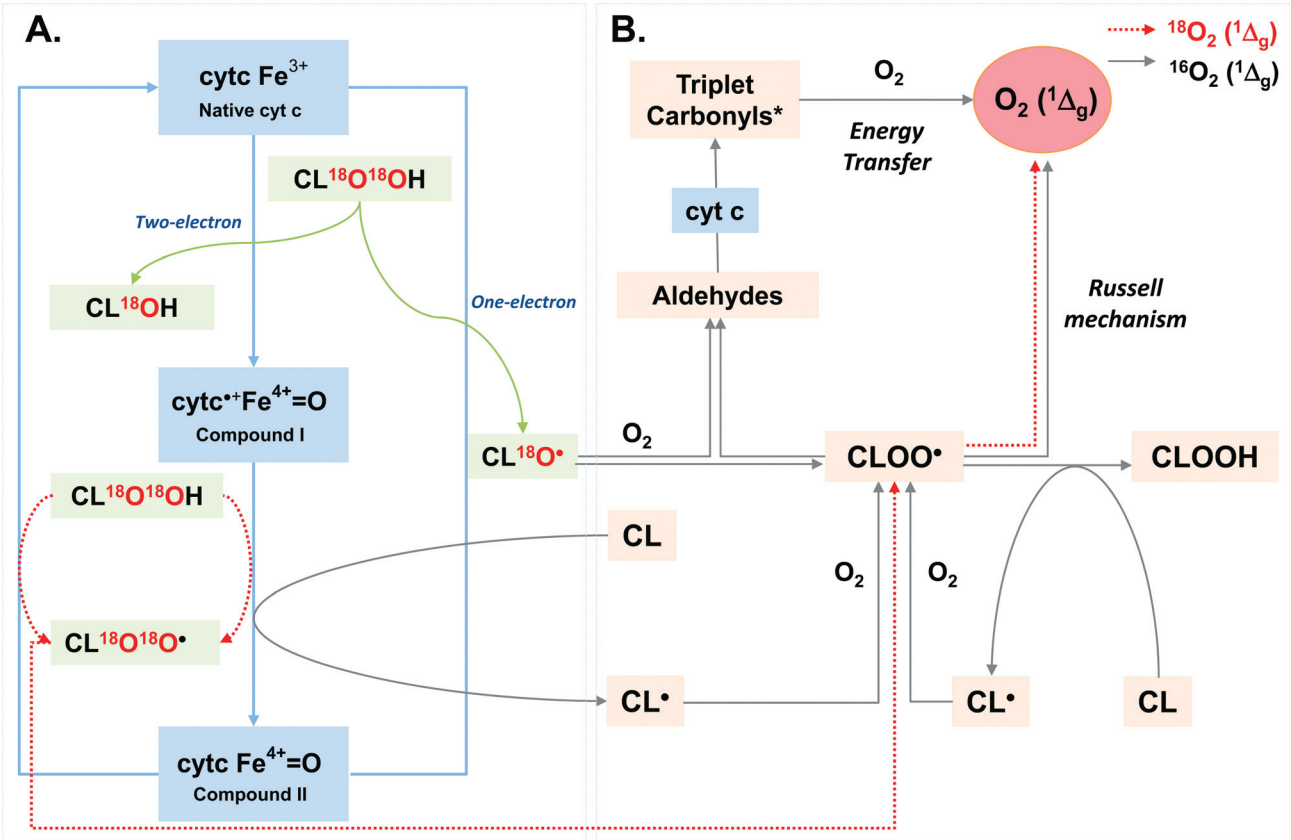
It is clear from chemiluminescence and chemical trapping assays that there are at least two pathways leading to singlet oxygen formation in the cyt c–CL system: one involving CL hydroperoxide decomposition (Scheme 1A) and another related to CL oxidation (Scheme 1B). Our results showed that the last pathway is the major route responsible for singlet oxygen in our model.

Although being minor, singlet oxygen can be directly generated from CL hydroperoxides as clearly evidenced by the detection of 18-oxygen labeled singlet oxygen in incubations containing 18-oxygen labeled CL hydroperoxides (Fig. 6). The generation of singlet oxygen directly from hydroperoxides is also supported by luminescence data showing a flash of light just upon cyt c mixing with CL liposomes containing increasing concentrations of CLOOH (Fig. 4). It is important to note that we have observed a similar emission signal for singlet oxygen generated in the reaction of fatty acid hydroperoxides with metal ions.[28] However the participation of free metal ions in our model can be discarded because reactions were conducted in the presence of a metal chelator. Thus, in analogy to our previous findings, singlet oxygen generation directly from CL hydroperoxides can be ascribed to reactions involving peroxyl radicals by the Russell mechanism[28,45] (eqn (1)). In this mechanism two peroxyl radicals react to generate a linear tetraoxide intermediate that decomposes to yield the corresponding ketone, alcohol and singlet oxygen. Indeed, the involvement of this type of mechanism was confirmed by the detection of labeled singlet oxygen in the reactions of cyt c in the presence of 18-oxygen labeled CL hydroperoxides.



(1)

Besides the formation of singlet oxygen directly from CL hydroperoxides which accounted for less than 5% of singlet oxygen generated by the cyt c–CL system, our data indicate that most of the singlet oxygen is generated by a pathway involving CL oxidation. This hypothesis is based on the fact that time-dependent formation of singlet oxygen generation was followed by a parallel consumption of TLCL, reaching a saturation level when CL was totally consumed (Fig. 1C and 5B). The decrease in CL content was accompanied by the formation of CL monohydroperoxides especially at the first few minutes of the incubation, indicating that CL is being oxidized. This oxidation was probably activated by small amounts of CL hydroperoxides present in liposome preparations. It is important to note that Kagan's group reported an enhancement of up to 1000-fold in



Scheme 1 Activation of the cyt c peroxidase cycle in the presence of CLOOH and the generation of singlet oxygen. A. Cyt c reacts with $CL^{18}O^{18}OH$ by one- and two-electron mechanisms. The two-electron reduction of hydroperoxides to hydroxides yields cyt c compound I type species, which can oxidize hydroperoxides and CL to peroxyl radicals ($CL^{18}O^{18}O^{\cdot}$) and $CL^{\bullet}$ radicals, respectively. This reaction produces compound II which can be reduced back to the native state through a pathway involving one-electron oxidation of substrates, such as CL hydroperoxides. Alternatively, the one-electron reduction of $CL^{18}O^{18}OH$ converts it to alkoxyl radicals ($CL^{18}O^{\cdot}$). This radical can also yield peroxyl radicals through a number of pathways including internal rearrangements and reactions with oxygen. It can also react with CL yielding $CL^{\cdot}$. B. Possible routes leading to singlet oxygen formation. The peroxyl radical is a common intermediate formed from CL oxidation and CLOOH decomposition. This radical can generate singlet oxygen by the Russell mechanism. It is also an important intermediate responsible for the propagation of CL oxidation and can also undergo chain cleavage yielding aldehydes. These aldehydes can react with cyt c yielding excited triplet carbonyl species, which can generate singlet oxygen by an energy transfer mechanism. Red dashed lines show the route by which $CL^{18}O^{18}OH$ yields 18-oxygen labeled singlet oxygen molecules.

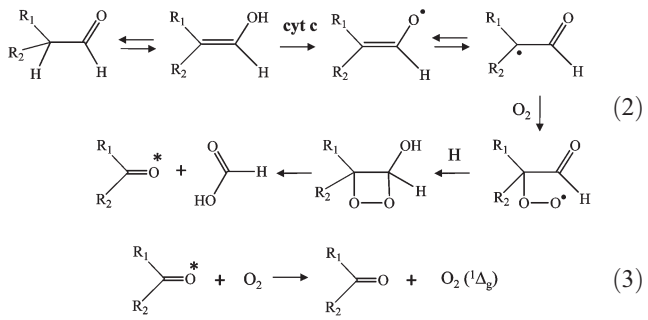
the peroxidase activity of cyt c–CL complexes in the presence of fatty acid hydroperoxides, a process analogous to what happens with COX-1 and 2.[40] Thus similarly to fatty acid hydroperoxides, CL hydroperoxides can be used as a substrate necessary to activate cyt c peroxidase activity.

The cyt c reaction with hydroperoxides has been extensively studied.[46,47] This process induces the formation of radical intermediates (*e.g.* tyrosyl radicals[48]) capable of promoting CL peroxidation.[40] Scheme 1 summarizes the routes for cyt c reaction with hydroperoxides, the oxidation of CL and the possible pathways leading to singlet oxygen generation. As a peroxidase, cyt c can react with hydroperoxides by two-electron or one-electron mechanisms (Scheme 1). In the two-electron mechanism, cyt c–Fe^{3+} catalyzes the heterolytic reduction of the hydroperoxide to its corresponding alcohol with the generation of a ferryl cation radical intermediate (cytc$^{\cdot+}$–Fe^{4+}=O, compound I). In the one-electron mechanism, hydroperoxides undergo homolytic cleavage yielding alkoxyl radicals ($CLO^{\cdot}$) and the ferryl form of cyt c (cytc–Fe^{4+}=O, compound II). The fate of cyt c compound II is not clear. Nonetheless, it can be expected that this intermediate induces the formation of radicals from substrates during its reduction back to the native state.

Although there is no clear consensus regarding the main mechanism by which hydroperoxides react with cyt c, both one- and two-electron pathways generate reactive intermediates (*e.g.* alkoxyl radicals, compound I and compound II) that are able to induce CL oxidation and CL hydroperoxide decomposition. Both processes can lead to the generation of peroxyl radicals ($CLOO^{\cdot}$) (Scheme 1B). As mentioned above, peroxyl radicals generate singlet oxygen by the Russell mechanism. Alternatively, radical intermediates (*e.g.* alkoxyl and peroxyl radicals) can be cleaved yielding a series of secondary products, such as aldehydes. Indeed, a 2-fold increase in malonaldehyde content has been detected in incubations of cyt c with CL-containing liposomes.[49] Importantly, a number of studies showed that aldehydes can be converted to long-lived excited triplet carbonyl species upon reaction with peroxidases,[50–52] and the latter is known to yield singlet oxygen by an energy transfer mechanism.

The reaction of aldehydes with peroxidases has been characterized in detail. Briefly, the enol form of aldehydes reacts with

cyt c generating an enoxyl radical, which undergoes rearrangements and reaction with oxygen to yield a dioxetane intermediate[52,53] (eqn (2)). Fragmentation of dioxetanes yields carbonyl compounds, one of them in its excited state.[54] Finally, triplet excited carbonyls can transfer energy to molecular oxygen yielding singlet oxygen (eqn (3)). The involvement of excited triplet carbonyl species in cyt c–CL incubations is supported by an experiment showing an enhancement in the visible light emission in the presence of 9,10-dibromoanthracene sulphonate (Fig. S4, ESI†). Thus, in addition to the Russell mechanism, our study indicates that singlet oxygen generation by the cyt c–CL system can also involve triplet–triplet energy transfer from excited carbonyl species.



(2)

(3)

Conclusions

In summary, cyt c–CL interaction generates singlet oxygen at a yield of approximately 15% relative to the initial concentration of CL. The generation of singlet oxygen was dependent on a number of factors, including the type of unsaturated fatty acids esterified to CL, the amounts of pre-formed hydroperoxides and pH. For instance, the generation of singlet oxygen was dependent on both electrostatic and hydrophobic interactions between cyt c and CL, and occurred only with CL species containing tetralinoleoyl species. This specificity is possibly related to the ability of linoleoyl acyl chains to induce structural rearrangements required for maximal activation of cyt c peroxidase activity[43] and also to the higher oxidizability of this fatty acid compared to oleoyl acyl chains. Using 18-oxygen-labeled CL hydroperoxides we found that only a minor fraction of singlet oxygen is directly formed from hydroperoxide decomposition into peroxyl radicals. On the other hand, singlet oxygen formation was paralleled by CL consumption, suggesting that a major fraction of singlet oxygen is formed through a mechanism involving cyt–CL binding and CL oxidation. Altogether we propose a mechanism of singlet oxygen generation involving cyt c–CL complex formation, followed by the activation of the cyt c peroxidase catalytic cycle that uses CL hydroperoxides as a source of oxidizing equivalents to promote CL oxidation. This process induces the formation of peroxyl radicals as well as excited triplet states, which are all known well established sources of singlet oxygen. The biological significance of singlet oxygen generated by the cyt c–CL complex needs to be further investigated. Nonetheless, our findings point to a potential role of singlet oxygen as an additional oxidant possibly involved in the pathway leading to cyt c release from mitochondria and induction of cell death.

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