Cardiolipin modulates allosterically peroxynitrite detoxification by horse heart cytochrome *c*

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ABSTRACT

Upon interaction with bovine heart cardiolipin (CL), horse heart cytochrome *c* (cyt*c*) changes its tertiary structure disrupting the heme-Fe-Met80 distal bond, reduces drastically the midpoint potential out of the range required for its physiological role, binds CO and NO with high affinity, and displays peroxidase activity. Here, the effect of CL on peroxynitrite isomerization by ferric cyt*c* (cyt*c*-Fe(III)) is reported. In the absence of CL, hexa-coordinated cyt*c* does not catalyze peroxynitrite isomerization. In contrast, CL facilitates cyt*c*-Fe(III)-mediated isomerization of peroxynitrite in a dose-dependent fashion inducing the penta-coordination of the heme-Fe(III)-atom. The value of the second order rate constant for CL-cyt*c*-Fe(III)-mediated isomerization of peroxynitrite (k_{on}) is $(3.2 \pm 0.4) \times 10^5 \, M^{-1} \, s^{-1}$. The apparent dissociation equilibrium constant for CL binding to cyt*c*-Fe(III) is $(5.1 \pm 0.8) \times 10^{-5} \, M$. These results suggest that CL-cyt*c* could play either pro-apoptotic or anti-apoptotic effects facilitating lipid peroxidation and scavenging of reactive nitrogen species, such as peroxynitrite, respectively.

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1. Introduction

Cytochrome *c* (cyt*c*) is a peripheral membrane heme-protein functioning in-between the inner and the outer membrane of the mitochondrion mediating electron transfer between different proteins of the respiratory chain. Moreover, cyt*c* plays a primary role in apoptosis [1–5]; indeed, in response to a variety of apoptosis inducing agents, cyt*c* is released into the cytosol where it actively participates in the execution of programmed cell death by binding to the apoptosis protease activation factor (*i.e.*, APAF-1) with the subsequent activation of pro-caspase-9 [6–9].

Approx 15% of mitochondrial cyt*c* is tightly bound to the inner mitochondrial membrane being involved in the peroxidase activity, an event considered crucial for initiating the apoptotic process. In contrast, free cyt*c* participates in the electron transfer process and inhibits reactive oxygen species formation (thus preventing cell oxidative stress). Among the phospholipids constituting the mitochondrial membrane, cardiolipin (CL) is considered the preferential interacting partner with cyt*c*; this phospholipid (which represents about 20% of total lipids of the membrane) possesses a unique structure containing four (instead of two) fatty acid tails [10].

The CL-cyt*c* interaction plays an important role in modulating the protein function and, consequently, the cell fate. At the early stage of apoptosis, the CL-cyt*c* affinity decreases leading to the dissociation of the CL-cyt*c* complex and the release of the free protein in the cytosol [4,11]. CL-bound cyt*c* shows a non-native tertiary structure, a disrupted heme-Fe–Met80 distal bond [12–17], and a drastically reduced midpoint potential, which falls out of the range required for its physiological role [18]. Further, the cleavage of the distal Fe–Met80 bond endows cyt*c* with a high affinity for CO and NO [19,20] and peroxidase activity [21–27].

The peroxidase activity acquired by mitochondrial CL-bound cyt*c* is critical during the early stages of the apoptotic process generating CL hydroperoxides. These compounds favor the dissociation of the CL-cyt*c* complex and the release of free soluble cyt*c* from the mitochondrial membrane in the cytosol [11,23,28]. Remarkably, the affinity of CO and NO for the CL-cyt*c* complex is high enough to envisage an antiapoptotic effect of nanomolar CO and NO concentrations via inhibition of the cyt*c* peroxidase activity [19,20].

The high reactivity of the penta-coordinated CL-cyt*c* complex [11,19,20,26] prompted us to investigate the effect of CL on peroxynitrite isomerization by ferric cyt*c* (cyt*c*-Fe(III)). In the absence of CL, hexa-coordinated cyt*c* does not catalyze peroxynitrite isomerization, even though it has been shown that peroxynitrite may

induce the very slow nitration (30 min) of the solvent-exposed Tyr74 residue; this leads to the cleavage of the Fe–Met80 bond, which is substituted by a weak Fe–Lys72 ligation [29]. In contrast, CL facilitates the fast cytc-Fe(III)-mediated isomerization of peroxynitrite in a dose-dependent fashion, inducing the penta-coordination of the heme-Fe(III)-atom. These results suggest that the CL-cytc complex could display multiple pro- and anti-apoptotic roles interacting with reactive oxygen and nitrogen species.

2. Materials and methods

Ferric horse heart cytochrome c (cytc-Fe(III)) and bovine heart CL were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the other products were purchased from Merck AG (Darmstadt, Germany). The cytc-Fe(III) concentration was determined spectrophotometrically at 410 nm ($\varepsilon_{410\ nm} = 1.06 \times 10^5\ M^{-1}\ cm^{-1}$) [30]. Aqueous dispersions of CL liposomes were prepared as previously reported [17].

Peroxynitrite was synthesized from both KO_2 and NO or HNO_2 and H_2O_2 and stored at $-80.0\ °C$. The concentration of peroxynitrite was determined spectrophotometrically by measuring the absorbance at 302 nm ($\varepsilon_{302\ nm} = 1.705 \times 10^3\ M^{-1}\ cm^{-1}$) [31,32].

Kinetics of peroxynitrite isomerization by cytc-Fe(III), in the absence and presence of CL (final concentration, $2.0 \times 10^{-5}\ M \leqslant$ [CL] $\leqslant 1.6 \times 10^{-4}\ M$), was recorded at 302 nm ($\varepsilon_{302\ nm} = 1.705 \times 10^3\ M^{-1}\ cm^{-1}$) by rapid mixing either the cytc-Fe(III) solution (final concentration, $1.0 \times 10^{-6}\ M \leqslant$ [cytc-Fe(III)] $\leqslant 5.0 \times 10^{-6}\ M$) or the CL-cytc-Fe(III) solution (final concentration, $2.0 \times 10^{-5}\ M \leqslant$ [CL] $\leqslant 1.6 \times 10^{-4}\ M$, and $1.0 \times 10^{-6}\ M \leqslant$ [cytc-Fe(III)] $\leqslant 5.0 \times 10^{-6}\ M$) with the peroxynitrite solution (final concentration, [peroxynitrite] = $1.0 \times 10^{-4}\ M$). The light path of the observation cuvette was 10 mm, and the dead-time was 1.4 ms. No gaseous phase was present [33].

Kinetics of peroxynitrite isomerization by cytc-Fe(III), in the absence and presence of CL, was analyzed in the framework of the minimum reaction Scheme 1 [33].

Values of the pseudo-first-order rate constant for cytc-Fe(III)-mediated peroxynitrite isomerization (k_{obs}), in the absence and presence of CL, have been determined from the analysis of the time-dependent absorbance decrease at 302 nm, according to Eq. (1) [33]:

$$[\text{peroxynitrite}]_t = [\text{peroxynitrite}]_i \times e^{-kobs \times t} \tag{1}$$

Values of the second-order rate constant for CL-cytc-Fe(III)-mediated peroxynitrite isomerization (k_{on}) and of the first-order rate constant for peroxynitrite isomerization in the absence of either cytc-Fe(III) or CL (k_0) have been determined from the linear dependence of k_{obs} on the (CL-)cytc-Fe(III) concentration, according to Eq. (2) [33]:

$$k_{obs} = k_{on} \times [\text{cyt}c\text{-Fe(III)}] + k_0 \tag{2}$$

The value of the apparent dissociation equilibrium constant for CL binding to cytc-Fe(III) (K) has been determined from the dependence of k_{on} on the CL concentration (i.e., [CL]), according to Eq. (3):

$$k_{on} = k_{on(top)} \times [\text{CL}]/(K + [\text{CL}]) \tag{3}$$

where $k_{on(top)}$ is the asymptotic value of k_{on} under conditions where [CL] $\gg K$.

All data were obtained at pH 7.0 ($3.0 \times 10^{-2}\ M$ Hepes buffer) and 20.0 °C. The effect of 1.0 M KCl on values of k_{on} and k_0 has been also investigated.

NO_2^- and NO_3^- analysis was carried out spectrophotometrically at 543 nm by using the Griess reagent and VCl_3 to catalyze the conversion of NO_3^- to NO_2^-, as described previously [33].

Kinetic and thermodynamic data were analyzed using the MatLab program (The Math Works Inc., Natick, MA, USA). The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation.
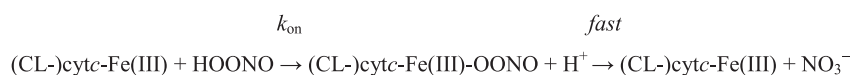
3. Results and discussion

Kinetics of peroxynitrite isomerization by (CL-)cytc-Fe(III) was fitted to a single-exponential decay for more than 90% of its course (Fig. 1, panel A). This indicates that no intermediate species (e.g., (CL-)cytc-Fe(III)-OONO; see Scheme 1) accumulate(s) in the course of peroxynitrite isomerization, the formation of the transient (CL-)cytc-Fe(III)-OONO species representing the rate limiting step in catalysis.

In the absence of CL, values of k_{obs} for peroxynitrite isomerization are unaffected by cytc-Fe(III) over the whole concentration range explored (1.0×10^{-6}–$5.0 \times 10^{-6}\ M$). Indeed, values of k_{obs} are independent of the cytc-Fe(III) concentration and correspond to k_0 (i.e., the first-order rate constant for the spontaneous isomerization of peroxynitrite) within the experimental error (Fig. 1, panel B). This indicates that cytc-Fe(III) does not catalyzes peroxynitrite isomerization. In contrast, values of k_{obs} for CL-cytc-Fe(III)-mediated isomerization of peroxynitrite increase with the CL-cytc-Fe(III) concentration (Fig. 1, panel C), indicating that the CL-cytc-Fe(III) complex catalyzes peroxynitrite isomerization. The analysis of data according to Eq. (2) allowed the determination of values of k_{on} (corresponding to the slope of the linear plots) and k_0 (corresponding to the y intercept of the linear plots) for CL-cytc-Fe(III)-catalyzed isomerization of peroxynitrite at $2.0 \times 10^{-5}\ M \leqslant$ [CL] $\leqslant 1.6 \times 10^{-4}\ M$. Under all the experimental conditions, values of k_0 are in good agreement with those reported in the literature [33–38]. According to literature [24], high ionic strength (i.e., 1.0 M KCl) inhibits the CL-cytc-Fe(III)-catalyzed isomerization of peroxynitrite impairing the formation of the CL-cytc-Fe(III) complex (Fig. 1, panel B).

As shown in Fig. 1 (panel D), CL facilitates cytc-Fe(III)-mediated isomerization of peroxynitrite in a dose-dependent fashion. Indeed, values of k_{on} increase on increasing the CL concentration. The analysis of data according to Eq. (3) allowed the determination of both values of K (= $(5.1 \pm 0.8) \times 10^{-5}\ M$) and $k_{on(top)}$ (= $(3.2 \pm 0.4) \times 10^5\ M^{-1}\ s^{-1}$).

As shown in Table 1, CL-cytc-Fe(III) catalyzes peroxynitrite isomerization, NO_3^- and NO_2^- yields being 91% and 8%, respectively. In contrast, the NO_3^- and NO_2^- yield decreases (75%) and increases (26%), respectively, in the presence of cytc-Fe(III) or CL or KCl. As expected [24], peroxynitrite isomerization yields 72% NO_3^- and 26% NO_2^- in the presence of CL-cytc-Fe(III) and 1.0 M KCl.

Catalytic isomerization of peroxynitrite appears to be a common feature of penta-coordinated ferric heme-proteins, i.e., CL-cytc-Fe(III) (present study), HSA-heme-Fe(III) [33], horse Mb [34], sperm whale Mb(III) [35], and human Hb(III) [34]. Notably, hexacoordinated human neuroglobin does not catalyze peroxynitrite isomerization [39].

$$
\text{(CL-)cyt}c\text{-Fe(III)} + \text{HOONO} \xrightarrow{k_{on}} \text{(CL-)cyt}c\text{-Fe(III)-OONO} + \text{H}^+ \xrightarrow{fast} \text{(CL-)cyt}c\text{-Fe(III)} + \text{NO}_3^-
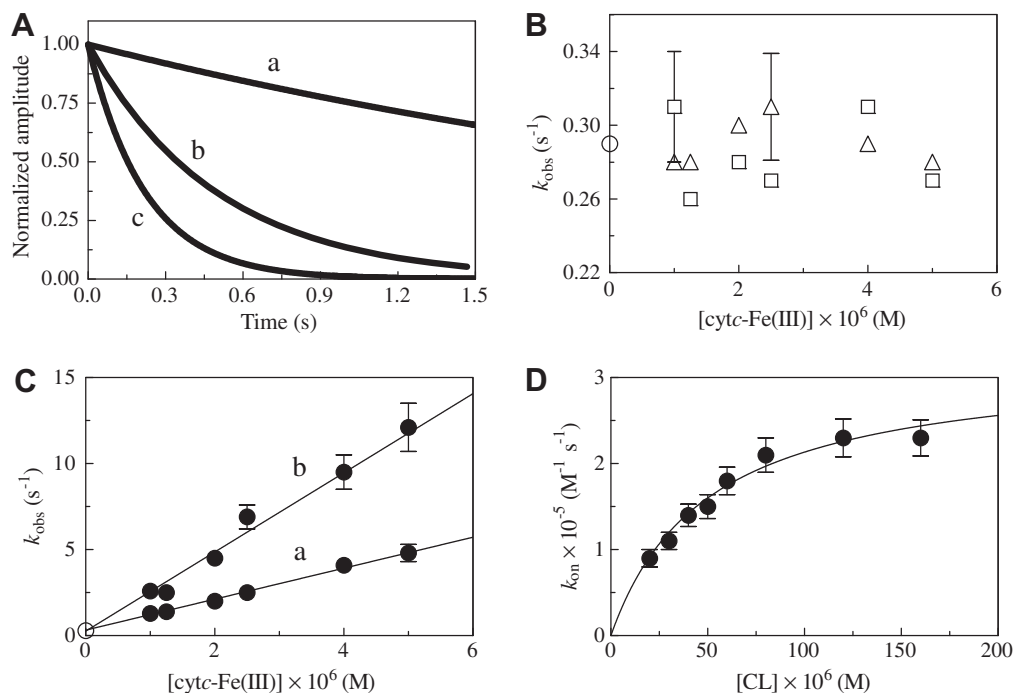$$

Scheme 1.

Fig. 1. Peroxynitrite isomerization by (CL-)cyt$c$-Fe(III). Normalized averaged time courses of peroxynitrite isomerization by (CL-)cyt$c$-Fe(III) (A). The cyt$c$-Fe(III) concentration was 0.0 M (trace a) and 2.0×10^{-6} M (traces b and c). The CL concentration was 0.0 M (trace a), 2.0×10^{-5} M (trace b), and 1.2×10^{-4} M (trace c). The time course analysis according to Eq. (1) allowed the determination of the following values of k_{obs}: 2.8×10^{-1} s^{-1} (trace a), 2.0 s^{-1} (trace b), and 4.5 s^{-1} (trace c). Dependence of k_{obs} on the cyt$c$-Fe(III) concentration in the absence of CL and KCl (squares) and in the presence of 2.0×10^{-5} M CL and 1.0 M KCl (triangles) (B). The open circle on the ordinate indicates the average k_0 value (= 2.9×10^{-1} s^{-1}). For clarity, the standard deviation is shown for two k_{obs} values. Dependence of k_{obs} on the CL-cyt$c$-Fe(III) concentration (C). The CL concentration was 2.0×10^{-5} M (a) and 1.2×10^{-4} M (b). The continuous lines were calculated according to Eq. (2) with the following parameters: a - k_{on} = 9.0×10^{4} M^{-1} s^{-1} and k_0 = 3.2×10^{-1} s^{-1}; b - k_{on} = 2.3×10^{5} M^{-1} s^{-1} and k_0 = 2.6×10^{-1} s^{-1}. The open circle on the ordinate indicates the average k_0 value (= 2.9×10^{-1} s^{-1}). Dependence of k_{on} on the CL concentration (D). The continuous line was calculated according to Eq. (3) with $k_{on(top)}$ = $(3.2 \pm 0.4) \times 10^{5}$ M^{-1} s^{-1} and K = $(5.1 \pm 0.8) \times 10^{5}$ M. All data were obtained at pH 7.0 and 20.0 °C. Where not shown, standard deviation is smaller than the symbol. For details, see text.

Table 1
NO_3^- and NO_2^- distribution of peroxynitrite isomerization in the absence and presence of cyt$c$-Fe(III), CL, and KCl, at pH 7.0 and 20.0 °C.[a]

cyt$c$-Fe(III) (M)	[CL] (M)	KCl (M)	$[NO_3^-]$ (%)	$[NO_2^-]$ (%)	$[NO_3^-] + [NO_2^-]$ (%)
–	–	–	76 ± 6	25 ± 4	101
4.0×10^{-5}	–	–	73 ± 6	26 ± 4	99
–	3.0×10^{-4}	–	74 ± 7	27 ± 3	101
–	–	1.0	75 ± 7	25 ± 3	100
4.0×10^{-5}	3.0×10^{-4}	–	91 ± 8	8 ± 3	99
4.0×10^{-5}	3.0×10^{-4}	1.0	72 ± 8	26 ± 3	98

[a] The peroxynitrite concentration is 1.0×10^{-4} M.

Table 2
Values of k_{on} for peroxynitrite scavenging by ferric heme-proteins.

	k_{on} (M^{-1} s^{-1})	pH	T (°C)	Ref.
CL-cyt$c$[a]	3.2×10^{5}	7.0	20.0	Present study
HSA-heme-Fe	4.1×10^{5}	7.2	22.0	[33]
Horse heart Mb	2.9×10^{4}	7.0	20.0	[34]
Sperm whale Mb	1.6×10^{4}	7.5	20.0	[39]
Human Hb	1.2×10^{4}	7.5	20.0	[34]

[a] [CL] >> K; see text.

Note that the k_{on} value for peroxynitrite isomerization by CL-cyt$c$-Fe(III) is similar to that for HSA-heme-Fe(III) action [33,37,38] and is higher by about one order of magnitude than those reported for horse Mb(III) [34], sperm whale Mb(III) [35], and human Hb(III) [34] (Table 2).

Peroxynitrite isomerization by cyt$c$-Fe(III) (present study) and HSA-heme-Fe(III) [33] is modulated allosterically by CL and drugs,
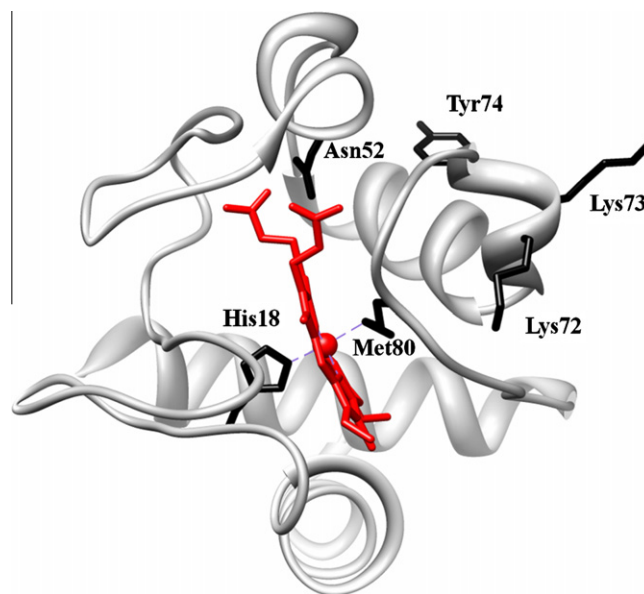

Fig. 2. Ribbon structure of horse heart cyt c. The heme is shown in red. Residues His18 and Met80 coordinate the heme-Fe-atom (bonds are represented as dashed lines). Residues Asn52, Lys72, and Lys73 could play a critical role in the interaction with CL. The Lys72 residue replaces Met80 as the sixth coordination ligand of the heme-Fe-atom upon the very slow peroxynitrite-induced nitration of the solvent-exposed Tyr74 residue. The molecular graphics image (PDB: 1HRC) was produced using the UCSF Chimera package [40]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. In particular, CL acts as an allosteric activator of cyt$c$-Fe(III) by binding to specific regions of the protein (see Fig. 2);

upon the CL-cytc complex formation, the heme pocket region is altered and penta-coordination of the heme-Fe(III)-atom is facilitated. In contrast, HSA-heme-Fe(III)-mediated peroxynitrite isomerization is allosterically inhibited by drug binding to both Sudlow's site I and II (e.g., warfarin and ibuprofen, respectively). Indeed, drug binding to HSA-heme-Fe(III) induces the distortion of the heme geometry and the hexa-coordination of the heme-Fe(III)-atom, impairing HSA-heme-Fe(III) reactivity (e.g., peroxynitrite isomerization) [33,37,38].

In conclusion, it appears evident that CL binding to cytc induces tertiary changes facilitating the cleavage of the distal heme-Fe–Met80 bond, and favoring the enzymatic activity of CL-bound cytc. This structural change(s) seems different from that observed upon the very slow peroxynitrite-induced nitration (30 min) of Tyr74 in hexa-coordinated cytc [29]. Therefore, data obtained so far suggest that the CL-cytc complex could act as either a pro- or an anti-apoptotic factor, depending on the conditions under which it operates. In particular, the CL-cytc complex functions as a pro-apoptotic factor catalyzing the peroxidative reduction of H_2O_2 which leads to CL peroxidation. In contrast, the CL-cytc complex exerts an anti-apoptotic action facilitating the isomerization of peroxynitrite to nitrate with the consequent scavenging of reactive nitrogen species. Further, the reaction of NO with the ferrous CL-cytc complex impairs the cytc enzymatic activity, thus exerting a protecting effect from programmed cell death [20].

As a whole, these examples envisage the potentialities of CL-bound cytc to act as a modulator of the apoptotic process, according to the levels and the type of oxidizing species present in the cellular microenvironment. The capability of triggering either pro- and/or anti-apoptotic processes renders the CL-cytc complex a crucial element for the regulation of the cell life program, thus opening a new (although complex) scenario where the role of cytc appears to be significantly more extensive than what thought so far.

Acknowledgments

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