Biochemical and Biophysical Research Communications 415 (2011) 463–467

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Peroxynitrite detoxification by horse heart carboxymethylated cytochrome *c* is allosterically modulated by cardiolipin

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ARTICLE INFO

Article history:
Received 17 October 2011
Available online 29 October 2011

Keywords:
Horse heart cytochrome *c*
Carboxymethylated cytochrome *c*
Cardiolipin-bound carboxymethylated cytochrome *c*
Peroxynitrite isomerization
Kinetics
Allostery

ABSTRACT

Carboxymethylation of equine heart cytochrome *c* (cyt*c*) changes its tertiary structure by disrupting the heme-Fe-Met80 distal bond, such that carboxymethylated cyt*c* (CM-cyt*c*) displays myoglobin-like properties. Here, the effect of cardiolipin (CL) on peroxynitrite isomerization by ferric CM-cyt*c* (CM-cyt*c*-Fe(III)) is reported. Unlike native ferric cyt*c* (cyt*c*-Fe(III)), CM-cyt*c*-Fe(III) catalyzes peroxynitrite isomerization, the value of the second order rate constant (k_{on}) is $6.8 \times 10^4\ M^{-1}\ s^{-1}$. However, CM-cyt*c*-Fe(III) is less effective in peroxynitrite isomerization than CL-bound cyt*c*-Fe(III) (CL-cyt*c*-Fe(III); $k_{on} = 3.2 \times 10^5\ M^{-1}\ s^{-1}$). Moreover, CL binding to CM-cyt*c*-Fe(III) facilitates peroxynitrite isomerization ($k_{on} = 5.3 \times 10^5\ M^{-1}\ s^{-1}$). Furthermore, the value of the dissociation equilibrium constant for CL binding to CM-cyt*c*-Fe(III) ($K = 1.8 \times 10^{-5}$ M) is lower than that reported for CL-cyt*c*-Fe(III) complex formation ($K = 5.1 \times 10^{-5}$ M). Although CM-cyt*c*-Fe(III) and CL-cyt*c*-Fe(III) display a different heme distal geometry and heme-Fe(III) reactivity, the heme pocket and the CL cleft are allosterically linked.

1. Introduction

Cytochrome *c* (cyt*c*) is one of the most widespread and highly conserved peripheral membrane heme-protein mediating electron transfer between different proteins of the respiratory chain and playing a primary role in apoptosis [1–3].

In native cyt*c*, the heme-Fe-atom is hexa-coordinated, displaying two axial ligands, namely His18 and Met80 [4–6]. The heme-Fe-Met80 bond can be cleaved by binding of heme-Fe-atom ligands, such as CO (at acidic pH values), NO, azide, cyanide, fluoride, imidazole, and pyridine, all displaying unfavorable thermodynamic and kinetic parameters [7–16]. Moreover, native cyt*c* does not show peroxidase activity [3,17] and it does not facilitate peroxynitrite detoxification [18]. However, peroxynitrite may induce the very slow nitration (30 min) of the solvent-exposed Tyr74 residue; this leads to the cleavage of the heme-Fe-Met80 bond, which is substituted by the weak heme-Fe-Lys72 ligation [19].

At neutral pH, Met80 can be carboxymethylated, leading to the cleavage of the heme-Fe-Met80 bond with the displacement of Met80 by an intrinsic ligand, possibly His26 or His33 or Lys79 [20–23]. In contrast, Met80 carboxymethylation at acidic pH leads to a mixed population constituted by hexa- and penta-coordinated species (i.e., His18-heme-Fe-His26 or His18-heme-Fe-His33, His18-heme-Fe-H_2O, and His-heme-Fe, respectively). Remarkably, the non-native distal axial bond of the heme-Fe-atom of carboxymethylated cyt*c* (CM-cyt*c*) is weak [20–23]. Thus, like myoglobin (Mb), CM-cyt*c* binds ligands at the sixth coordination position of the heme-Fe-atom with favorable thermodynamic and kinetic parameters and shows peroxidase activity [10–13,23–28].

Very recently, cardiolipin (CL) binding to native cyt*c* has been reported to modulate the protein functions and, consequently, the cell fate [3,29–31]. CL-bound cyt*c* (CL-cyt*c*) shows a non-native tertiary structure, a disrupted heme-Fe-Met80 distal bond [32–36] and a drastically reduced midpoint potential [37]. The cleavage of the distal heme-Fe-Met80 bond endows CL-cyt*c* with a high affinity for CO and NO [38,39], peroxidase activity [1,17,40–44], and peroxynitrite detoxification properties [18].

The Mb-like reactivity of CM-cyt*c* [10–13,24–27] prompted us to investigate the effect of ferric CM-cyt*c* (CM-cyt*c*-Fe(III)) on peroxynitrite isomerization, in the absence and presence of CL. Met80 carboxymethylation induces cyt*c*-Fe(III)-mediated peroxynitrite isomerization. Moreover, CL binding to CM-cyt*c*-Fe(III) facilitates peroxynitrite isomerization. These results support the role of cyt*c* as a peroxynitrite scavenger, displaying multiple pro- and anti-apoptotic roles [31].

Abbreviations: CL, cardiolipin; CL-cyt*c*-Fe(III), CL-bound cyt*c*-Fe(III); CL-CM-cyt*c*-Fe(III), CL-bound CM-cyt*c*-Fe(III); CM-cyt*c*-Fe(III), carboxymethylated-ferric cytochrome *c*; cyt*c*-Fe(III), ferric cytochrome *c*; HSA-heme-Fe(III), ferric human serum heme-albumin.

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doi:10.1016/j.bbrc.2011.10.094

2. Materials and methods

Ferric equine heart cytochrome *c* (cyt*c*-Fe(III)) and bovine heart cardiolipin (CL) were obtained from Sigma–Aldrich (St. Louis, MO, USA). CM-cyt*c*-Fe(III), carboxymethylated at positions 65 and 80, was prepared from the native form as detailed elsewhere [25]. The cyt*c*-Fe(III) and CM-cyt*c*-Fe(III) concentration was determined spectrophotometrically at 410 nm ($\varepsilon_{410\ nm} = 1.06 \times 10^5\ M^{-1}\ cm^{-1}$) [45] and 530 nm ($\varepsilon_{530\ nm} = 9.4 \times 10\text{-}3\ M^{-1}\ cm^{-1}$) [20], respectively.

Peroxynitrite was synthesized from either 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 ($\varepsilon_{302\ nm} = 1.705 \times 10^3\ M^{-1}\ cm^{-1}$) [18].

Kinetics of peroxynitrite isomerization by cyt*c*-Fe(III) and CM-cyt*c*-Fe(III), in the absence and presence of CL (final concentration, $2.0 \times 10^{-5}\ M \leq [CL] \leq 1.6 \times 10^{-4}\ M$) and/or cyanide (final concentration, 2.0×10^{-4} M), was recorded at 302 nm ($\varepsilon_{302\ nm} = 1.705 \times 10^3\ M^{-1}\ cm^{-1}$) by rapid mixing the cyt*c*-Fe(III) solution (final concentration, $1.0 \times 10^{-6}\ M \leq [cyt\text{-}Fe(III)] \leq 5.0 \times 10^{-6}\ M$) or the CM-cyt*c*-Fe(III) solution (final concentration, $1.0 \times 10^{-6}\ M \leq [CM\text{-}cyt\text{-}Fe(III)] \leq 5.0 \times 10^{-6}\ M$) or the CL-CM-cyt*c*-Fe(III) solution (final concentration, $2.0 \times 10^{-5}\ M \leq [CL] \leq 1.6 \times 10^{-4}$ M, and $1.0 \times 10^{-6}\ M \leq [CM\text{-}cyt\text{-}Fe(III)] \leq 5.0 \times 10^{-6}\ M$) with the peroxynitrite solution (final concentration, $2.5 \times 10^{-5}\ M \leq [peroxynitrite] \leq 2.5 \times 10^{-4}\ M$) [18].

Kinetics of peroxynitrite isomerization by cyt*c*-Fe(III), CM-cyt*c*-Fe(III), and CL-CM-cyt*c*-Fe(III) was analyzed in the framework of the minimum reaction Scheme 1 [18].

Values of the pseudo-first-order rate constant for cyt*c*-Fe(III)-, CM-cyt*c*-Fe(III)- and CL-CM-cyt*c*-Fe(III)-mediated peroxynitrite isomerization (k_{obs}) have been determined from the analysis of the time-dependent absorbance decrease at 302 nm, according to Eq. (1) [18]:

$$[peroxynitrite]_t = [peroxynitrite]_i \times e^{-kobs \times t} \quad (1)$$

Values of the second-order rate constant for CM-cyt*c*-Fe(III)- and CL-CM-cyt*c*-Fe(III)-mediated peroxynitrite isomerization (k_{on}) and of the first-order rate constant for peroxynitrite isomerization in the absence and presence of cyt*c*-Fe(III) (k_0) have been determined from the linear dependence of k_{obs} on the CM-cyt*c*-Fe(III), CL-CM-cyt*c*-Fe(III), and cyt*c*-Fe(III) concentration, according to Eq. (2) [18]:

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

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

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

where $k_{on(top)}$ is the asymptotic value of k_{on} under conditions where $[CL] \gg K$, and $k_{on(bottom)}$ corresponds to the value of k_{on} in the absence of CL.

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 [46].

All data were obtained at pH 7.0 (3.0×10^{-2} M Hepes buffer) and 20.0 °C. The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation.

3. Results

Kinetics of peroxynitrite isomerization by cyt*c*-Fe(III), CM-cyt*c*-Fe(III), and CL-CM-cyt*c*-Fe(III) was fitted to a single-exponential decay for more than 90% of its course (Fig. 1, panel A; see Eq. (1)). This indicates that no intermediate species (i.e., (CL-CM-)cyt*c*-Fe(III)-OONO; Scheme 1) accumulates in the course of peroxynitrite isomerization, the formation of the transient cyt*c*-Fe(III)-OONO, CM-cyt*c*-Fe(III)-OONO, and CL-CM-cyt*c*-Fe(III)-OONO species representing the rate limiting step in catalysis. This behavior is similar to that reported for CL-cyt*c*-Fe(III), ferric human serum heme-albumin (HSA-heme-Fe(III)), equine heart Mb (Mb-Fe(III)), sperm whale Mb-Fe(III), and human hemoglobin (Hb-Fe(III)) [18,46–48].

According to literature [18], values of k_{obs} for peroxynitrite isomerization are unaffected by cyt*c*-Fe(III) (Fig. 1, panel B), corresponding to k_0 (i.e., the first-order rate constant for spontaneous peroxynitrite isomerization) (Fig. 1, panel B). In contrast, values of k_{obs} for CM-cyt*c*-Fe(III)-mediated peroxynitrite isomerization increase linearly with the CM-cyt*c*-Fe(III) concentration (Fig. 1, panel C). Remarkably, CL binding to CM-cyt*c*-Fe(III) facilitates peroxynitrite isomerization, k_{obs} values increasing linearly with the CL-CM-cyt*c*-Fe(III) concentration (Fig. 1, panel C). The analysis of data according to Eq. (2) allowed the determination of values of k_{on} for CM-cyt*c*-Fe(III)- and CL-CM-cyt*c*-Fe(III)-mediated peroxynitrite isomerization (corresponding to the slope of the linear plots; $6.8 \times 10^4\ M^{-1}\ s^{-1}$ and $5.3 \times 10^5\ M^{-1}\ s^{-1}$, respectively; $[CL] = 1.6 \times 10^{-4}$ M) and k_0 for peroxynitrite isomerization in the absence and presence of cyt*c*-Fe(III) (corresponding to the *y* intercept of the linear plots; $2.8 \times 10^{-1}\ s^{-1}$ and $3.1 \times 10^{-1}\ s^{-1}$, respectively). Values of k_0 here obtained are in good agreement with those reported in the literature [18,46–51].

Since the hexa-coordinated species CM-cyt*c*-Fe(III)-CN and CL-CM-cyt*c*-Fe(III)-CN do not affect peroxynitrite isomerization kinetics (Fig. 1, panel B), the acceleration of the peroxynitrite isomerization rate by CM-cyt*c*-Fe(III) and CL-CM-cyt*c*-Fe(III) appears to be due to the reaction of peroxynitrite with a penta-coordinated heme-Fe(III) atom, as reported for human Hb-Fe(III) [47], horse heart Mb-Fe(III) [47], and HSA-heme-Fe(III) [46].

To confirm the catalytic effect of CM-cyt*c*-Fe(III) and CL-CM-cyt*c*-Fe(III) on peroxynitrite isomerization, the dependence of k_{obs} and k_0 on the peroxynitrite concentration was determined in the absence and presence of cyt*c*-Fe(III) derivatives (Fig. 2). Under all the experimental conditions, k_{obs} and k_0 values for peroxynitrite isomerization slightly decrease upon increasing peroxynitrite concentration. In contrast, the amplitude of kinetics increases as a function of the peroxynitrite concentration (data not shown). The decrease of k_{obs} and k_0 values upon increasing peroxynitrite concentration at fixed cyt*c*-Fe(III) concentration (Fig. 2) has been proposed to reflect the occurrence of the peroxynitrite/peroxynitrous acid adduct at $[peroxynitrite] > 5.0 \times 10^{-5}$ M, around neutrality [47]. Accordingly, the decrease of k_{obs} and k_0 values may reflect either the slow CM-cyt*c*-Fe(III)- and CL-CM-cyt*c*-Fe(III)-mediated decomposition of the peroxynitrite/peroxynitrous acid adduct or the slow dissociation of the peroxynitrite/peroxynitrous acid adduct preceding the CM-cyt*c*-Fe(III)- and CL-CM-cyt*c*-Fe(III)-catalyzed peroxynitrite isomerization [47].

As shown in Fig. 3, CL facilitates CM-cyt*c*-Fe(III)-mediated peroxynitrite isomerization; indeed, values of k_{on} increase upon

$$(CL\text{-}CM\text{-})cyt\text{-}Fe(III) + HOONO \xrightarrow{k_{on}} (CL\text{-}CM\text{-})cyt\text{-}Fe(III)\text{-}OONO + H^+ \xrightarrow{fast} (CL\text{-}CM\text{-})cyt\text{-}Fe(III) + NO_3^-$$

Scheme 1.