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To cite this version:

Paolo Ascenzi, Roberto Santucci, Massimo Coletta, Fabio Polticelli. Cytochromes: Reactivity of the "dark side" of the heme. Biophysical Chemistry, 2010, 152 (1-3), pp.21. 10.1016/j.bpc.2010.09.008 . hal-00694281

HAL Id: hal-00694281

https://hal.science/hal-00694281v1

Submitted on 4 May 2012

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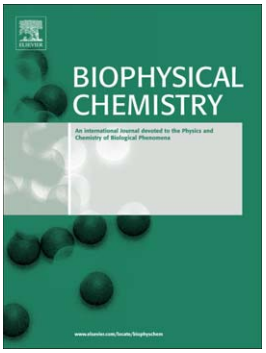

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PII: S0301-4622(10)00232-2
DOI: doi: 10.1016/j.bpc.2010.09.008
Reference: BIOCHE 5434

To appear in: *Biophysical Chemistry*

Received date: 2 August 2010
Revised date: 27 September 2010
Accepted date: 27 September 2010

Please cite this article as: Paolo Ascenzi, Roberto Santucci, Massimo Coletta, Fabio Polticelli, Cytochromes: Reactivity of the "dark side" of the heme, *Biophysical Chemistry* (2010), doi: 10.1016/j.bpc.2010.09.008

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Cytochromes: reactivity of the "dark side" of the heme [#]

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Running title: NO binding to the heme proximal side

Keywords: Heme-proteins; *Alcaligenes xylosoxidans* cytochrome *c´*; Horse heart cytochrome *c*; Nitrogen monoxide; Carbon monoxide; Ligand binding; Peroxidase activity; Heme-ligand geometry, Allostery.

Abbreviations: Axcyt *c'*, cytochrome *c´* from *Alcaligenes xylosoxidans*; CL, cardiolipin; CL-hhcyt *c*, cardiolipin-bound horse heart cytochrome *c*; cyt *c*, cytochrome *c*; cyt *c'*, cytochrome *c´*; hhcyt *c*, horse heart cytochrome *c*; Mb, myoglobin.

[#] Rephrasing of the title of the famous concept album "The Dark Side of the Moon" (1973) by the English progressive rock group Pink Floyd**.**

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Summary

Ligand binding to the heme distal side is a paradigm of heme-protein biochemistry, the proximal axial ligand being in most cases a His residue. NO binds to ferrous heme-Fe-atom giving rise to hexa-coordinated adducts (as in myoglobin and hemoglobin) with His and NO as proximal and distal axial ligands, respectively, or to penta-coordinated adducts (as in soluble guanylate cyclase) with NO as the axial distal ligand. Over the last decade, the ferrous derivative of *Alcaligenes xylosoxidans* cytochrome *c´* (Axcyt *c*') and of cardiolipin-bound horse heart cytochrome *c* (CL-hhcyt *c*) have been reported to bind NO to the "dark side" of the heme (*i.e.*, as the proximal axial ligand) replacing the endogenous ligand His. Conversely, CL-free hhcyt *c* behaves as ferrous myoglobin by binding NO to the heme distal side, keeping His as the proximal axial ligand. Moreover, the ferrous derivative of CL-hhcyt *c* binds CO at the heme distal side, the proximal axial ligand being His. Furthermore, CL-hhcyt *c* shows peroxidase activity. In contrast, CL-free hhcyt *c* does not bind CO and does not show peroxidase activity. This suggests that heme-proteins may utilize both sides of the heme for ligand discrimination, which appears to be modulated allosterically. Here, structural and functional aspects of NO binding to ferrous Axcyt *c*' and (CL-)hhcyt *c* are reviewed.

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The free radical NO is pivotal in many different biological functions (1-17), so much so that NO-synthase gene has been proposed in novel gene therapy approaches (18). Remarkably, heme-proteins are pivotal in the synthesis, trafficking, storage, and metabolism of NO. In fact, the formation of heme-NO complexes occurs in NO generation by NO-synthase (19-22), NO sensing by soluble guanylate cyclase (23), NO transport and storage by nitrophorins (24,25), and NO metabolism by NO-reductases and -oxidases (26,27).

As reported for O_2 and CO, NO binds to the distal side of the ferrous heme, the proximal axial ligand of the heme-Fe-atom being in most cases a His residue. Therefore, many NO-binding heme-proteins display hexa-coordinated heme adducts. However, in some heme-proteins, NO binding to the heme distal side induces the dissociation of the proximal His residue, leading to penta-coordinated complexes. Moreover, the temperature-dependent equilibrium mixture of the ferrous nitrosylated hexa- and penta-coordinated heme has been observed in some heme-proteins (see refs 23,25,28,29).

NO binding to the ferrous heme-Fe-atom not only affects the heme-nitrosyl electronic structure and the heme-Fe-atom coordination number, but also triggers conformational changes that could be linked to allosteric regulation in heme-proteins. The relevant role of ferrous nitrosylated heme adducts has stimulated a significant effort to understand the chemistry of penta- and hexa-coordinated species and to identify the driving force for the loss of the proximal His ligand leading to the formation of the penta-coordinated ferrous heme-Fe-NO adduct (see 23,35,28,29).

Recently, the possibility that NO could bind to the proximal heme side came to light. In fact, since the proximal heme ligand (His) can be lost during the penta-coordination of the ferrous heme-Fe-NO complex, it may be possible for the NO ligand to occupy the proximal heme coordination site. Indeed, the crystal structure of a periplasmic class IIa *c*-type cytochrome *c′* from the denitrifying bacterium *Alcaligenes xylosoxidans* (Axcyt *c*’) showed

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that distal to proximal heme-NO conversion can occur in heme-proteins (30-32). NO binding to the proximal heme side of Axcyt *c'* has been postulated to be relevant: (*i*) in NO-trafficking or defense against nitrosative stress (33-36), (*ii*) in discriminating between the gaseous ligands NO, CO, and O_2 (37), and (*iii*) in inducing specific gas-dependent conformational changes (30). NO binding to the cardiolipin-bound horse heart cytochrome *c* (CL-hhcyt *c*) is also consistent with the formation of a proximal heme-NO penta-coordinated species (33). Note that NO acts as a gas modulator in the mitochondria, triggering, among others, apoptosis (39). Lastly, NO binding to soluble guanylate cyclase shows some features that might suggest NO binding to the proximal side of the heme (30,40).

Here, the nitrosylation mechanisms of ferrous Axcyt *c*' and (CL-)hhcyt *c* are reviewed and functional implications examined.

Diatomic gas discrimination by *Alcaligenes xylosoxidans* cytochrome *c´*

Cytochromes *c'* (cyt *c'*) are a distinct family of class IIa cytochromes found in the periplasm of certain denitrifying, nitrogenfixing, photosynthetic, methanotrophic, and sulfur-oxidizing bacteria (31,37,41,42). Although it has long been assumed that cyt *c'* are involved in electron transfer (37,43), cyt *c'* present in the denitrifying bacteria have been suggested to bind NO, helping to alleviate nitrosative stress (33,37,44,45). Ferrous cyt *c'* are able to bind also CO, although they do not generate stable O_2 adducts (37,46-54).

Cyt *c'* are usually homodimers, each subunit being composed of an antiparallel four-helix bundle of approximately 130 residues containing a *c*-type heme. A conserved Cys-Xxx-Xxx-Cys-His motif provides the proximal His ligand and two Cys-thioether bonds to the heme (residues 116, 119, and 120 in Axcyt *c'*). The access to the vacant sixth co-ordination site on the distal face of the heme is mediated by the side chain of a hydrophobic residue (Leu16 in Axcyt *c'*). Indeed, the distal pocket is generally hydrophobic, sterically crowded,

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and less solvent-accessible than the proximal pocket, which is positively polarized, less crowded and readily accessible to the surrounding solvent (see 31).

In the physiological environment, cyt *c'* seems to function in the reduced oxidation state of the heme-Fe-atom (*i.e.*, in the ferrous form) (see 37). Ferrous Axcyt *c'* has been reported to bind NO and CO at the proximal and distal side of the heme, respectively, representing the first structurally-characterized example of exogenous ligand binding to both sides of the heme-Fe-atom (30,31,55,56).

Under steady-state conditions, ferrous Axcyt *c'* binds NO to the proximal side of the heme-Fe-atom as the proximal axial ligand, leading to a penta-coordinated NO adduct (Fig. 1). NO is present as two equally populated conformers with an average Fe-N-O angle of 128°. Overall the structure of the ferrous nitrosylated derivative of Axcyt *c'* overlaps with that of the ligand-free reduced form, as the positions of the heme, the Fe-atom and Leu16 are highly similar. In both structures, the heme-Fe-atom lies out of the heme plane, which is distinctly puckered, by about 0.3 Å towards the proximal axial ligand (*i.e.*, His120 and NO in the ligand-free and nitrosylated derivatives, respectively). The major differences between the ligand-free and nitrosylated derivatives of ferrous Axcyt *c'* are the ligation of NO (as the proximal axial ligand of the heme-Fe-atom), the displacement of the proximal His120 and the flipping of Arg124, which stakes against the heme plane and is hydrogen-bonded to conformer 2 of NO (30,31).

As shown in Figure 1, the binding geometry of NO to the heme-Fe-atom of ferrous Axcyt *c'* is completely different from that observed in ferrous nitrosylated sperm whale myoglobin (Mb) (57). In sperm whale Mb, NO is bound at the heme distal side, the proximal axial ligand being HisF8. Specifically, the Fe-N-O angle is 112°, and the Nε atom of the distal HisE7 residue is located 2.8 Å away from the nitrogen atom of the heme-bound NO, implying electrostatic stabilization of the heme-Fe-NO complex.

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Carbonylation of ferrous Axcyt *c'* leads to a hexa-coordinated adduct. CO binds to the distal face of the heme in a near-linear conformation (the Fe-C-O angle is 167°), thereby displacing the side chain of Leu16. In this latter case, the proximal axial ligand is His120. The heme-Fe-atom is within the plane of the heme, which is essentially flat. In addition, a second CO molecule is hydrogen bonded to the Nδ1 atom of His120 (30,31) (Fig. 1). This mimics the interaction seen in some heme-proteins between the proximal His residue and either a main-chain carbonyl or a side-chain carboxyl group (see refs 58,59). The positions of the proximal His120 residue and of the heme-Fe-atom in the ferrous ligand-free and carbonylated structures of Axcyt *c'* are essentially identical, and the movement and flattening of the heme restore the Fe-atom into the plane of the heme (30,31) (Fig. 1). This is in contrast with the situation in hemoglobin where the binding of gaseous diatomic ligands pulls the ferrous Fe-atom into the heme plane and the proximal His residue towards the heme, thereby triggering allosteric transition(s) (28).

The binding geometry of CO to the heme-Fe-atom of ferrous Axcyt *c'* is reminiscent of that reported for ferrous carbonylated sperm whale Mb (60) (Fig. 1). Indeed, in sperm whale Mb CO is bound at the heme distal side, the proximal axial ligand being HisF8. Specifically, the Fe-C-O angle is about 130° and the Nε atom of the heme distal HisE7 residue is located 3.3 Å away from the oxygen atom of the heme-bound CO, implying a weaker electrostatic stabilization of the Fe-CO complex with respect to the Fe-NO complex.

Nitrosylation of ferrous Axcyt *c'* involves at least three species (*i.e.*, A to C in Fig. 2), including the initial and final forms (*i.e.*, A and C, respectively) and one intermediate (*i.e.*, B) (30-32,55,56,61-63). NO binds to the distal side of the ferrous heme (*i.e.*, A in Fig. 2) to give the hexa-coordinated ferrous His120-Fe-NO adduct (*i.e.*, B in Fig. 2). The value of the second-order rate constant for the A to B reaction (*i.e.*, k_1 in Fig. 2) is 4.6×10^4 M^{-1} s^{-1}. Nitrosylation of ferrous Axcyt *c'* leads to the weakening and breaking of the His120-Fe bond

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(see Fig. 2). A second NO displaces the proximal His120 residue and binds to the proximal side of the heme, giving a transient hexa-coordinated ferrous NO-Fe-NO species (see Fig. 2), which evolves toward the dissociation of the distally-bound NO, yielding the final ferrous penta-coordinated proximal NO-Fe adduct (*i.e.*, C in Fig. 2). The value of the second order rate constant for the B to C reaction (*i.e.*, k_2 in Fig. 2) is 8.7×10^3 M^{-1} s^{-1}. The transient NO-Fe-NO complex was not observed, and thus it could be concluded that the dissociation of the distally-bound NO is not the rate limiting event. Values of k_1 and k_2 for NO binding to ferrous Axcyt *c'* are smaller by several orders of magnitude with respect to those reported for the nitrosylation of ferrous heme-proteins (*e.g.*, CL-hhcyt *c* (see below) and Mb) (38,64).

Denitrosylation of ferrous Axcyt *c'*-NO involves four species (*i.e.*, C to A in Fig. 2), including the initial and final forms (*i.e.*, C and A, respectively) and two intermediates (*i.e.*, D and E) (62). Photolysis of NO from the equilibrium state designated C (see Fig. 2) yields a tetra-coordinated species (*i.e.*, D in Fig. 2). NO rebinding to the transient form designated D (see Fig. 2) is fully geminate (≥ 99%) and the value of the rate constant (*i.e.*, k_3; see Fig. 2) is 1.4×10^{11} s^{-1}. Therefore, the rate constant for NO escape from the transient tetra-coordinated Axcyt *c'*···NO species (*i.e.*, D in Fig. 2; k_5) to the bulk solvent is ~ $\times10^9$ s^{-1}. This suggests that the proximal binding heme environment of Axcyt *c'* does not allow for accommodation of both NO and His120 in the proximity of the ferrous heme-Fe-atom, such that His120 can bind the heme only when NO leaves the proximal heme environment (65). The observed overall rate constant for NO dissociation from C to A (see Fig. 2) is 4.1×10^{-4} s^{-1}; in view of the high percentage of geminate recombination (*i.e.*, ≥ 99%, see above), this value indicates that the actual rate for NO dissociation (from C to B, see Fig. 2) in the absence of photolysis is ~ 4×10^{-2} s^{-1}. In this framework (see Fig. 2), the binding of His120 to the NO-free tetra-coordinated Axcyt c' species is about 100-fold slower (*i.e.*, ~ 10^9 s^{-1}) than the rebinding of NO, slowing the formation of species A in Fig. 2.

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The reactivity of ferrous Axcyt *c'* towards NO has been postulated to be modulated especially by Arg124 (32). Indeed, the retention of a basic residue at the 124 position is essential to stabilize by hydrogen-bonding the His120-Fe-NO transient adduct of Axcyt *c'*. Surprisingly, this indicates that the proximal Arg124 residue modulates the reactivity towards NO on the heme distal side (32). Moreover, the presence of a cationic residue at this proximal position is likely to be relevant for the physiological function of members of the cyt *c'* family containing invariantly either an Arg or a Lys residue (see HOMSTRAD database for a multiple sequence alignment: http://tardis.nibio.go.jp/cgi-bin/homstrad/showpage.cgi?family=cytprime&disp=str).

No kinetic and thermodynamic data concerning Axcyt *c'* carbonylation are available. However, it has been reported that the bimolecular rate constant for carbonylation of *Rhodopseudomonas palustris* cyt *c'*, homologous to Axcyt *c'*, ranges from ~ 1×10^{3} M^{-1} s^{-1}, in the folded form, to ~ 1×10^{6} M^{-1} s^{-1}, in the unfolded state. This suggests that electron transfer, occurring only in the folded conformer, triggers the folding-unfolding equilibrium of *Rhodopseudomonas palustris* cyt *c'* (66). Moreover, ferrous cyt *c'* do not generate stable O_2 adducts (see ref. 37).

Cardiolipin switches allosterically horse heart cytochrome *c* function from electron transport to gas sensing and peroxidation

Eukaryotic cytochromes *c* (cyt *c*) are water-soluble globular heme-proteins that are located within the compartment delimited by the inner and outer mitochondrial membranes. Horse heart cyt *c* (hhcyt *c*) is a single-chain heme-protein composed of 104 amino acids, containing the heme as a prosthetic group lying within a crevice and covalently attached to the polypeptide chain by two thioether bonds formed with residues Cys14 and Cys17; the heme is axially coordinated by His18 and Met80. The protein is relatively rigid (67-69), in

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agreement with its electron transfer function. Hhcyt *c* binds NO with low affinity (70), does not bind CO (71), and does not display peroxidase activity (72-74).

Hhcyt *c* is involved not only in electron transfer from the bc_1 complex to the terminal acceptor cytochrome *c* oxidase, but also in apoptosis (39,74-76). In this process, hhcyt *c* interacts with cardiolipin (CL) prior to be released from the mitochondrion (77-83). CL, which constitutes about 20% of total lipids of the mitochondrial membrane, is synthesized in the mitochondrion and possesses a unique structure, being composed of four (instead of two, as in most lipids) fatty acid tails. CL binding to hhcyt *c* plays an important role in determining the function of the heme-protein (and, thus, the cell fate). At the early stage of apoptosis, the CL-hhcyt *c* complex dissociates favoring hhcyt *c* release into the cytosol (84). Cytosolic hhcyt *c* plays an important pro-apoptotic role activating caspase-9, which is one of the proteases involved in the caspase cascade leading to cell death (85). CL peroxidation is indicated as one of the factors responsible for the CL-hhcyt *c* complex dissociation, although the mechanism governing the event remains still unclear (86). This hypothesis is based on the observation that oxidative degradation of CL occurs in the p53-induced apoptosis (87), and that CL hydroperoxides have lower binding affinity for hhcyt *c* with respect to CL (88). Thus, CL binding to hhcyt *c* is impaired by CL peroxidation and phospholipid peroxidation is prevented by BCL-2, the anti-apoptotic protein member of the Bcl-2 family known to inhibit hhcyt *c* release from mitochondrion (89,90).

CL binds to hhcyt *c* at two distinct regions, called the A-site and the C-site, characterized by different affinity for phospholipids (77,82). The binding reaction seems to proceed through the insertion of one acyl chain of CL in the hhcyt *c* interior (77,91). The so-called “extended lipid anchorage” hypothesis assumes that one acyl chain of CL accommodates into the protein while the other one points to the opposite direction from the head-group; this event seems to keep the protein firmly anchored to the membrane (91). The

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region of hhcyt *c* into which CL binds is uncertain and still matter of controversy (81,83,91); thus, possible models have been proposed. The first model to be formulated (91) suggests that upon CL binding to hhcyt *c* one acyl chain of CL protrudes into the protein interior through the hydrophobic channel located close to the invariant Asn52 residue. The CL chain insertion into the protein is assumed to be favored by H-bonding between Asn52 and the protonated phosphate group of CL. Subsequently, an alternative model has been proposed (81); it asserts that the binding of CL to the protein occurs in the region of the Met80-containing loop, and that the acyl chain protrudes into the protein between the hydrophobic strands formed by 67-71 and 82-85 residues. Anchoring of the phospholipid to the protein would occur *via* electrostatic interactions between the deprotonated phosphate group of the CL liposome and the Lys72 residue of hhcyt *c*. In addition to these two models, a very recent report considers the possibility that both sites of the protein may participate to CL binding (83); accordingly, CL would bind to hhcyt *c* by two (instead of one) acyl chains, such binding mode having been confirmed to be sterically feasible (Fig. 3).

Upon interaction with CL, hhcyt *c* has been shown: (*i*) to change its tertiary structure disrupting the heme-Fe-Met80 distal bond and, in some cases, to vary the spin state of the metal (the proximal axial ligand is His) (74,77,81-83,91,92), (*ii*) to reduce drastically the midpoint potential out of the range required for its role in the respiratory chain (80), (*iii*) to display peroxidase activity (78,93-96), also through the peroxidation of CL, thus inducing the permeabilization of the mitochondrial membrane (74,78,80), and (*iv*) to bind CO and NO with high affinity (38,97), modulating apoptosis (98-100) by inhibiting the CL-hhcyt *c* peroxidase activity (38,79.97). Interestingly, the CL-hhcyt *c* complex binds NO and CO at the proximal and distal side of the heme, respectively (38,97), as reported for Axcyt *c'* (see ref. 31).

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In the ferrous CL-hhcyt *c* complex, NO replaces the proximal His residue via an unusually complex set of kinetic steps, suggesting a remarkable mobility of the heme environment induced by CL (38).

Nitrosylation of ferrous CL-hhcyt *c* involves five species (*i.e.*, A to E in Fig. 4), including the initial and final forms (*i.e.*, A and E, respectively) and three intermediates (*i.e.*, B, C, and D) (38). The first very rapid [NO]-dependent kinetic process ($k_1 = 2\times10^7$ M^{-1} s^{-1}) has been assigned to the initial NO binding to the ferrous heme-Fe-atom. Interestingly, the second order rate constant is several orders of magnitude faster than that for nitrosylation of Axcyt *c'* (63) and Met80Ala hhcyt *c* mutant (101), in which NO binding is rate limited by steric hindrance provided by the tightly packed protein cage. The primary NO binding process (*i.e.*, the A to B reaction; see Fig. 4) leads to a penta-coordinated derivative (*i.e.*, the B species in Fig. 4). The transient NO-bound hexa-coordinated form was not observed, and thus it has been concluded that, once NO is bound to the ferrous heme-Fe-atom, the dissociation of the His18 residue is more rapid than the rapid-mixing stopped-flow dead time (*i.e.*, < 1 ms). Following the initial binding reaction a slower step occurs (*i.e.*, the B to C reaction; see Fig. 4), whose rate is essentially [NO]-independent ($k_2 = 7$ s^{-1}). This second process has been referred to the binding of an yet unidentified weak field ligand (*i.e.*, X in Fig. 4) trans to the ferrous heme-Fe-NO complex (*i.e.*, the C species in Fig. 4). The nature of the weak field ligand is still unclear, possibly being an intrinsic amino acid residue or a water molecule (38). However, at physiological NO concentrations this can be a long lived intermediate that could play a direct role in NO uptake and/or release in mitochondria, with obvious functional implications. Whatever is the nature of the proximal X ligand, it appears to be displaced by a second NO molecule (*i.e.*, C to D reaction; see Fig. 4). Three remarkable, but possible linked, features characterize this process: (*i*) the reaction is slow, (*ii*) the time courses are not exponential but zero order (typical for diffusive processes), this being particularly evident at

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high NO concentration, and (*iii*) the rates are proportional to the NO concentration. Therefore, it has been proposed that in the C to D reaction the NO in solution partitions favourably into the considerable lipid phase provided by CL, and from this phase NO diffuses to the site where it can displace the putative X ligand. The overall rate constant of the C to D reaction (*i.e.*, k_3; see Fig. 4) is 1×10^{-1} s^{-1}, clearly indicating that the access pathways of NO to the distal and to the proximal binding sites of ferrous CL-hhcyt *c* drastically differ. From the spectroscopic viewpoint the species designated D (see Fig. 4) is similar, but not identical, to the final product (*i.e.*, E in Fig. 4) and it has been assigned to a transient penta-coordinated ferrous heme-Fe-NO adduct. The last slow (k_4 = 0.1 s^{-1}), [NO]-independent step (D to E reaction; see Fig. 4) takes the protein to the final penta-coordinated ferrous heme-Fe-NO adduct (*i.e.*, E in Fig. 4). The nature of this final transition is unknown; it has been suggested that it reflects some slow structural relaxation of the lipid/protein complex bringing the heme environment to its final equilibrium state (*i.e.*, E in Fig. 4).

Denitrosylation of ferrous CL-hhcyt *c*-NO involves four species (*i.e.*, E to A in Fig. 4), including the initial and final forms (*i.e.*, E and A, respectively) and two intermediates (*i.e.*, F and G) (38). Photolysis of NO from the equilibrium state designated E (see Fig. 4) yields a tetra-coordinated species (*i.e.*, F in Fig. 4). NO rebinding to the transient form designated F (see Fig. 4) is largely geminate (89%) and the value of the rate constant (*i.e.*, k_5; see Fig. 4) is 1.3×10^{11} s^{-1}. The remainder of the NO (11%) moves away from the heme pocket reaching the bulk phase or moves to sites removed from the heme but within the CL-hhcyt *c* complex. Unlike Axcyt c', in the case of CL-hhcyt *c* the rate constant for His18 binding (k_6 = 1.6×10^{10} M^{-1} s^{-1}; see Fig 4) competes with NO binding, producing an appreciable amount of penta-coordinated species (G in Fig. 4), suggesting that the flexible proximal binding heme environment of CL-hhcyt *c* allows for accommodation of both NO and His18 in the proximity of the ferrous heme-Fe-atom. The rate at which NO recombines on the proximal side of the

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heme (reaction G to F in Fig. 4) is dictated by the rate of the His18 dissociation (*i.e.*, k_7 = 4×10^2 s^{-1}). The final step (*i.e.*, G to A in Fig. 4) counterbalances the slow structural rearrangement(s) that has been assigned to the transition within the nitrosylated ferrous CL-hhcyt *c* complex from D to E (see Fig. 4). The overall rate constant for NO dissociation from E (see Fig. 4) is 5×10^{-4} s^{-1}.

The CL-induced conformational change(s) is also reflected in CO binding properties of hhcyt *c*. Thus, while native hhcyt *c* is unable to bind CO (102), ferrous CL-hhcyt *c* interacts with CO by a simple second-order process (97), as reported for the Met80X hhcyt *c* mutants (101,103), carboxymethylated hhcyt *c* (102), and Mb (104). The value of the dissociation equilibrium constant for ferrous CL-hhcyt *c* carbonylation (~ 3×10^{-8} M) (97) is similar to that reported for carboxymethylated hhcyt *c* (102) and Mb (104). However, both combination and dissociation rate constants for (de)carbonylation of the ferrous CL-hhcyt *c* complex (~ 1×10^7 M^{-1} s^{-1} and 0.18 s^{-1}, respectively) are larger than those reported for the Met80X mutants (Silkstone et al., 2002), carboxymethylated hhcyt *c* (102), and Mb (104). This suggests a very open heme crevice in the CL-hhcyt *c* complex (accounting for the very fast CO association rate constant) (97), but also a weaker proximal Fe-His bond (as suggested by the faster CO dissociation rate constant) (105), which seems confirmed also by the very fast dissociation rate for the proximal His residue upon NO binding (38). Therefore, CL binding to hhcyt *c* seems to induce a gross conformational change(s) on both the proximal and distal sides of the heme, bringing about the loss of the electron transfer properties of hhcyt *c* and its transformation into a peroxidase (78,80).

Conclusion and Perspectives

As a whole, the unusual features of NO and CO binding to the heme-Fe-atom of ferrous Axcyt *c'* represent a new mechanism of heme-based gas sensors. These heme-

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proteins, including soluble guanylate cyclase, FixL, and CooA, play a central role in regulating important biological processes by sensing changes in the concentrations of NO, O_2, and CO, respectively. Indeed, heme-based gas sensors discriminate diatomic gaseous ligands by gas-dependent changes of: (*i*) the heme-Fe-atom oxidation state, (*ii*) the heme-Fe-atom coordination state and axial ligation, as well as (*iii*) either flattening or puckering of the porphyrin ring (31,38,97,106-109). These changes then bring about dramatic structural changes of the proximal side of the heme, which becomes more accessible to molecules from the bulk solvent, as indicated by the binding of NO to the proximal side of the heme in Axcyt *c'* (30) and the binding of imidazole to NO-bound soluble guanylate cyclase (110).

In the case of the CL-hhcyt *c* complex the high flexibility of hhcyt *c* renders it more suitable to be modulated and NO indeed may be very useful to induce a conformational change, which involves both the distal and the proximal side. It might then work as a messenger for a change in the function of hhcyt *c* once bound to CL, such that hhcyt *c* could act either as a gas sensor in apoptosis (38,97) and/or as a new protein with still unexplored enzymatic properties, among which peroxidase activity is only an "iceberg" summit (74,78,80) Thus, NO might modulate the appearance of the peroxidase activity through its interaction with the heme-Fe-atom and in this framework the proximal or distal interaction side might represent two different aspects of such a modulatory role. This being the case, an additional linkage between NO levels and apoptosis might emerge, opening a new scenario for the role of hhcyt *c* in the framework of the cellular apoptotic process.

Acknowledgements

This work was partially supported by grants from the Ministero dell'Istruzione, dell'Università e della Ricerca of Italy (PRIN 2007ECX29E_002 and University Roma Tre, CLAR 2010, to P.A.).

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Figure captions

Fig. 1. Structures of ferrous nitrosylated and carbonylated Axcyt *c'* and sperm whale Mb (swMb). In the final penta-coordinated nitrosylated derivative of ferrous Axcyt *c'*, the displacement of the proximal His120 residue and the flipping of the Arg124 side chain (which stakes against the heme plane and is hydrogen-bonded to conformer 2 of NO) occur. The carbonylation of ferrous Axcyt *c'* displaces the Leu16 side chain, the proximal ferrous heme-Fe-atom axial ligand is His120. In addition, a second CO molecule is hydrogen bonded to Nδ1 of His120 (green line). In the nitrosylated derivative of sperm whale Mb, NO is bound at the heme distal side with the nitrogen atom 2.8 Å away from the Nε atom of the distal HisE7 residue, implying electrostatic stabilization of the Fe-NO complex (green line). In ferrous carbonylated sperm whale Mb, CO is bound at the heme distal side as well, with its oxygen atom located 3.3 Å away from the Nε atom of the distal HisE7 residue, suggesting a weaker electrostatic stabilization of the Fe-CO complex (green line) with respect to the Fe-NO adduct. Atomic coordinates have been taken from PDB entries 1E85, 1E86, 1HJT, and 1VXH (30,57,60).

Fig. 2. Simplest reaction mechanism for nitrosylation and denitrosylation of ferrous Axcyt *c'*(-NO). Putative reaction intermediates are shown within brackets. A to C reactions (left and bottom) were investigated by rapid-mixing. C to A reactions (right and top) were investigated by laser photolysis (30-32,55,56,61-63).

Fig. 3. Molecular model of the putative CL-hhcyt *c* complex. The CL molecule (shown in orange) binds to hhcyt *c* by insertion of two of the four acyl chains in the vicinity of Asn52 and Met80. This binding mode allows the tethering of hhcyt *c* to the membrane as CL can still

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remain membrane-bound through the other two acyl chains. The insertion of a single acyl chain into hhcyt *c* molecule is unlikely due to stereochemical constrains which would force the adjacent CL acyl chain to be solvent exposed (83).

Fig. 4. Simplest reaction mechanism for nitrosylation and denitrosylation of ferrous CL-hhcyt *c*(-NO). Putative reaction intermediates are shown within brackets. A to E reactions (left and bottom) were investigated by rapid-mixing. E to A reactions (right and top) were investigated by laser photolysis (38).

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Figure 1

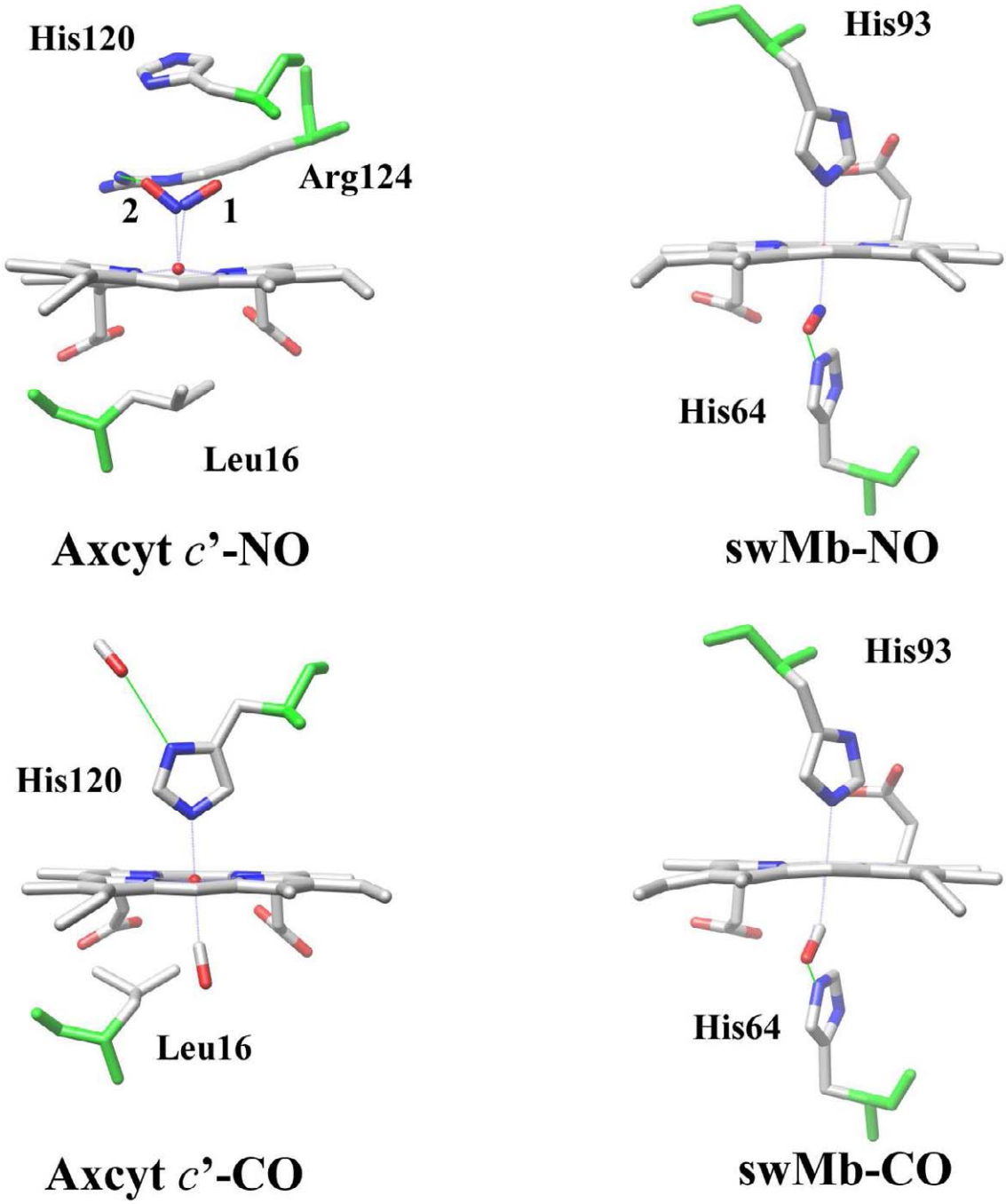

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Figure 2

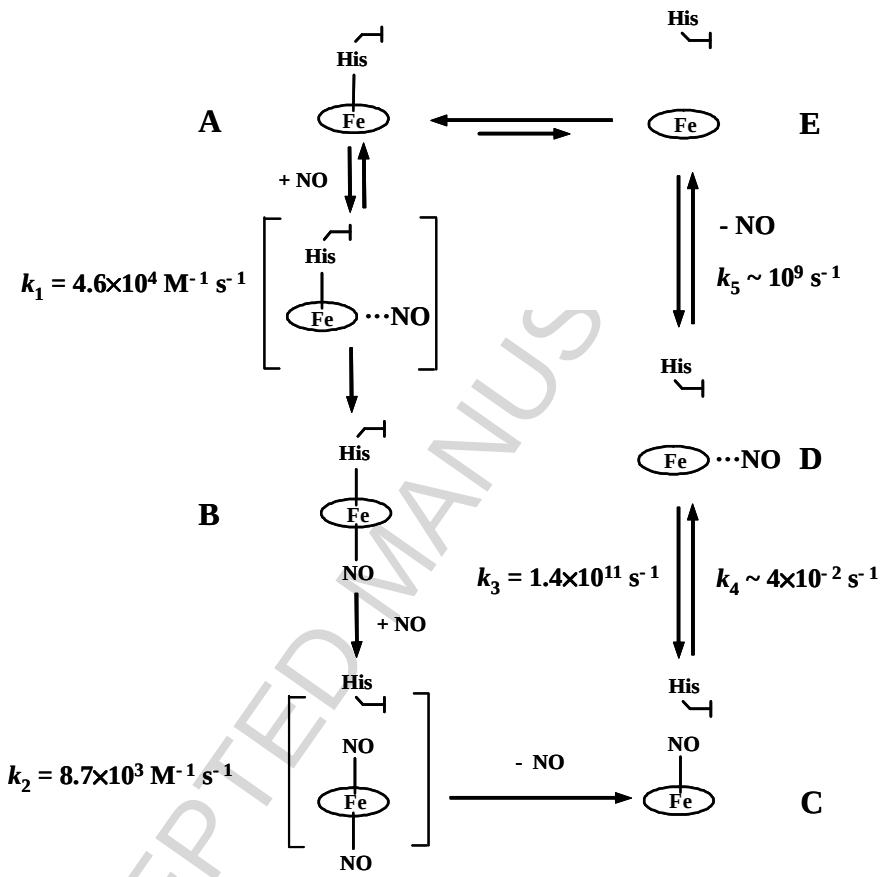

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Figure 3

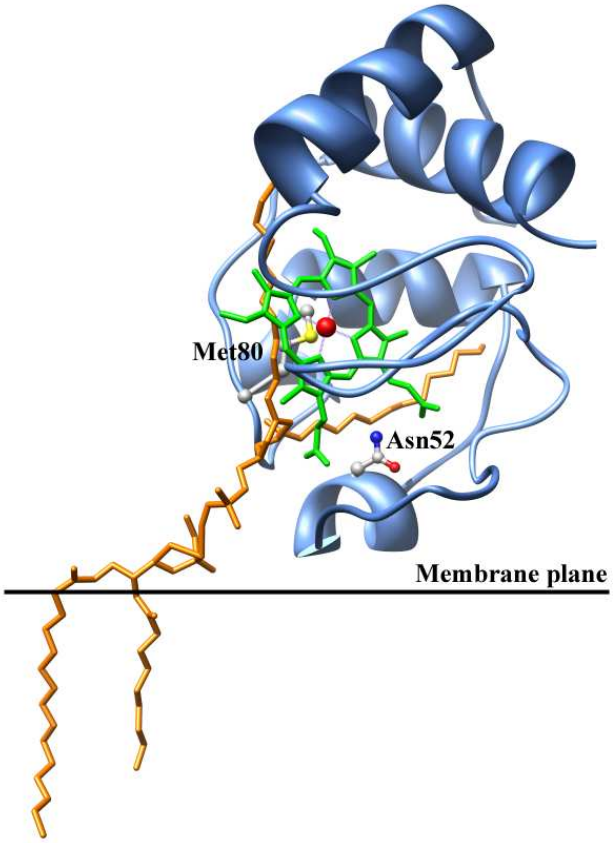

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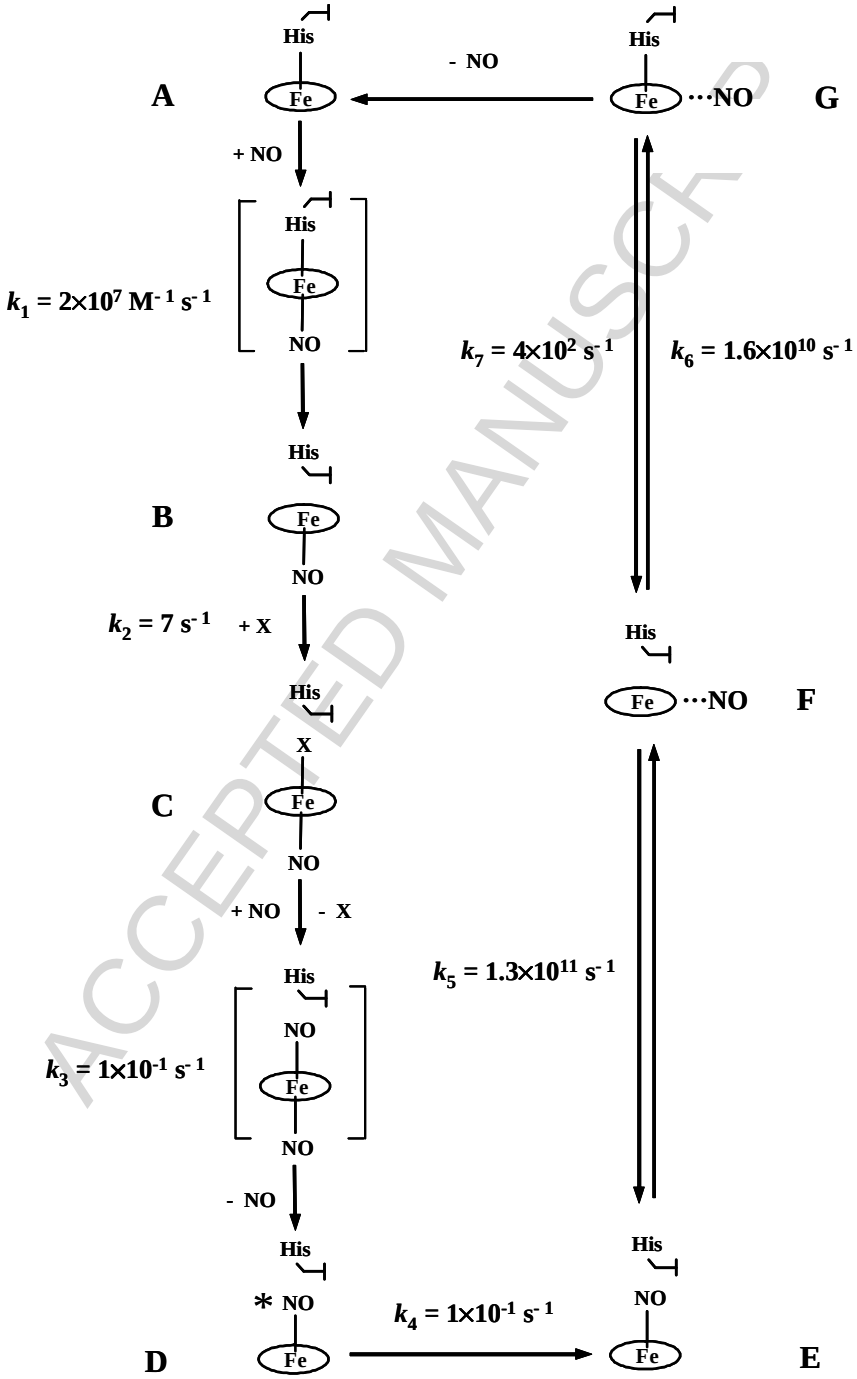
Figure 4



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Research Highlights

- **Cytochromes bind NO at the "dark side" of the heme**
- **NO and CO discrimination by *Alcaligenes xylosoxidans* cytochrome *c´***
- **Cardiolipin is an allosteric modulator of horse heart cytochrome *c***
- **NO and CO discrimination by cardiolipin-bound horse heart cytochrome *c***
- **Cardiolipin, NO and CO modulate horse heart cytochrome *c* roles in apoptosis**

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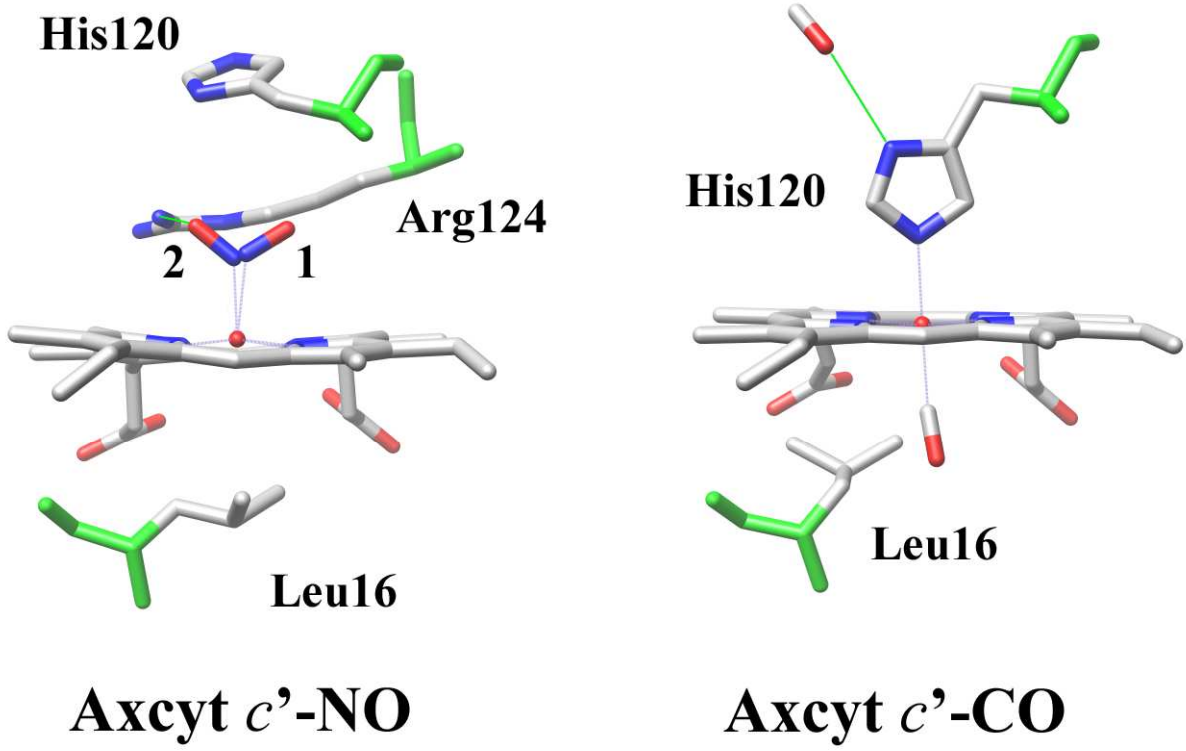
Graphical abstract



NO binds to the "dark side" of the heme