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Review

Cytochrome *c* oxidase and nitric oxide in action: Molecular mechanisms and pathophysiological implications ☆

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ABSTRACT

Background: The reactions between Complex IV (cytochrome *c* oxidase, CcOX) and nitric oxide (NO) were described in the early 60's. The perception, however, that NO could be responsible for physiological or pathological effects, including those on mitochondria, lags behind the 80's, when the identity of the endothelial derived relaxing factor (EDRF) and NO synthesis by the NO synthases were discovered. NO controls mitochondrial respiration, and cytotoxic as well as cytoprotective effects have been described. The depression of *OXPHOS* ATP synthesis has been observed, attributed to the inhibition of mitochondrial Complex I and IV particularly, found responsible of major effects. *Scope of review:* The review is focused on CcOX and NO with some hints about pathophysiological implications. The reactions of interest are reviewed, with special attention to the molecular mechanisms underlying the effects of NO observed on cytochrome *c* oxidase, particularly during turnover with oxygen and reductants. *Major conclusions and general significance:* The NO inhibition of CcOX is rapid and reversible and may occur in competition with oxygen. Inhibition takes place following two pathways leading to formation of either a relatively stable nitrosyl-derivative (CcOX-NO) of the enzyme reduced, or a more labile nitrite-derivative (CcOX-NO_2^-) of the enzyme oxidized, and during turnover. The pathway that prevails depends on the turnover conditions and concentration of NO and physiological substrates, cytochrome *c* and O_2. All evidence suggests that these parameters are crucial in determining the CcOX *vs* NO reaction pathway prevailing *in vivo*, with interesting physiological and pathological consequences for cells. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Most of the biological oxygen reduction is catalyzed by the so-called terminal oxidases, the last enzymatic complexes in the respiratory electron transport chain of aerobic organisms. Terminal oxidases encompass the large superfamily of heme-copper oxidases, subdivided into the A-, B-, and C-families [1], widespread among the different Life Kingdoms, and the Cu-lacking *bd*-type oxidases [2,3], that have been identified only in prokaryotes. The heme-copper oxidases all perform the same oxygen reduction and proton pumping chemistry [1,4], and accept electrons either from cytochrome *c* (cytochrome *c* oxidase, CcOX — E.C. 1.9.3.1) or from quinols [5,6], both substrates being in turn reduced by the respiratory complexes preceding in the chain. In the aerobic world of Eukaryotes, there is the mitochondrial aa_3-type CcOX, containing heme *a* and heme a_3.

Mitochondrial CcOX is reduced by cytochrome *c* and reduces, in turn, O_2 to water. The electron transfer is electrogenic and coupled to proton translocation across the inner mitochondrial membrane; the overall process contributes to build up and maintenance of the proton electrochemical gradient, $\Delta\mu_H^+$ ~200 mV, used by the ATP synthase to drive ATP synthesis [7–16].

The overall reaction is:

$$4\ \text{cyt.}\ c^{2+} + O_2 + 8\ H^+_{in} \rightarrow 4\ \text{cyt.}\ c^{3+} + 2\ H_2O + 4\ H^+_{out}$$

Since the late 80's there have been more than 20 years of intense research on the reactions between terminal oxidases and the nitrogen monoxide, NO (common name *nitric oxide*). As other classical respiratory chain inhibitors, such as cyanide, NO also inhibits CcOX, but with the major difference that NO inhibition reverts completely and quickly upon removal of this gaseous inhibitor from solution [17–20]. This peculiarity suggests that the NO-inhibition of mitochondrial CcOX can be better described as a functional control of cell respiration [21–26].

In this paper, we will provide un updated picture of the structural and functional properties of CcOX, suitable to figure out how NO reacts with the enzyme. The detailed molecular mechanisms of the reactions between CcOX and NO will then follow. Two

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alternative chemical reactions of NO with the metal ions in the active site of CcOX have long been known; these yield either a nitrosyl- or a nitrite-CcOX derivative [27,28]. In 2000, using detergent-solubilised CcOX in turnover, Sarti et al. [29] demonstrated that these two derivatives accumulate to different extents depending on the redox steady-state level of the enzyme. As shown therein, accumulation reflects the rate of electron donation by cytochrome c^{2+} and withdrawal by O_2, a finding confirmed by the same group on mitochondria and cells [21,30], and later on further investigated and substantiated [31–33]. A point that should be kept in mind is that NO is widely distributed among cells and tissues, and is also highly permeable to membranes [34]. It is therefore likely that the NO cell destiny and its body clearance, as such or as nitrite/nitrate (the main catabolites of higher nitrogen oxides, NO_x), have predictable bioenergetic implication.

At mechanistic level, the experimental conditions favoring one reaction mechanism or the other, i.e., CcOX nitrosylation or formation of the nitrite-derivative, may acquire patho-physiological meaning and have been therefore analyzed in detail.

2. Snapshot of structural and functional properties of CcOX

In mammals, a single monomer of CcOX is made of 13 different subunits identified by roman numbering [35], whereas in the more elementary bacterial enzymes the number of subunits is smaller, i.e., ≤4 [36,37]. Of these 13 subunits, subunits I, II and III are encoded by the mitochondrial DNA and form the functional core of the enzyme. These subunits are the largest, ranging from ~30 kDa to 60 kDa [38], and include all the redox sites, providing also the structural scaffold essential to the proton pumping machinery. The remaining 10 subunits are smaller and encoded in the nucleus [39], performing not fully understood regulatory roles [40,41]. In particular, Subunits IV and VIa were proposed to mediate a phosphorylation-dependent mechanism regulating CcOX activity (reviewed in [42]).

As purified from beef heart (typically after solubilisation in detergents such as lauryl-maltoside) and crystallized, CcOX exists as a homo-dimer [35]. The total surface area at the monomer-monomer interface is ~500 Å. A single monomeric functional unit of the enzyme contains 5 redox active metal ions, 2 Fe and 3 Cu, forming 4 interacting sites, see Fig. 1. These are organized as follows: at the electron accepting pole of the enzyme, 2 coppers in the bimetallic Cu_A site and 1 Fe-heme *a*, whereas in the O_2-binding active site, 1 Fe-heme a_3 coupled to 1 Cu_B. This structure is embedded into the inner mitochondrial membrane making the intramolecular electron transfer processes within the protein moiety particularly complex, as the electrons and protons transferred by/through the enzyme contribute to formation and maintenance of the H^+ electrochemical gradient $\Delta\mu_H^+$ used by the ATPase (see pdb Id n# 2OCC, for mammalian CcOX and n#1AR1, for *Paracoccus denitrificans*). The protein also contains 1 Mg^{2+} and 1 Ca^{2+} (reversibly exchanging with Na^+ in mammalian oxidase [43]); moreover 1 Zn^{2+}, 5 phosphatidyl ethanolamines and 3 phosphatidyl glycerols as well as 2 cholate molecules co-purify with the protein [35].

The Mg^{2+} ion detected in the bovine enzyme is close to the heme a_3-Cu_B site, at the interface between subunits I and II [35] and is substituted by Mn in bacteria [44,45] As recently confirmed [46], the Mg/Mn site is probably involved in the exit pathway for protons/water molecules [47,48].

Nowadays, the 3D structure of a number of cytochrome oxidases native or mutated and in different redox and liganded states has been solved by X-ray crystallography. The analysis has been performed on the enzyme purified from mammals [35,49,50], and bacteria [36,51–54], the latter in the native or the recombinant form [55]. The structure of a bo_3-type ubiquinol oxidase (from *Escherichia coli*) is also available [56], along with that one of the divergent cbb_3-type oxidase from *Pseudomonas stutzeri* [57]. The original structural studies

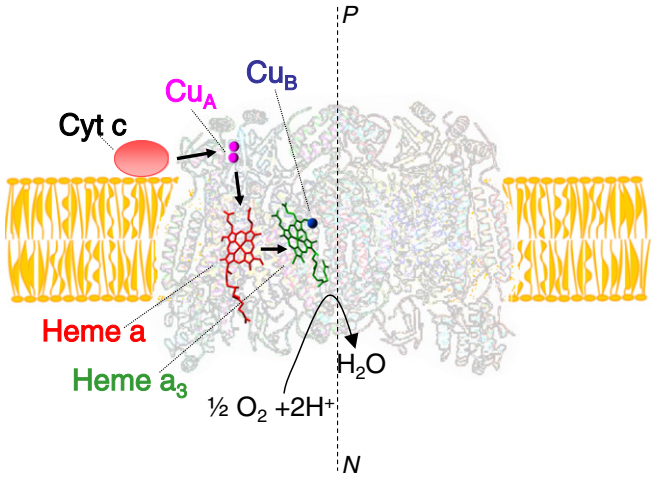

Fig. 1. CcOX the dimer assembly and the redox active metals. The overall 3D structure of the mammalian enzyme is accommodated into a bilayer (sketched), and oriented with the cytochrome *c* binding site on the external surface of the inner mitochondrial membrane, positively (*P*) charged compared to the mitochondrial matrix, negatively (*N*) charged. The Fe-hemes and coppers in a single monomeric unit (half of the dimer) are reported in different colors; these redox sites are crucial to the enzymatic and ligand binding activity of the enzyme. The electron transfer pathway from cytochrome *c* to the heme$_{a3}$-Cu_B via the Cu_A site and heme *a* is indicated with small arrows. From the Protein Data Bank coordinates of the oxidized beef heart CcOX (PDB Id: 2OCC), deposited by Yoshikawa et al. [50].

carried out on the wild-type fully oxidized aa_3-type enzymes have been extended to mutants of the K and D proton channels in *Rhodobacter sphaeroides* [58], and to different redox and ligation states of the enzyme [59,60].

As schematically drawn in Fig. 1, at the level of the electron accepting pole of CcOX, the Cu_A site contains two copper ions, tightly coupled. This site, embedded in the subunit II domain protruding into the mitochondrial intermembrane space (the periplasm in bacteria), undergoes a one-electron reduction by cytochrome *c* [61–64]. The reduced Cu_A center rapidly donates intramolecularly the electron to heme *a*, that is located 19 Å away (Cu_A-to-Fe_a distance), buried within the membrane-embedded domain of the enzyme, such that its reduction brings the electron close to the midplane of the membrane. The structural transition triggered by the reduction of heme *a*, observed by X-ray crystallography at 1.8/1.9 Å resolution [49] and confirmed by FTIR measurements [65], has been proposed by Yoshikawa and co-workers to be crucial for proton pumping, involving D51[1] in subunit I [49,50,66].

Electrons donated by the reducing substrates are transferred intra-molecularly from heme *a* to heme a_3 and Cu_B. In the fully reduced state, at 1.9 Å resolution, CcOX displays a trigonal planar coordination of Cu_B by three histidine residues, one of which is covalently linked to a tyrosine residue of subunit I (Y244) thereby taking an important part in the O_2 reduction cycle [67,68]. CcOX is targeted and inhibited by a number of small molecules/ions, such as CN^-, CO, $HCOO^-$, H_2S, N_3^- and NO, that can be accommodated into the active site of the enzyme [69]. The pathophysiological implications are quite different and, nowadays, better focussed owing to a clearer understanding of the mechanisms by which these small ligands react with the enzyme. Nitric oxide particularly has been shown to be of interest, since it is ubiquitous and able to reversibly inhibit mitochondrial respiration (see [17,18,20,21,29,70]).

[1] Unless otherwise stated, the amino acid numbering is based on the bovine heart cytochrome *c* oxidase sequence.

All structural data support the idea that CcOX from quite dissimilar organisms are almost identical in their key electrostatic and thermodynamic properties, all of them performing at least the same redox chemistry in the presence of reducing substrates and oxygen [4]. This similarity among terminal oxidases becomes less evident when considering the reaction with NO: CcOX, for instance, is unable to reduce efficiently NO to dinitrogen monoxide (N_2O) [71], differently from *T. thermophilus* ba_3 oxidase, *E. coli* bo_3 quinol oxidase or the cbb_3-type enzyme from *P. stutzeri* [72–74]. Structural analysis indicates that CcOX is a rigid protein, undergoing small conformational changes upon redox changes. Taken together, the structural data suggest that heme-copper oxidases all share a conserved bimetallic active site (binuclear center) made of a high-spin heme (a_3, o_3 or b_3, depending on the identity of the enzyme) and a copper ion (called Cu_B). This site is where O_2 binds and is processed, but also where NO binds and undergoes redox chemistry [21,22].

In the 3D structure, the fully oxidized enzyme, regardless of whether from beef heart, *P. denitrificans*, *R. sphaeroides* or *T. thermophilus*, displays an electron density between the iron of heme a_3 and Cu_B. This density has been related to the presence of different ligands bridging the two metal ions in the active site, namely it has been assigned to a (hydro)peroxo species [50] or to H_2O and OH^- [51]. In the fully reduced state, or after re-oxidation (*pulsing* [75]) of the enzyme, the electron density between the two metals vanishes, indicating ligand dissociation [49,52]. At higher resolution, 1.95 Å, the 3D structure of the *as isolated* bovine heart CcOX appears consistent with the presence of a peroxo group bridging the two metals [76]. This is also suggested by resonance Raman measurements showing a band at 755 cm^{-1} fully compatible with the O–O stretching mode of a peroxide, bridging the Fe and Cu in the active site of a fully oxidized, *as isolated* CcOX [77], but see also Kaila et al. [78] for an alternative interpretation.

3. The intermediates populated by CcOX during turnover with O_2

The assessment of the chemistry of the catalytic CcOX intermediates has been hard, and in some cases is still a matter of debate, particularly owing to difficulties in assigning the redox state of the metals in the active site in each species observed during the catalytic cycle. Although the issue is out of the scope of this paper, a schematic representation of the species populated during turnover is provided to help the understanding of the reaction mechanisms by which NO reacts with CcOX during turnover.

Following electron donation by cytochrome *c*, electrons are transferred intramolecularly to the binuclear heme a_3-Cu_B catalytic site. This site can accept up to two electrons starting from a ferric/cupric ground state; it can thereby exist in a fully oxidized ($[Fe^{3+}_{a3}Cu^{2+}_B]$, **O**), in a single-electron reduced ($[Fe^{2+}_{a3}Cu^{2+}_B]$ or $[Fe^{3+}_{a3}Cu^{+}_B]$, **E**) and in a two-electron reduced ($[Fe^{2+}_{a3}Cu^{+}_B]$, **R**) state. O_2 binds rapidly to the **R** active site yielding the so-called compound **A** [79]. Compound **A** is short-lived, and the very fast delivery of electrons to bound O_2 leads to formation of an intermediate, originally proposed to be a peroxo species ($[Fe^{3+}_{a3}$–O–O–$Cu^{2+}_B]$. This intermediate was thus called **P**, but it rather proved to be a ferryl derivative of heme a_3 (Fe^{4+}_{a3}=O) with Y244 in the radical form [80–82]. **P** is in turn converted to the **F** intermediate, that is also a ferryl adduct of heme a_3 but, differently from **P**, has Y244 reduced and protonated. **F** eventually converts to the fully oxidized state **O** upon arrival of an additional electron from Cu_A/heme *a*.

These intermediates react differently with NO, leading to the accumulation of CcOX derivatives characterized by different functional properties and destiny (see below).

4. The 3D structure of the NO-bound CcOX

In 2010 Muramoto et al. [59] published the 3D structure of the fully reduced NO-bound CcOX obtained at 100 K, to be compared with the structure obtained at 50 K and under illumination by Ohta K. et al. [83]. As shown in Fig. 2, in the dark and at 100 K, NO binds to the Fe^{2+} of heme a_3 in a bent end-on coordination structure (131° in the Fe–N–O angle), whereas under illumination and at 50 K an electron density is detected in between the Cu_B and the Fe_{a3}, nicely fitting the NO molecule as drawn by difference with the reduced CcOX structure [50,83]. As pointed out by the authors and shown in the figure, after photodissociation the NO molecule is close, but not bound, to either Fe_{a3} or Cu_B indicating that, at least under these conditions, re-binding is negligible. The structure also confirms that the chemical bond of NO to the active site, responsible for formation of the nitrosyl derivative, and assigned by spectroscopy to the reduced heme a_3 iron, is photolabile [29,84]; moreover, under the conditions explored, NO appears closer to Cu_B rather than to Fe_{a3} [83].

A pre-requisite for O_2 and CO, but not for NO binding, is the complete reduction of the active site (see below). It is worth noticing that, when the enzyme is purified in the presence of chloride, there is plenty of time for this ion to bind the binuclear site, where it hinders (slows down) some reactions [85,86]. Interestingly, upon reduction of the active site, chloride is released and the site promptly becomes reactive toward other anions and ligands.

5. CcOX and NO during catalysis: the two-mechanisms frame

The reactions of NO with CcOX have long been known [27,28] and are schematically represented in Fig. 3. The NO produced enzymatically by the NO synthases or exogenously added to respiring cells, organelles or purified CcOX, proved to be able to inhibit O_2 consumption by CcOX [17–20,87], as well as ATP production [88]. Since first observed by time resolved stopped flow spectroscopy [29], it was suggested that inhibition occurs following two different reaction pathways, leading to formation of a relatively stable [Fe^{2+} NO Cu^{+}_B] nitrosyl-derivative, or a labile [Fe^{3+} NO^-_2 $Cu^{?}_B$] nitrite-bound derivative; in the latter derivative the redox state of Cu_B, transiently reduced by NO, is uncertain (see [21] and references therein). It may be worth to clarify that when O_2 and NO are allowed to react at the same time with CcOX, inhibition by NO may or may not occur in competition with O_2, depending on the fractional distribution of the catalytic intermediates of CcOX. Since O_2 can only bind to the fully reduced, **R** CcOX binuclear site [89], **R** is the only intermediate that can react with both O_2 and NO, whereas the **O**, **E**, **P** and **F** intermediates can solely react with NO. Therefore, the reaction of NO with these latter intermediates does not occur in competition with O_2, at least strictly speaking. Indeed, at bulk level, O_2 and NO by reacting one with the other do reciprocally lower their concentration [90]. Based on these observations, the mechanism by which NO reacts with CcOX depends on the relative fraction of the catalytic CcOX intermediates populated at a given time [91], and in turn on the electron flux level through CcOX [29]. This original observation has been later on reproduced, extended and quantified using different CcOX-containing systems, at different O_2 tensions [31,92], and down to submicromolar NO concentration values [33].

Common to both mechanisms, the inhibited CcOX recovers its activity spontaneously in the presence of O_2, as NO is slowly but progressively degraded (oxidized) in solution. Reversal of inhibition is clearly enhanced in the presence of NO scavengers, such as myoglobin, hemoglobin or reduced glutathione (GSH), physiologically present in the cell or exogenously added to the system; the same result is obtained by specifically inhibiting the cellular endogenous NO production by NO-synthases (NOSs) [93,94].

Given the involvement of NO as a signaling molecule in many patho-physiological processes, a large number of investigations has been carried out (reviewed in [21,22,26,32,33,95,96]), showing that the NO inhibition of the enzyme can occur at all levels, from the isolated enzyme to mitochondria, cells and tissues [17–20,93,94,97–103].

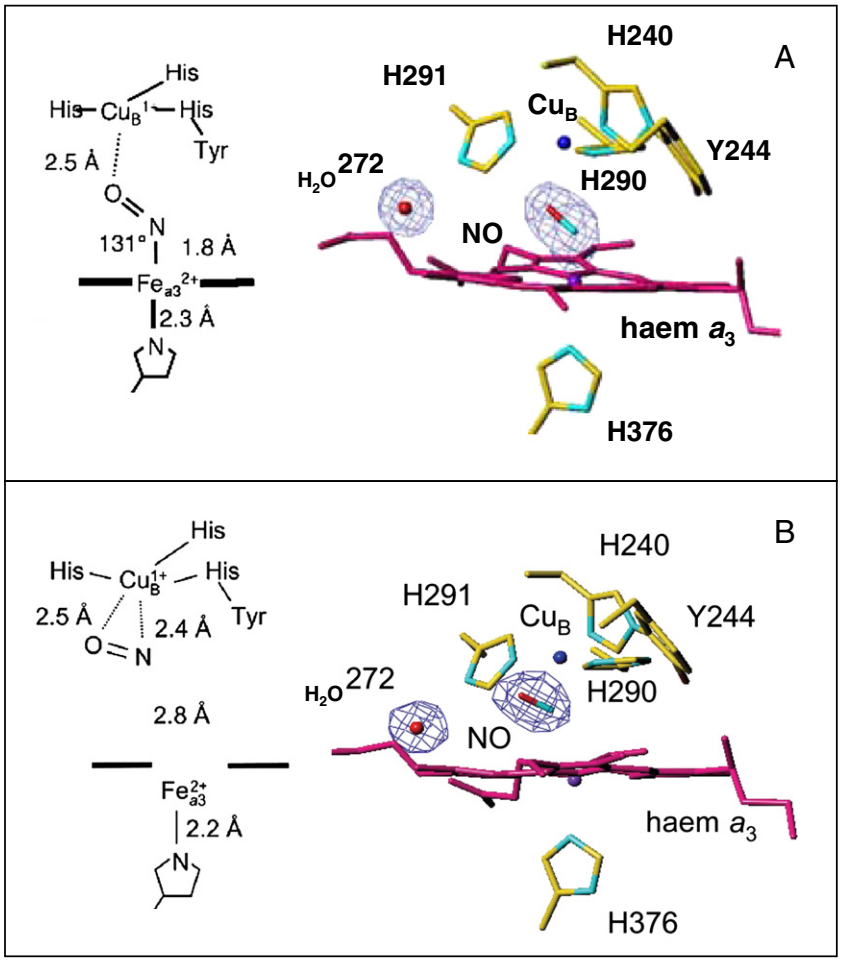

Fig. 2. X-ray crystallographic structure of the NO-treated bovine heart CcOX. Enzyme fully reduced and NO bound, at 100 K and in the dark (panel A, reproduced from [59]) or at 50 K and under light illumination (panel B, reproduced from [83] with IUCr's copyright permission).

5.1. The nitrosylation pathway

The reaction of NO with the fully reduced (**R**) binuclear site yields a characteristic [Fe^{2+} NO Cu_B^+] nitrosyl adduct and occurs at a rate ($k = 0.4-1\times10^8\ M^{-1}\ s^{-1}$, [27,104]) similar to that of O_2. A first important consequence is that, in the presence of NO, all conditions favoring the reduction of the CcOX catalytic site will also favor its stabilization in the nitrosylated state whose structure has been reported by Muramoto et al. [59]. The CcOX nitrosyl-adduct is relatively stable and, only upon scavenging free NO in solution, NO is thermally released unaltered by the reduced enzyme [29,105]. An important corollary of this finding is that in eukaryotes the mitochondrial CcOX has lost competence in reducing NO to N_2O [71]. As reported above, this ability is retained by some bacterial terminal oxidases showing a slow, but measurable NO-reductase activity [72–74]; a finding consistent with the hypothesis that heme-copper oxidases and bacterial heme b_3-containing NO reductases share a common phylogenesis [106,107].

The stability of the nitrosyl adduct is peculiar: compared, to hemoglobin ($k_{off} \approx 10^{-4}$–$10^{-5}\ s^{-1}$ at 20 °C), the rate of NO dissociation from reduced CcOX is unexpectedly high ($k_{off} = 3.9\times10^{-3}\ s^{-1}$ at 20 °C, [29]) as it is the activity recovery of the enzyme from inhibition. Interestingly, the nitrosyl adduct is photosensitive and NO is promptly released from CcOX under (white light) illumination [29,83,84]. The light-induced NO displacement from CcOX enables us to study the kinetics of recovery of respiration and to gain insight into the mechanism of CcOX inhibition by NO, particularly under experimental conditions unfavorable to UV–visible absorption spectroscopy, such as in the presence of scattering samples, as intact cells or mitos [30,108,109].

In the presence of NO, under the redox conditions compatible with full reduction (**R**) of the catalytic site, the inhibition is set promptly and with high affinity ($K_d = 0.2$ nM [31]). Under these turnover conditions, inhibition is set in competition with O_2 (because **R** can react with both NO and O_2) and, upon scavenging NO and/or exposing the enzyme to O_2, the activity is recovered at the rate of NO dissociation from reduced heme a_3, without additional redox chemistry at the site or involvement of superoxide ($O_2^{-}\bullet$) formation [105,110].

The NO dissociation is much faster from the nitrosyl adduct of the fully reduced Cu-lacking cytochrome *bd* oxidase from *E. coli* ($k = 0.133 \pm 0.005\ s^{-1}$ at 20 °C, [111,112]), pointing to a role of Cu_B in this reaction, in line with the recent observation by Ohta et al. [83]. It is worth mentioning that the remarkably higher NO dissociation rate measured for cytochrome *bd* suggests that this enzyme may represent, for those microorganisms able to express it, a defense mechanism ensuring a higher respiratory resistance to nitrosative stress [112–115].

The reduction kinetics of heme a_3 in the K354M of the *Paracoccus denitrificans* is extremely slow so that the O_2 reduction activity of this enzyme is severely impaired. Unexpectedly, however, this mutant binds NO at the reduced heme a_3 about two orders of magnitude more rapidly than O_2, suggesting that NO can react also with the single-electron reduced (**E**) binuclear site [116] in line with previous proposals [117,118]; it remains to be elucidated whether NO directly targets heme $a_3{}^{2+}$ or primarily reacts with Cu_B^+ to be subsequently transferred intramolecularly to the nearby heme. The whole issue is

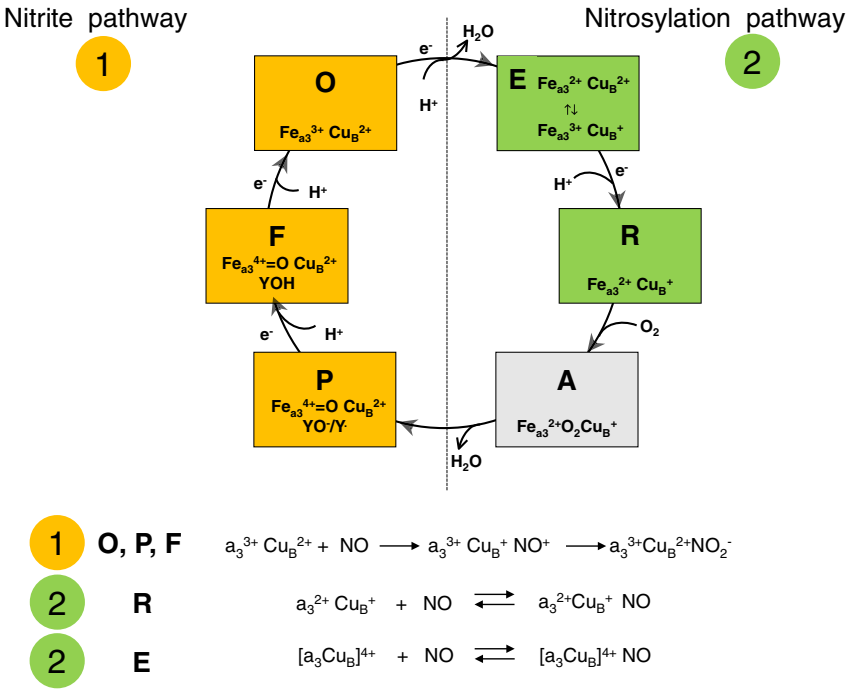

Fig. 3. CcOX catalytic intermediates and the two pathways of reaction with NO. The catalytic cycle of CcOX is schematically reported with the indication of the redox and the oxygen ligation state of the metals in the active site. The reactions of the intermediates with NO leads to production of the nitrite-bound derivative (1) or the nitrosylated adduct (2). The Y244 residue, tightly interacting with Cu_B, is also indicated (see text). The oxidized **O** species is fully reduced to **R**, *via* formation of the half-reduced intermediate **E**, in which the electron is either on Fe_{a3} or on Cu_B. Upon reaction with O_2, **R** converts into **A**, which decays to **F** *via* formation of either $\mathbf{P_R}$ or $\mathbf{P_M}$ (depending on whether the initial species is fully reduced, leading to $\mathbf{P_R}$, or mixed valence, leading to $\mathbf{P_M}$) reviewed in [69]. Y244 should have radical character in the $\mathbf{P_M}$ intermediate only. The oxidized **O** state is restored from **F** through further electron transfer. When reacting with the oxidized Cu_B (intermediates **O**, **P** and **F**), NO yields the (light-insensitive) CcOX-NO_2^-, nitrite-derivative. Upon binding to reduced heme a_3 (intermediate **R**) yields the (light-sensitive) CcOX-NO, nitrosyl-derivative.

still debated since, according to others [31,119], the reaction of NO with **E** is unnecessary to account for steady-state data.

5.2. The 'nitrite' pathway

Alternatively to the redox-inert binding to the reduced binuclear site, upon reacting with CcOX, NO can also be oxidized by the binuclear site of intermediates **O** [86,120], **P** and **F** [91,121], generated during turnover: in all these cases the reaction end-product proved to be the nitrite-bound enzyme.

The slow oxidation of NO to nitrite by the Cu_B in the fully oxidized (**O**) CcOX was first reported in the early 80's [28]. These authors assayed the reactivity toward NO of the *as isolated*, *resting* CcOX, a (*slow*) form [122] of the enzyme displaying different redox and ligand binding properties, compared to the so-called *pulsed* active (*fast*) enzyme [75], generated upon exposing the fully reduced *resting* enzyme to O_2. Cooper et al. [120] found that NO reacts with the *pulsed* CcOX much more rapidly (ms) than the *resting* enzyme; following the bimolecular collision, one electron is injected by NO into Cu_B, thereby rapidly re-equilibrating with heme *a via* reverse electron transfer [120]. In the fast reaction (ms), NO is oxidized to nitrosonium ion (NO^+) to be subsequently hydroxylated (or hydrated) to nitrous acid/nitrite. Eventually the enzyme displays the spectroscopic features of the NO_2^--bound ferric heme a_3 and is inhibited. Later on, Giuffrè et al. [86] confirmed those findings and measured the bimolecular rate constant of the reaction of Cu_B^{2+} with NO ($k = 2\times10^5\ M^{-1}\ s^{-1}$ at 20 °C); these authors also showed that the oxidized enzyme *as isolated*, reacts slowly with NO due to the presence of an external anionic ligand (chloride) bound at the oxidized binuclear site during the purification procedure [86]. The CcOX *pulsing* process, as performed in the experiments reported by Cooper et al. [120], removes chloride and other ligands from the enzyme [76,77], thereby allowing fast reaction with NO [86]. In agreement with the proposal that NO preferentially reacts with the oxidized Cu_B, the oxidized *E. coli* cytochrome *bd*, lacking Cu_B, reacts with NO very slowly ($k = 1.5\times10^2\ M^{-1}\ s^{-1}$ at 20 °C) and without significant nitrite production [123].

The *in situ* formation of NO_2^- is eventually followed by NO_2^- binding to the (nearby) oxidized heme a_3. The nitrite-complex formed under these conditions is inhibited, as shown by standard cytochrome *c* oxidation assays, and is optically and functionally undistinguishable from the complex obtained by addition of a large excess nitrite (>mM) to the oxidized enzyme [91]. Notably from the functional point of view, the affinity of nitrite for reduced heme a_3 is low [91]. Accordingly, while nitrite dissociation from the oxidized enzyme is very slow ($t_{1/2}$ ~80 min, at 25 °C), upon reduction of the active site, dissociation of NO_2^- occurs and activity is promptly restored [91,124].

Relevant to the discussion about the reciprocal control between CcOX and NO [125], upon reduction of heme a_3 during turnover, dissociation of nitrite from the catalytic intermediates is significantly faster (by one order of magnitude) than NO-dissociation from the same site in the reduced state [29,91].

In conclusion, Torres et al. [121] as well as Giuffrè et al. [91] reported that NO reacts fairly quickly not only with (chloride-free) CcOX in the oxidized **O** state, but also with the enzyme in the **P** and **F** state, all independently generated. The final adduct was always the nitrite-bound CcOX, though the reaction of NO with **O** proved to be slightly faster than the same reaction with intermediates **P** and **F** ($k = 2\times10^5\ M^{-1}\ s^{-1}$ *vs* k ~ $10^4\ M^{-1}\ s^{-1}$ at 20 °C). A point that remains to be clarified is whether formation of the nitrite-bound intermediates necessarily involves Cu_B^{2+}, as originally proposed [86,120]. Interestingly, the ferryl intermediate **F** of the *Azotobacter vinelandii* cytochrome *bd* oxidase, though lacking Cu_B, causes the oxidation of NO to nitrite at a high rate ($k = 1.2\pm0.1\times10^5\ M^{-1}\ s^{-1}$ at 20 °C, [126]). Thus, as reported for other hemeproteins, it is also possible that NO reacts directly with the ferryl heme a_3 of CcOX, without involving Cu_B [126–128].

6. The interplay between the NO *vs* CcOX reaction pathways

The two reaction pathways between NO and CcOX were independently discovered and described more than 30 years ago [27,28,129]. The two pathways coexist and one prevails over the other, depending on the electron flux level at the CcOX site, as first proposed in 2000 by Sarti et al. [29].

Based on this first observation, strengthened by further experimental evidence [30,31,96] and theoretical analyses [31,119,125], it is agreed that CcOX can react with NO according to two different reaction pathways: the first reaction yields the nitrosyl NO-bound enzyme, while the second one produces nitrite-bound CcOX. As just recalled, the reaction of NO with **O**, **P** and **F** is approximately 10^3 fold slower than NO binding to the fully reduced **R** binuclear center (and perhaps to **E**). It is worth to keep in mind that the slower kinetics of the reaction of NO with intermediates **O**, **P** and **F**, is compensated by their predominance under turnover conditions, when **R** is expected to be populated only at negligible level [91]. As expected, the accumulation of the intermediates at any given time depends on the rate of electron donation by substrates *vs* the rate of electron withdrawal by O_2, thus ultimately on the overall mitochondrial metabolic state, see Antunes et al. [125].

In synthesis, both reaction pathways lead to reversible CcOX inhibition, and activity is restored either by thermal dissociation of NO from the nitrosyl enzyme [a_3^{2+} *NO* Cu_B^+], the process being slow (and light-sensitive), or by expelling nitrite from heme a_3^{3+} [a_3^{3+} NO^- Cu_B^{2+}], following the electron transfer from heme *a* to the active site [91,124,125]. Owing to the different reactivity of CcOX intermediates toward O_2 and NO, the competition between NO and O_2 is expected only when the 'nitrosyl' pathway prevails, and not when the nitrite-bound enzyme is accumulated [29,31,32]. A conclusion compatible with this synthesis has been recently achieved by Aguirre et al. [33]. These authors, by using as a model system iNOS-transfected HEK 293 cells, and high-resolution respirometry over a wide range of O_2 concentrations, down to nanomolar, confirmed that the NO inhibition of respiration is always reversible. In agreement with the original proposal [21,29], later on substantiated [31], Aguirre et al. [33] did not observe a linear dependence of the IC_{50} of NO on [O_2]. They rather reported a parabolic dependence of the $IC_{50,NO}$ that was interpreted as the evidence for the existence of an enzyme adduct accommodating both a NO and an O_2 molecule at the binuclear center. Such an adduct has been put forward by Pearce et al. [110,130], who suggested that NO bound to reduced heme a_3 in the presence of O_2 is first metabolized to nitrate (by reaction with superoxide anion formed at Cu_B) and then to nitrite prior to dissociation into the bulk. This hypothesis, however, was not confirmed by others [105] so that the experimental evidence for such a CcOX adduct with both a NO and an O_2 molecule bound at the active site is still lacking.

7. The biology of the CcOX reaction with NO

The information so far collected on the interaction of CcOX with NO raises a number of questions relevant to cell biology and, in turn, to human pathophysiology. Depending on the availability of NO, O_2 and, particularly, reducing equivalents (electrons) entering the respiratory chain, thus on the reduction level of all the metal sites in CcOX, the mechanism by which NO reacts with CcOX may be different, and changes in mitochondrial signaling and metabolism are plausible.

In this respect, an important issue is whether we can predict which one of the two reaction pathways described predominates in intact cells respiring on endogenous substrates upon exposure to exogenous/endogenous NO. Working with SH-SY5Y neuroblastoma cells, Mastronicola et al. [30] found that, in the presence of [O_2]> 100 μM and under otherwise identical conditions, the 'nitrite' pathways predominate, unless the electron flux through CcOX is artificially enhanced (e.g. by tetramethyl-p-phenylenediamine, TMPD), Fig. 4. As shown in the figure, the respiration of cells cultured under standard 'basal conditions', i.e., in the presence of (not limiting) O_2 and endogenous reducing substrates, is rapidly inhibited following the addition of μM NO. Most notably, upon addition of HbO_2 efficiently scavenging excess NO, activity recovers immediately: the kinetics of functional recovery is fully compatible with the nitrite dissociation from reduced heme a_3 [29]. The addition to the same cells of TMPD, in the presence of ascorbate, is able to induce, in a concentration dependent manner, the appearance of a delay in the kinetics of recovery of respiration, fully compatible with the monomolecular NO dissociation reaction from reduced heme a_3. The existence of the nitrosyl-adduct is confirmed by the observation that in the absence of excess NO (i.e. in the presence of HbO_2) recovery can be accelerated shading light on the respiring cells, owing to the light sensitivity of the NO – Fe^{2+} bond (see Fig. 4).

In line with this view, Palacios-Callender et al. [23] reported that human embryonic kidney cells, while respiring toward hypoxia, if pulsed with small amounts of NO (nM), i.e., under conditions closer to the physiological ones, do not lose respiratory efficiency, while CcOX becomes significantly more reduced indicating that a fraction of the enzyme is inhibited since involved in the NO oxidation reaction. This phenomenon of compensation, able to keep a suitable respiration level, was described as 'cushioning' effect more than 40 years ago by Britton Chance [131] and was related to the increase in the reduction level of cytochrome *c* due to partial inhibition of CcOX [23,132]. When [O_2] becomes limiting, respiration slows down and eventually stops [23,132].

Thus, the conditions favoring the pathway leading to accumulation of nitrite-CcOX, though variable, must therefore include the presence of O_2 at a concentration compatible with a cell normoxic state (~10 μM) and of limited amounts of NO (~10^2 nM) [23]. Under these conditions, besides the partial inhibition of CcOX that might even occur without an evident inhibition of respiration, one might expect a mitochondrial response due to the higher reduction level of the components of the mitochondrial electron transport chain [23,132], leading to superoxide and, in turn, hydrogen peroxide and other ROS production, triggering cell signaling [133].

It is therefore feasible to propose that the overall picture, at least under the conditions leading to nitrite formation, is more consistent with a mitochondrial control function exerted by the reaction of NO with CcOX, a frame more complex than a *simple* reversible O_2-sensitive inhibition. This issue has been clearly addressed by Antunes et al. [125], who suggested that the oxidation of NO to nitrite by the oxidized CcOX actually prevents the inhibitory heme a_3 nitrosylation and, perhaps more importantly, lowers the NO bio-availability.

A completely different behavior is observed when nitrosylation of CcOX prevails, with accumulation of the nitrosyl [a_3^{2+} *NO* Cu_B^+] derivative. Experiments aimed at understanding conditions favoring formation of the CcOX-nitrosyl derivative and its functional recovery were performed using the enzyme differently integrated, i.e., purified and detergent solubilized or *in situ* as in mitochondria and intact cells [30]. As shown in Fig. 4, in all the experiments, the parameter critical to drive the CcOX NO-inhibition pathway toward the enzyme nitrosylation was the concentration of reductants (cytochrome c^{2+}) provided in the assay. In the presence of soluble CcOX this parameter was varied by increasing directly the concentration of cytochrome c^{2+}. In the presence of cells or mitochondria the fraction of cytochrome c^{2+} was increased by using membrane permeable TMPD and excess ascorbate, as reductants [30,108].

For the mechanistic implications, particularly interesting are the experiments carried out using a suspension of lymphoblastoid cells collected from patients affected by Ataxia Telangiectasia (AT), a severe genetic disease characterized by massive radical chemistry

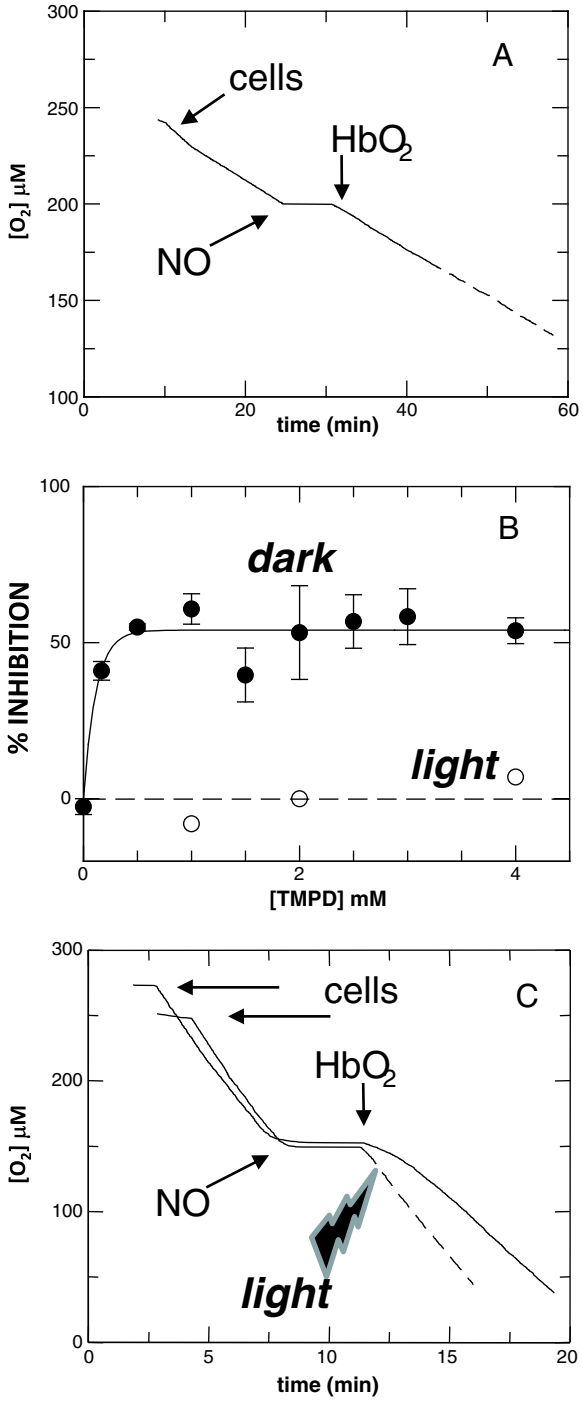

Fig. 4. Dependence of the CcOX NO inhibition pathway on availability of reductants. A) Neuroblastoma cells are allowed to respire using endogenous reductants (i.e., lower cytochrome c^{2+} concentration) in the dark. Inhibition of respiration is induced by addition of 1 μM NO; thereafter, 1 μM HbO_2 is added to scavenge free NO and recovery of the O_2 consumption activity is immediately observed, suggesting that inhibition occurred through formation of the CcOX-NO_2^- derivative. B) Experiments as in panel A, but carried out upon artificially increasing the concentration of cytochrome c^{2+} with ascorbate and TMPD. The extent of CcOX inhibition, observed (in the dark) at a fixed time after NO scavenging, increases with TMPD concentration. C) Typical oxygraphic time course observed in the presence of 1 mM TMPD in the dark (solid) or after illumination (dashed). Experimental conditions as in panel B. Notice the delay of recovery after addition of HbO_2, in the dark. Inhibition promptly vanishes upon shading light, due to light-sensitivity of the nitrosyl Fe^{2+}NO adduct [30].

[134]. From the bioenergetic point of view, compared to parental controls and everything else being virtually identical, the AT cells are characterized by an intrinsically higher O_2 consumption rate [108]. The higher respiration matches a significantly higher (~70%) cytochrome *c* concentration detected in AT cells (Fig. 5). Suggestive of a higher propensity of AT cells to undergo CcOX nitrosylation, experiments of TMPD titration in the presence of ascorbate (similar to those presented in Fig. 4), carried out in parallel using AT and control cells, have shown that AT cells form the CcOX-nitrosyl derivative at a concentration of TMPD approximately 5 times smaller than controls [108].

A common finding in the observations has been that in the presence of O_2 and NO (μM) the pathway of the CcOX inhibition by NO, in the presence of endogenous reducing substrates, was always nitrite-like [30]. Upon artificially increasing the electron flux through the respiratory chain, however, and/or reducing the O_2 concentration [31], the O_2-competitive 'nitrosyl' pathway tends to take over [29,30]. Mathematical models [119, 125] further confirmed that experimental data on CcOX inhibition by NO can be simulated only if both the competitive and uncompetitive pathways are included, with the latter prevailing at lower

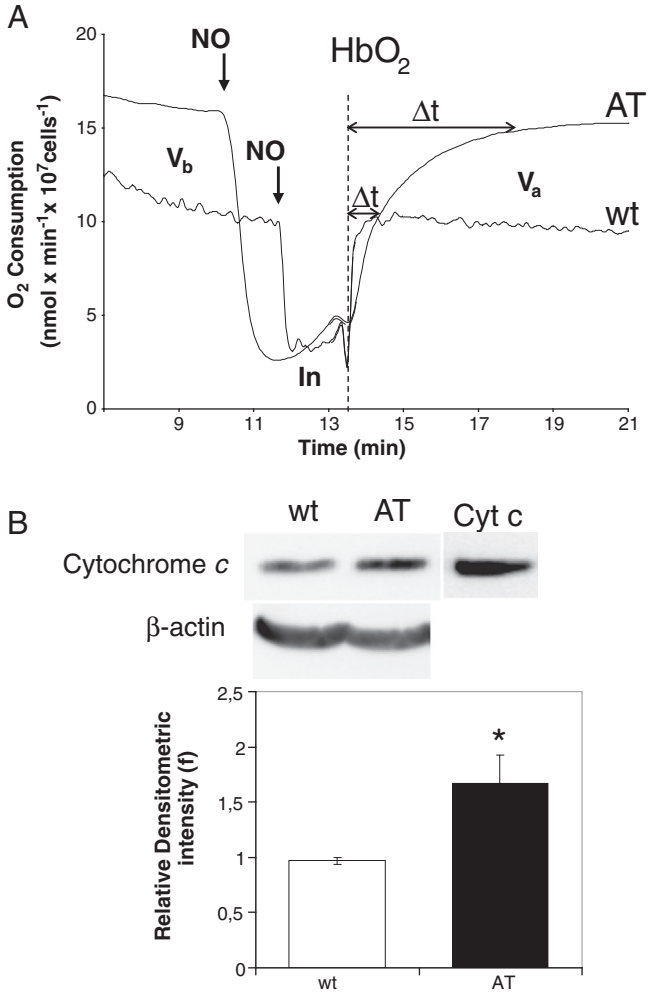

Fig. 5. NO inhibition of respiration in lymphoid cells (Ataxia Telangiectasia patient) expressing different amounts of cytochrome *c*. A) Oxygraphic profiles along with their first derivative, of lymphoid cells collected from a patient affected by Ataxia Telangiectasia (AT) and from controls (wt). As in Fig. 4, cells are allowed to respire in the dark, in the presence of 1 mM TMPD and ascorbate. Inhibition (In) of respiration is induced by 1 μM exogenous NO; thereafter, 1 μM HbO_2 is added to scavenge free NO. *Notice*: i) the higher rate of O_2 consumption by AT cells before NO inhibition (Vb) and after its release (Va) matches the higher cytochrome *c* content of these cells; ii) the time necessary for AT cells to recover activity (Δt) after addition of HbO_2 is longer than for control cells. B) Cytochrome *c* immunoblotting assays on cell lysates. Relative Densitometric Intensity (RDI) value of AT and control cells (wt), showing that everything else being virtually identical, AT cells contain a higher amount of cytochrome *c*. Modified from [108].

electron flux and higher [O_2]. The whole picture is compatible with simulations reported by Giuffrè et al. [91] showing that, at low electron flux, the overall occupancy of intermediates **O**, **P** and **F** at steady-state should increase, thereby favoring the 'nitrite' uncompetitive pathway, but see also Mason et al. [135]. As already mentioned, the O_2-competitive inhibition pathway prevails under conditions of high electron flux (turnover), when the overall occupancy of **R** and **E** tends to increase [91].

The CcOX nitrosyl-derivative is fairly stable at the body temperature (NO dissociation occurs at $k_{off} \sim 10^{-2}\ s^{-1}$, at 37 °C), thus CcOX inhibition becomes more severe when nitrosylation is favored. It is tempting to speculate that all conditions leading to accumulation of the CcOX nitrosyl-derivative are compatible with a less efficient OXPHOS-mediated ATP production that can be compensated, or not, by production of glycolytic ATP (Warburg effect) [136]. Interestingly, as pointed out by Almeida et al. [137], different cell lines have different ATP compensatory efficiencies: for instance, astrocytes can compensate glycolytically, whereas neurons cannot. For this very same reason it has been reasonable to propose that the higher propensity to CcOX nitrosylation of cells from ataxia telangiectasia patients may contribute to the severe systemic symptoms of this disease [108].

8. NO/nitrite recycling

The bioavailability of NO to tissues and cells is an important issue. Besides the NO catalytic production by the NOSs, the chemical or the catalytic reduction of higher nitrogen oxides (NO_x) is also considered an important route to maintain suitable steady state levels of NO. In human plasma nitrite is approximately 200 nM and, in equilibrium with cells and tissues, represents an important buffering source of NO [138–140]. When in tissues the oxygen tension decreases, the ability of the NOSs to generate NO is compromised by the lack of the O_2 substrate. Under these conditions, the anoxic environment promotes tissue acidification, favoring the acidic disproportionation of nitrite [141,142], as well as the enzymatic reduction of nitrite to NO [139,143–145]. The question we wish to pose, however, is whether CcOX takes an active part in the nitrite cell-cycling. The data collected over the past 20 years, or more, have clearly demonstrated that CcOX produces nitrite by reacting with NO, particularly under low turnover (electron flux) conditions and in the presence of O_2. This evidence, along with the observation that CcOX is endowed with a nitrite-reductase activity [146–148], suggests that complex IV participates in maintaining the mitochondrial nitrite physiological level. Thus, as pointed out by Antunes et al. [125] it is feasible to assign to the reaction of NO with the oxidized CcOX a biologic role more complex than the inhibition of the mitochondrial respiratory chain.

In conclusion, as a *side effect* of more than 20 years investigation on the reactions between NO and CcOX, we are facing the intriguing, provocative, idea that the official classification of the enzyme cytochrome *c* oxidase (EC. 1.9.3.1.) should be updated, since all the experimental evidence shows that CcOX is both an O_2 reductase and a NO oxidase, depending on mitochondrial availability of these, both physiological substrates.

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