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Review

Nitric oxide and cellular respiration

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Abstract. The role of nitric oxide (NO) as a signalling molecule involved in many pathophysiological processes (e.g., smooth muscle relaxation, inflammation, neurotransmission, apoptosis) has been elaborated during the last decade. Since NO has also been found to inhibit cellular respiration, we review here the available information on the interactions of NO with cytochrome *c* oxidase (COX), the terminal enzyme of the respiratory chain. The effect of NO on cellular respiration is first summarized to present essential evidence for the fact that NO is a potent reversible inhibitor of in vivo O_2 consumption. This information is then correlated with available experimental evidence on the reactions of NO with purified COX. Finally, since COX has been proposed to catalyze the degradation of NO into either nitrous oxide (N_2O) or nitrite, we consider the putative role of this enzyme in the catabolism of NO in vivo.

Key words. Cytochrome oxidase; respiration; inhibition; intermediates; mechanism; NO degradation.

Nitric oxide and mitochondrial function

Immunochemical studies on mitochondria from a variety of sources have revealed the existence of a mitochondrial membrane-bound NO synthase (NOS) [1–4]. This discovery suggests that NO produced in these organelles may be responsible for the control of mitochondrial function. Consistently, rat liver mitochondria supplemented with saturating amounts of L-arginine were shown to produce NO [5] at a concentration (micromolar) expected to induce a significant inhibition of cytochrome *c* oxidase (COX) activity [6]. Besides short-term control of respiration at the level of COX (discussed below), long-term inhibition of the respiratory chain in response to persistent (several hours) high intracellular NO concentrations has been reported, particularly at the level of complex I [7]. Examination of these long-term effects of NO on the respiratory chain is outside the scope of this review, and will not be discussed further. We will focus on the evidence indicating that COX is a primary target of NO.

Cleeter et al. [8] first reported that the NO-releaser S-nitrosoglutathione can reversibly inhibit mitochondrial respiration, and suggested COX as the primary target of NO. Brown and Cooper [9] demonstrated the photolytic production of NO and inhibition of respiration in an elegant experiment in which they illuminated brain synaptosomes respiring in the presence of nitroprusside. Interestingly, in these experiments, and in others carried out with purified COX, inhibition reverted spontaneously in the dark. Consistently, upon addition of NO to respiring rat liver mitochondria, ATP synthesis was promptly depressed, but was restored in the presence of O_2 [10]. In all cases, the degree of inhibition depends on the NO/O_2 molar ratio, showing competition between these two ligands for the same target.

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Inhibition of respiration by NO was shown in 1990 by Carr and Ferguson [11] in submitochondrial particles, and later confirmed with mitochondria [8, 12], synaptosomes [9], astrocytes [13, 14], cardiac myocytes [15] and dog skeletal muscle [16]. Moreover, Brown et al. [17] using cell cocultures have shown that NO generated by activated macrophages can depress the respiration of adjacent fibroblasts. Induction of mitochondrial de-energization by exogenous NO, either as a gas in solution or released by donors, was also detected by fluorimetric techniques [18–20], and observed also with hepatocytes in coculture with Kupffer cells, upon stimulation of NOS with lipopolysaccharide [21].

On the basis of this large body of evidence, one may conclude that (i) NO reversibly inhibits mitochondrial respiration, (ii) this effect is prevented by inhibition of NOS or by using NO scavengers, and (iii) COX is a primary target of NO because of the competition with O_2.

Inhibition of respiration in situ

The effect of NO on mitochondrial energization has been measured by Sarti et al. [22] on single cells by fluorescence microscopy, thus avoiding possible specimen preparation artifacts. These experiments were designed to assess whether NO exerts a stationary control of respiration under normal cell culture growing conditions. In this study, rhabdomyosarcoma or neuroblastoma cells were incubated either with exogenous NO or with modulators of NOS activity.

Mitochondrial respiration was clearly inhibited using (E)-methyl - 2 - [(E) - hydroxy - imino] - 5 - nitro - 6 - methoxy-3-hexeneamide (NOR_1), which in water releases stoichiometric NO with a half-time of 1.7 min. In the presence of excess NOR_1 (fig. 1), the cells rapidly (~ 1 min) lose their ability to import rhodamine 123 (RD_{123}), a cationic dye which in the presence of nigericin [converting ΔpH into $\Delta\Psi$; ref. 23] is accumulated electrophoretically in the mitochondrial matrix. As shown in figure 1, following incubation with NOR_1, mitochondrial activity recovered rapidly in air; however, a second addition of NOR_1 restored the observed de-energization [22].

The fairly quick recovery of activity could be accounted for because of fast NO dissociation from reduced COX as measured in vitro using the solubilized beef heart enzyme [$k_{off} = 0.13\ s^{-1}$; ref. 24]; thus, as bulk NO is oxidized to nitrite in the aerated solution, oxidase activity is recovered. The recovery rate constant measured on intact cells by Sarti et al. [22] ($k = 0.01\ s^{-1}$) using fluorescence microscopy is similar to the values reported for other biological systems (such as rat liver and brain mitochondria or INS-1 pancreatic cells) and using different methods to supply NO (table 1).

Does endogenous NO control mitochondrial energization?

This question has been addressed by investigating the response of cultured neuroblastoma SY5Y or rhabdomyosarcoma cells to the NOS inhibitors L-nitroso-arginine (LNA) or 7-nitroindazole (7N). LNA is able to target most of the NOS isoforms, while 7N inhibits more selectively the neuronal NOS (nNOS) [25]. After incubation with LNA, stationary growing rhabdomyosarcoma cells display an approximately two-fold enhanced ability to import RD_{123}, compared to the control, while incubation of neuroblastoma cells with 7N induced a 1.4-fold enhancement of mitochondrial fluorescence. Therefore, inhibition of NOS in stationary growing cells enhances the ability of mitochondria to build up the membrane potential. Treatment of neuroblastoma cells with suitable concentrations of N-methyl-D-aspartate (NMDA), an activator of NOS [26, 27], induced a clear decrease of mitochondrial import of RD_{123} (with only 20% residual fluorescence with respect to the control) [22]. Thus, on the assumption that NMDA does not induce mitochondrial depolarization by some other mechanism, NOS activation depresses mitochondrial energization. These observations strongly suggest that even in the absence of stimulation, these cells in culture steadily produce NO at a concentration suitable to control respiration.

The competition between O_2 and NO and the evidence of a stationary basal level of NO controlling respiration suggest that a finite fraction of COX is normally inhibited by NO. As proposed by Brown [6], this may provide an explanation for the fact that the K_M for O_2 measured in vivo (5–10 μM) is higher than that determined in vitro (0.5 μM) [12, 28]. What would be the NO concentration necessary to account for the higher K_M for O_2 observed in vivo? An estimate may be attempted using the oversimplified reaction scheme depicted in figure 2. The complex two-substrate [O_2 and cytochrome *c* (cyt *c*)] catalytic cycle of COX is simplified to a one-substrate Michaelis scheme, assuming that the electron supply under physiological conditions is not rate limiting [29]. The value of K_M for this scheme is given by:

$$K_{M,O_2} = \frac{(k_2 + k_3) \times (k_5 + k_4[NO])}{k_1 \times k_5}$$

Assuming the internal electron transfer rate constant [$k_3 = 100\ s^{-1}$; ref. 29] to be rate limiting during turnover, a steady-state concentration of NO ~ 60 nM (consistent with estimates reported in the literature) would account for the $K_{M,O_2} = 10$ μM observed in vivo [28]. In

conclusion, the NO flux in intact cells leads to a significant control of cellular respiration, and the inhibited enzyme is COX because of the competition between NO and O_2. Can this competition be understood on the basis of the known structural information available on COX?

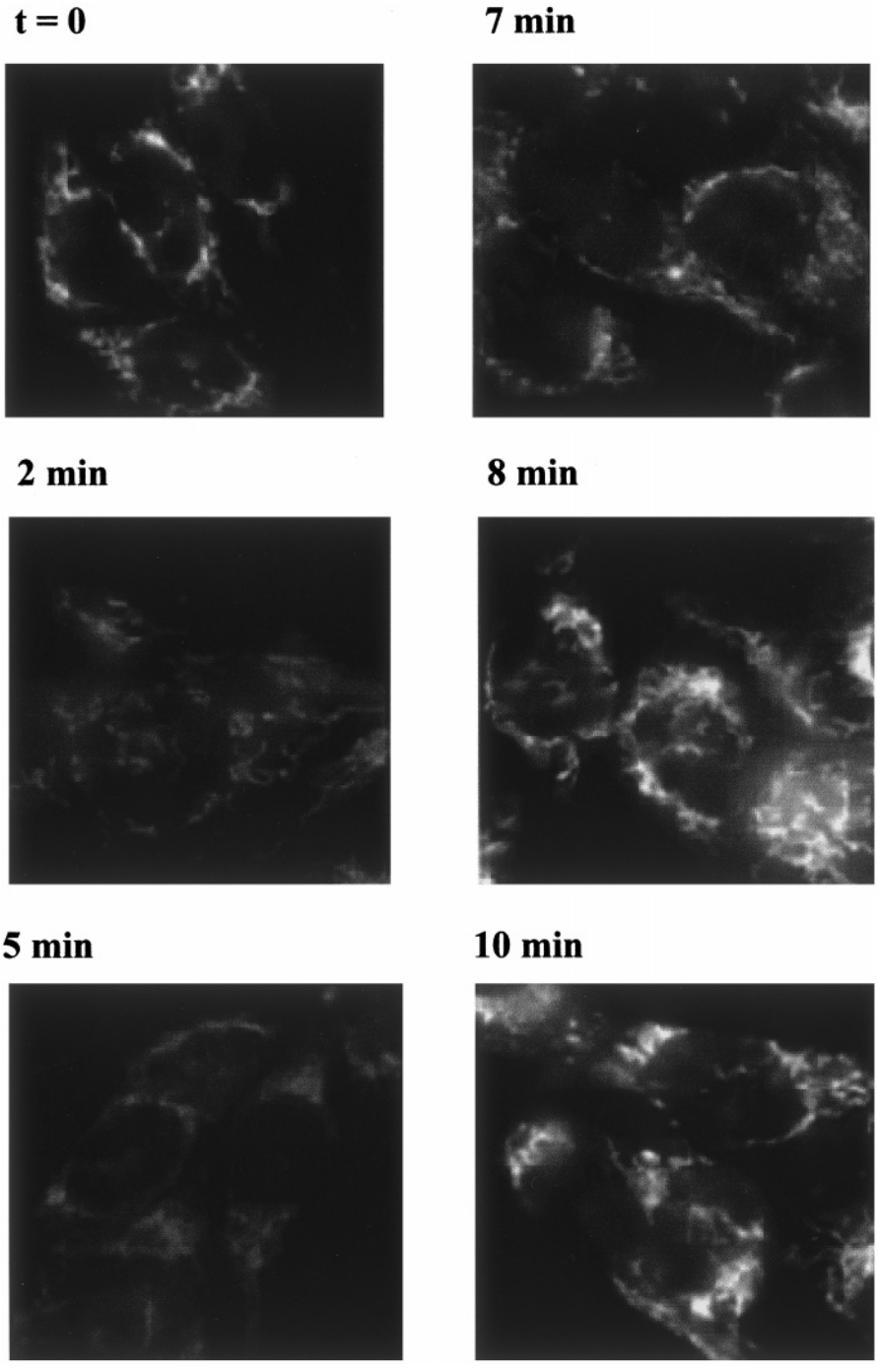

Figure 1. Import of rhodamine 123 by living cells: time-dependent changes in the mitochondrial fluorescence pattern induced by NOR_1. In the presence of 2 μM nigericin, the NO releaser NOR_1 was added to the air-equilibrated cell culture medium at time = 0, and fluorescence microscopy images taken at the indicated times after addition.

Table 1. Kinetics of reversal of NO inhibition.

k′ (s^{-1})	Biological system	Reference
0.013	rat brain synaptosomes	9
0.007	rat liver mitochondria	19
0.023	INS-1 pancreatic cells	20
0.009	rat heart mitochondria	54
0.13	beef heart COX	24
0.005	rat brain astrocytes	14

Structure and ligand binding to COX

The three-dimensional structure of COX purified from beef heart mitochondria and *Paracoccus denitrificans* is now available [30–32]. A large body of kinetic and structural evidence indicates that reduced cyt *c* reacts with Cu_A (the mixed-valence Cu-Cu binuclear center), which is in rapid redox equilibrium with cyt *a*; the heterobinuclear O_2-binding site (cyt a_3-Cu_B) is then reduced by cyt *a*. The structure of the binuclear center (fig. 3) shows that the Fe^{3+} of cyt a_3 and $Cu_B{}^{2+}$ are close (4.9 Å) to one another, but move slightly apart on complete reduction (5.2 Å) [33]. The coordination of Cu_B is independent of the oxidation state, and a small ligand (peroxide) probably bridges the two metals in the crystalline oxidized beef heart enzyme [33].

The reactions of the reduced enzyme with O_2, CO, and NO have been extensively characterized [see refs 34–36 for reviews] and the relevant rate constants for binding to the binuclear center are reported in table 2. A peculiar feature of the O_2-binding kinetics is that the apparent rate constant seems to display a saturation dependence on O_2 concentration, suggesting that binding to cyt $a_3{}^{2+}$ is preceded by binding to reduced Cu_B^+, which acts as a 'gate;' from these data, an apparent K_d (in the micromolar range) was assigned to the formation of a (low-affinity) adduct between Cu_B^+ and O_2 [37]. Although this interpretation has been criticized by Bailey et al. [38], it is accepted that NO is the only other ligand which binds to fully reduced COX with a rate constant similar to that of O_2 (though probably somewhat smaller).

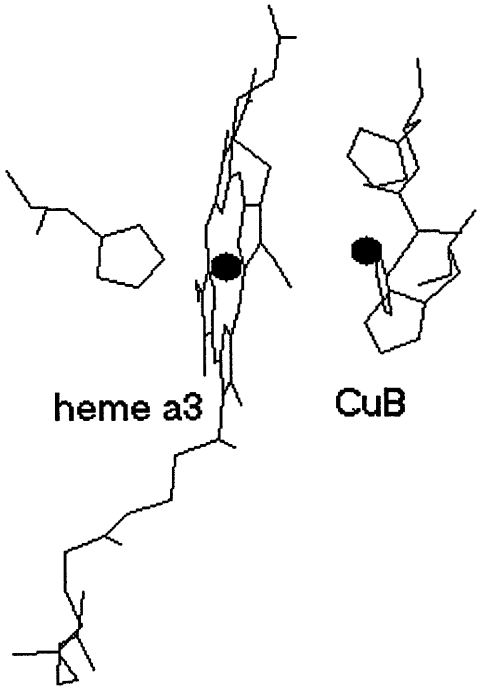

Figure 3. Structure of the cytochrome a_3-Cu_B binuclear site. From the Protein Data Bank coordinates deposited by Tsukihara et al. [31, 32]. The figure shows only the amino acid side chains coordinating the iron of cyt a_3 (on the proximal side at the left) and Cu_B (three histidines on the right). The structure refers to the fully oxidized beef heart COX.

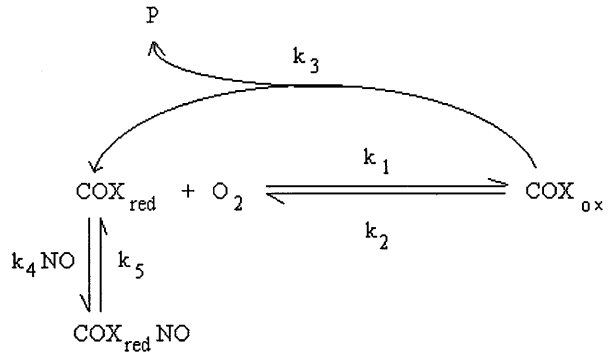

Figure 2. Effect of NO on the K_M for O_2 in vivo. k_1 is the bimolecular rate constant for O_2 binding ($k_1 = 2 \times 10^8\ M^{-1}\ s^{-1}$); k_2, the monomolecular rate constant for O_2 dissociation (which is essentially nil); k_3, the constant of the rate-limiting step in the catalytic cycle (which includes internal electron transfer, binding of cytochrome c^{2+} and release of cytochrome c^{3+}); k_4, the second-order rate constant for combination with NO, the competitive inhibitor of O_2 at the binuclear site ($k_4 = 4 \times 10^7\ M^{-1}\ s^{-1}$) and k_5 the first-order rate constant for the dissociation of bound NO ($k_5 = 0.1\ s^{-1}$).

Table 2. Rate constants and equilibrium constants for the reactions of reduced cytochrome *c* oxidase with O_2, CO and NO.

	O_2	CO	NO
k_{on} ($M^{-1}\ s^{-1}$)	$\sim 2 \times 10^8$	8×10^4	$0.4–1 \times 10^8$
k_{off} (s^{-1})	?	0.023	0.01–0.1
K_d (mM)	7.7[a]	3×10^{-4}	$0.1–2 \times 10^{-6}$

[a] As determined by Bailey et al. [38]. See reference for details.

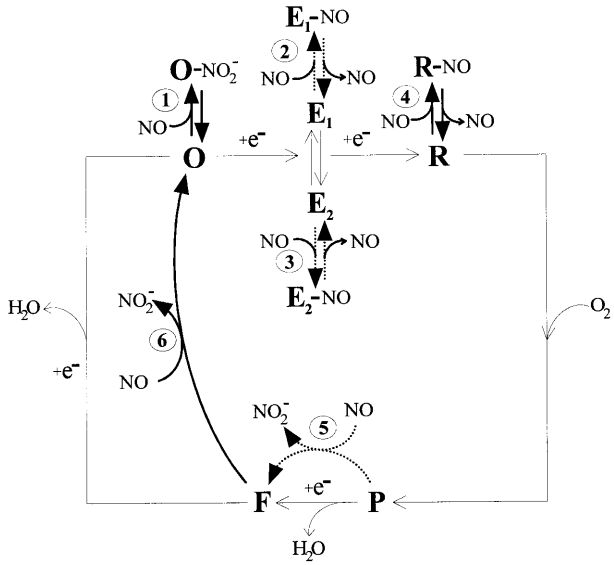

Figure 4. Reactions of different cytochrome *c* oxidase species with NO. ① Reaction with the fully oxidized binuclear site (**O**): $k_f = 2 \times 10^5\ M^{-1}\ s^{-1}$ [47, 48]. Nitrite dissociation takes hours in the absence of reductant [unpublished observations]. Reactions ② and ③ refer to the half-reduced binuclear site ($\mathbf{E}_1$ and $\mathbf{E}_2$), postulated by Torres et al. [51] and Giuffrè et al. [24], respectively. ④ Reaction with the fully reduced site (**R**): $k_f = 1 \times 10^8\ M^{-1}\ s^{-1}$ [44]; $k_r = 0.1\ s^{-1}$ [24]. Reactions ⑤ and ⑥ are with **P** and **F**, respectively: both reactions transform each of these intermediates into the following one in the catalytic cycle. Reaction with **F** ⑥ proceeds at $k_f \sim 1 \times 10^4\ M^{-1}\ s^{-1}$ [47].

On the basis of extensive transient spectroscopy experiments, detailed reaction schemes have been proposed for the catalytic cycle of COX [see refs 34 and 39 for reviews]. According to a consensus scheme shown in figure 4, the cyt a_3-Cu_B binuclear center can bind O_2 only in the fully reduced state (**R**). Formation of **R** was reported to be the rate-limiting step in the catalytic cycle, proceeding at a rate 20–25 s^{-1}) which has been assigned either to a slow proton diffusion [40] or to an intrinsically slow electron transfer [29]. Oxygen binding to **R** yields, initially, a reversible complex called compound **A**, which subsequently decays to the **P** (= peroxy) intermediate by electron delivery to bound O_2, and then to the **F** (= ferryl) species by additional electron transfer. Finally, **F** decays to the fully oxidized **O** species upon arrival of a fourth electron. It is important to note that oxidation of the enzyme from **R** to **O** upon O_2 binding proceeds with relaxation times in the microsecond range, whereas restoration of **R** from **O** occurs on a millisecond time scale.

This background information will acquire significance when discussing the next item, i.e., the mechanism of inhibition by NO and the states of the enzyme which are potential targets for NO.

COX: one enzyme, multiple possible targets for NO

Although several putative targets for NO can be envisaged in COX, including metals, thiol-containing and aromatic residues, at present the only metals shown to react with NO are at the cyt a_3-Cu_B binuclear center. Over the past 35 years, different reactions between NO and COX have been described (see fig. 4). NO can either bind to Fe^{2+} of cyt a_3 at the active site, or donate one electron to $Cu_B{}^{2+}$ with formation of the nitrosonium cation (NO^+), which by combination with hydroxide yields nitrite (NO_2^-) at neutral pH [41, 42]. In addition, it was suggested that NO can be reduced by COX to nitrous oxide (N_2O); this reaction, however, is essentially undetectable within tens of minutes according to Stubauer et al. [43]. Finally, some of the oxidation intermediates of COX were also proposed to react directly with NO.

The fully reduced cyt a_3-Cu_B site (**R**) binds NO to the heme-Fe, very rapidly ($k_{on} = 0.4–1 \times 10^8\ M^{-1}\ s^{-1}$) [44, 45] and with very high affinity ($K_a > 10^9\ M^{-1}$; table 2). More surprising was the finding that the NO dissociation rate constant from cyt $a_3{}^{2+}$ was unexpectedly fast ($k = 0.1\ s^{-1}$) [24], if compared with other heme proteins (for example NO dissociation from ferrous hemoglobin proceeds at $k = 10^{-3}–10^{-4}\ s^{-1}$ [46]).

The fully oxidized cyt a_3-Cu_B site (**O**) is also able to react with NO quickly ($k = 2.2 \times 10^5\ M^{-1}\ s^{-1}$) and with high affinity, if the purified enzyme is in the *fast* state and with no bound Cl^- [42, 47, 48]. This anion, once removed by reduction of the enzyme, rebinds to the oxidized cyt a_3-Cu_B very slowly (hours), preventing its reaction with NO (fig. 5). In the reaction with the oxidized Cl^--free *fast* enzyme, one electron is extracted from NO and back-transferred to cyt *a* and Cu_A, while NO is oxidized to nitrite, which seems to remain trapped in the active site. In contrast, if the enzyme is in the presence of chloride and/or in the so-called *slow* form, NO reacts with the binuclear center (possibly at $Cu_B{}^{2+}$) very slowly indeed [49].

Wilson and coworkers [41, 47] have extended their initial observations to the reaction of NO with the **P** and **F** intermediates (see above). Interestingly, they showed that both **P** and **F** are able to accept one electron from NO similarly to the **O** species (see fig. 4); these reactions (**P** → **F** and **F** → **O**) are associated with the formation of nitrite (see fig. 4). Torres et al. [47] reported that the reaction of NO with **F** ($k \approx 1 \times 10^4\ M^{-1}\ s^{-1}$) is even slower than that with **O** ($k = 2.2 \times 10^5\ M^{-1}\ s^{-1}$) [48].

In conclusion, several oxidation states of COX can react with NO, some of which can be reduced by this ligand with formation of nitrite (see fig. 4), a very low affinity inhibitor of COX [$K_I = 1.5$ mM; ref. 50]. However, since the reactivity of the various states is different

and their populations under turnover conditions variable, a unique assessment of the mechanism of inhibition of COX by NO is not straightforward, as discussed in the next section.

The mechanism of inhibition of COX by NO

The mechanism by which NO inhibits COX has been thoroughly investigated by spectroscopic and polarographic techniques, both on the purified and the membrane-bound enzyme [8–10, 24, 51]. In all cases, NO was confirmed as a potent inhibitor of COX, consistently with the inhibition constant measured with synaptosomes ($K_I = 270$ nM NO at $[O_2] = 140$ μM [9]). The onset of inhibition measured by the rate of cyt *c* oxidation is quite fast [24], and mitochondrial respiration shows the pattern of competition between NO and O_2 [10], pointing to COX as a primary target of NO.
Analysis of the optical spectra after addition of NO to purified COX in turnover showed that the inhibited state of the enzyme is the complex of NO with reduced cyt a_3 [24, 51]. In the presence of O_2, inhibited COX recovers activity because NO is removed from solution by reaction with O_2 [9]. The rapid recovery observed in experiments carried out with cells and tissues [seconds to minutes; ref. 22] is not surprising because of the relatively fast dissociation rate of NO from cyt a_3^{2+} [see above and ref. 24].

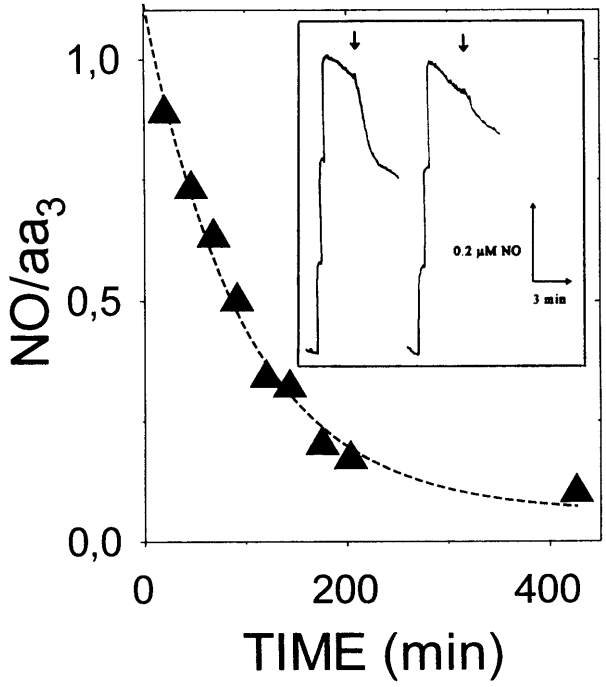

Figure 5. The effect of Cl^- binding on the reaction of NO with the oxidized enzyme. NO reacting with the oxidized enzyme after addition of 100 mM KCl, as measured amperometrically [48]: the fraction of NO reacting with the enzyme drops as a function of time after addition of KCl to a Cl-free enzyme. Inset: NO (three additions of 250 nM each) was added to degassed buffer prior to the addition of the oxidized enzyme (arrows). Compare the NO reaction with the Cl-free enzyme (left trace) with the measurement on the Cl-bound enzyme (right trace).

Given the rate constants for O_2 and NO binding (table 2), understanding the inhibition of respiration is not a straightforward task. The combination rate constant to the fully reduced binuclear center (**R**) favors O_2 (see table 2), against an inhibition constant which favors NO by a factor of 100–1000. This apparent inconsistency demands peculiar differences in the reaction of O_2 and NO with the two metals in the binuclear center. This point has been addressed by two groups [24, 51], with the proposal of two different mechanisms, both invoking a special role for the half-reduced state of the cyt a_3-Cu_B center species ($\mathbf{E}_1$ and $\mathbf{E}_2$). Giuffrè et al. [24] proposed a mechanism involving preferential binding of NO to reduced cyt a_3 in a half-reduced binuclear center, which is known to be non-reactive towards O_2 [52]; on the other hand, Torres et al. [51] proposed that NO displays a unique reactivity for Cu_B^+, with a rapid transfer of NO to cyt a_3^{2+} eventually generated by internal electron transfer. According to both models, one single NO binds to the active site, which at face value seems in contrast with the observations reported by Koivisto et al. [12]. Using mitochondria from brown adipose tissue, they observed a square dependence of the IC_{50} of NO on O_2 tension. This finding was taken by the authors as evidence that COX binds one O_2 molecule as electron acceptor, but can bind two NO molecules, with degradation of NO to N_2O. On the basis of what is summarized below, we believe that the interpretation provided by Koivisto et al. [12] is unlikely, but at present no alternative explanation for the square dependence of the IC_{50} can be offered. This intriguing result will have to be examined carefully.
The observation that, over and above binding, NO can also deliver an electron to species **O**, **F**, and **P** (see fig. 4), yielding possibly nitrite, opened new possibilities. The reaction of NO with these species may indeed lead to formation of an inactive nitrite-COX adduct, accounting for enzyme inhibition. In this context, the observation that the nitrite-bound enzyme, produced by the fast reaction of NO with the fully oxidized enzyme **O**, is inhibited [unpublished observations] is obviously relevant. Unfortunately, at present it is not known if the reaction of NO with **P** and **F** generates an inhibited nitrite-bound complex (similarly to **O**) or if nitrite dissociates rapidly from the enzyme.
A crucial question is therefore whether the reaction of NO with **O**, **P**, and **F** under turnover is sufficiently fast to compete with the very fast binding of NO to reduced

cyt a_3^{2+} [$k = 0.4–1 \times 10^8\ M^{-1}\ s^{-1}$; refs 44, 45], taking into account the relaxation time and the population of each species at steady-state. This information should clarify whether the reaction of Cu_B^{2+} with NO may provide a feasible alternative to the mechanisms of inhibition proposed by Torres et al. [51] and by Giuffrè et al. [24]. A conclusive answer to this question is presently not available, also in view of the fact that a quantitative description of the steady-state populations of COX in its different states is still lacking. Nevertheless, it is clear that only the reaction of NO with the (partially or totally) reduced binuclear center can account for the competition between O_2 and NO, which is the characteristic feature of the inhibition of respiration by NO.

Is there a metabolic degradation of NO catalyzed by COX?

It has been postulated that in vivo COX may metabolize NO. This topic is of considerable pathophysiological interest because, while the NO biosynthetic pathway is at present known in some detail, information on enzymatic in vivo degradation of NO is essentially lacking.

As discussed above, there is convincing experimental evidence that turnover species of COX (**O**, **P**, and **F**) are able to transform NO into nitrite. An appealing feature of this reaction is that it may represent a pathway of oxidative degradation of NO to the less toxic nitrite. Since NO rapidly reacts with superoxide O_2^- leading to the dangerous peroxynitrite $ONOO^-$, a pathway which may efficiently dispose of NO overproduction may be welcome by the cell. However, as already addressed in the previous section, it is not easy at this stage to assess to what extent COX catalyzes the formation of nitrite at steady-state. In addition, nitrite formed in the reaction of the fully oxidized enzyme (**O**) with NO binds and inhibits the enzyme; but if reversal of COX inhibition proceeds via rereduction of bound nitrite to NO and dissociation of the latter, this would be a 'futile' metabolic pathway for the disposal of NO. However, since the fate of nitrite formed by the reaction of NO with **P** and **F** is still unknown, the possibility of an oxidative degradation of NO mediated by COX is not ruled out.

A stimulating hypothesis, put forward by two groups [53, 54], envisaged that COX may catalyze the reduction of NO to N_2O, according to the following reaction:

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$$

This postulated function demands that COX, under reducing conditions, may bind and reduce 2 mol of NO, thereby acting as a bona fide NO reductase [see ref. 55 for a review].

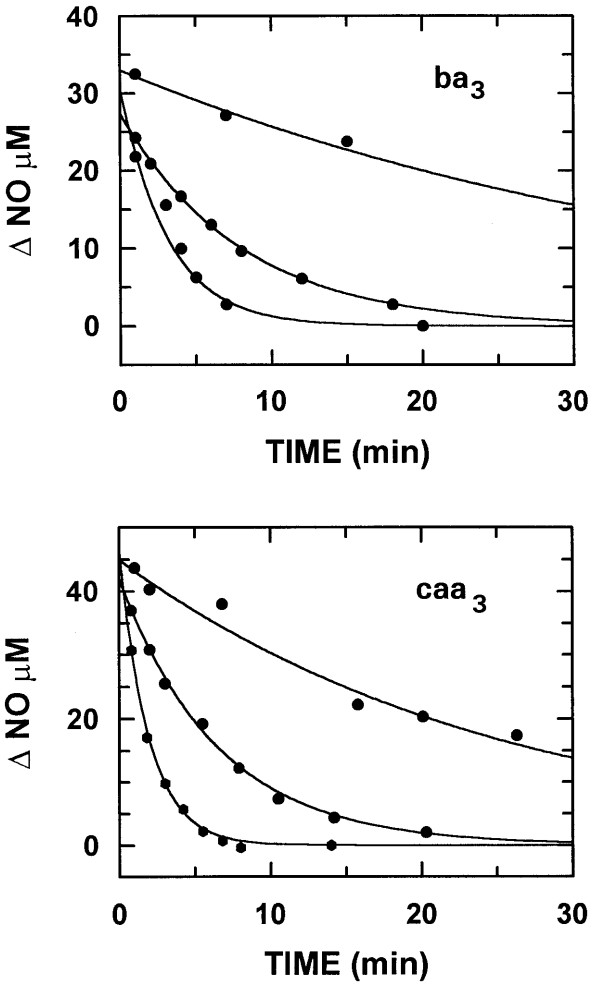

Figure 6. NO reductase activity of ba_3 and caa_3 oxidases of *Thermus thermophilus*. NO consumption by ba_3 and caa_3 oxidases from *T. thermophilus* in the presence of excess reductant. Enzyme concentrations (traces from bottom left to top right): 4.6, 1.5 and 0.5 μM for ba_3 and 1, 0.3 and 0.1 μM for caa_3. These experiments show that the NO reductase activity is proportional to the enzyme concentrations, under reducing conditions and excess NO.

An evolutionary theory which proposes that NO and O_2 reductases share a common phylogenic origin [56, 57] provided an appealing biological connection to this hypothesis. According to Stubauer et al. [43], however, beef heart COX does not catalyze the formation of N_2O to any significant extent. Based on optical and amperometric studies, these authors showed that there is no measurable NO reductase activity of COX either purified or in mitochondria. Moreover they also suggested that the effect of cyanide on the rate of NO disappearance (reported by Borutaitè and Brown [54]) could be understood on the basis of a simple competition of the two ligands for the reduced binuclear center.

Thus beef heart COX seems not to contribute to a reductive metabolism of NO, even under strongly reducing conditions.

Unexpectedly, we recently found [A. Giuffrè, G. Stubauer, P. Sarti, M. Brunori, W. G. Zumft, G. Buse, T. Soulimane, unpublished data] that two phylogenetically distant heme-copper oxidases, the ba_3 and the caa_3 COXs, expressed by the extreme thermophilic eubacterium *Thermus thermophilus*, can catalyze N_2O production from NO, at variance with the bovine enzyme (fig. 6). Although the catalytic efficiency of these bacterial enzymes at room temperature is very far from that of bona fide NO reductases, this result seems noteworthy, since to the best of our knowledge, these are the first ever described heme-copper oxidases displaying measurable NO reductase activity. This finding strongly supports the hypothesis of a common evolutionary origin of the respiration and denitrification electron transfer chains. In the context of this review, the discovery of a new activity of the *T. thermophilus* oxidases acquires significance because it suggests a strategy to engineer measurable NO reductase activity in eukaryotic COXs by site-directed mutagenesis.

In conclusion, although more than one species of COX has been shown to react with NO, at present there is no clear experimental evidence supporting the involvement of mitochondrial COX in an oxidative or a reductive degradation of NO. The problem of in vivo NO scavenging is, therefore, still an open question.

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