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The mechanism of cytochrome *C* oxidase inhibition by nitric oxide

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1. ABSTRACT

The basic biochemistry of the inhibition of cytochrome oxidase by NO is reviewed. Three possible mechanisms that include the binding of NO to the fully reduced Fe_{a3}-Cu_B site, to the semi-reduced Fe_{a3}-Cu_B site, and to the fully oxidized Fe_{a3}-Cu_B site are confronted with the experimental data. Mathematical models are used to facilitate the analysis and to solve puzzling observations concerning the NO inhibition of cytochrome oxidase. It is concluded that the inhibition of cytochrome oxidase by NO is mixed, having both competitive and uncompetitive components, but under physiological electron flows the competitive component is largely predominant. The physiological and pathological relevance of this inhibition is briefly discussed.

2. INTRODUCTION

The mitochondrial respiratory chain is essential to obtain energy from nutrients in a form that can be used by the cell. In this chain, electrons from the nutrients are fed in the respiratory chain in the form of NADH and $FADH_2$, reducing O_2 to H_2O, and generating a membrane potential across the inner mitochondrial membrane, which drives the formation of ATP from ADP. ADP, O_2, and more recently NO, are considered to be the main regulators of this process (1). Concerning NO, it reversibly inhibits cytochrome oxidase (COX), but there is an apparent paradox between the observed inhibition and the known kinetic data. NO inhibits COX in competition with O_2 rapidly, within a time scale of seconds, with half-inhibition of respiration attained at O_2 to NO ratios in the range 40-

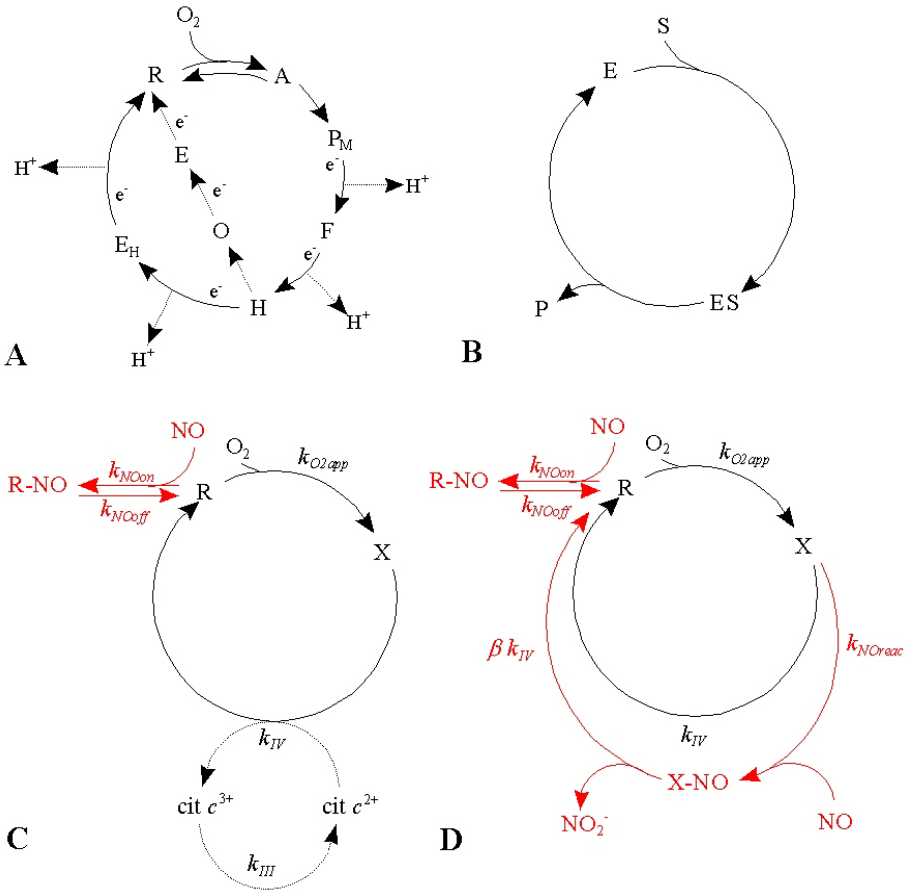

Figure 1. (A) Simplified scheme for the catalytic cycle of COX according to reference (5) is shown. The fully reduced Fe_{a3}-Cu_B site, denoted by R, reacts reversible with O_2 forming an adduct (A), which reacts rapidly and irreversibly to form the intermediate P_M, thus trapping O_2. This part of the cycle can be lumped as a single process characterized by the apparent binding rate constant k_{O2app} (C). The intermediate P_M is reduced to R via a sequential four electron reduction, coupled with vectorial pumping of protons and involving intermediates F, H, and E_H. Alternatively and in the absence of electron donors, H may relax into state O, which can be reduced back to R in a two sequential electron reduction not coupled with proton pumping and involving the intermediate E. The conversion of P_M to R, can be lumped as a single process characterized by k_{IV}, if all intermediates are considered to be near a steady-state (C). Because these intermediates relax with a time scale of millisecond or faster (20), for a time scale of seconds the steady-sate approximation is valid. (B) Classic scheme showing an enzyme following the Henri-Michaelis-Menten mechanism. (C) The simplified scheme used as a minimal mathematical model of COX catalytic cycle that includes the inhibition of COX by NO through binding to the fully reduced Fe_{a3}-Cu_B site (shown in red); this minimal model was extended by including explicitly the role played by cytochrome *c* (dotted line) (6). (D) Minimal model in (C) was extended to include the reaction of NO with oxidized forms of COX, forming NO_2^-, leading to hyperbolic uncompetitive inhibition.

00 (1). This seems to indicate that the interaction of COX with NO is stronger than that with O_2 and very fast. But: (a) the rate constants for the binding of O_2 and NO with the fully reduced binuclear center Cu_B/Fe_{a3} of COX are similar, respectively 1.4×10^8 $M^{-1}s^{-1}$ (2) and 4×10^7-1×10^8 $M^{-1}s^{-1}$ (3); (b) NO dissociates slowly from this center (0.01-0.13 s^{-1}) (4); while (c) the apparent dissociation rate constant of O_2 with COX is virtually zero, since during the initial steps of reduction of O_2 by COX, O_2 is kinetically trapped (2), and subsequently is converted to H_2O, the end product of the reaction. In this review we critically analyse in detail these puzzling observations, as well as the various alternative mechanisms that have been proposed, confronting them with experimental observations.

3. THE CATALYTIC CYCLE OF CYTOCHROME C OXIDASE

To understand the inhibition of COX by NO, it is important to describe the basic biochemistry of the catalytic cycle of COX. Although the details of this cycle and its integration with the generation of the membrane potential during mitochondrial respiration are not fully understood, the available knowledge is sufficient to understand the interactions of NO with COX. COX has two binuclear sites formed by a heme and a copper ion: the Fe_{a3}-Cu_B site where the actual reaction with O_2 occurs, and the Fe_a-Cu_A, which channels the electrons from cytochrome *c* to the Fe_{a3}-Cu_B site. In Figure 1A, a simplified scheme of COX

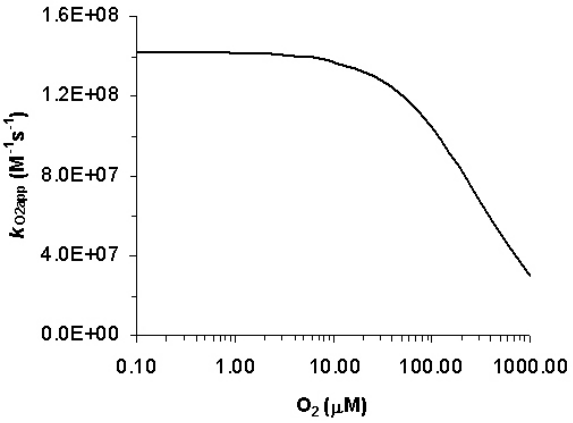

Figure 2. Estimation of the rate constant for the kinetic trapping of O_2 to COX, k_{O2app}, as a function of O_2 concentration. According to Wikstrom and colleagues (2), k_{O2app} is an apparent second order rate binding constant that depends on O_2 concentration and is defined as $k_{O2app} = k/(K_1K_2K_3 + K_2K_3[O_2] + K_3[O_2] + [O_2])$, where k is a rate constant for the inter-heme electron transfer between Fe_a and Fe_{a3} ($k = 2.4\times10^5$ s^{-1}) (2), K_1, K_2, and K_3 are equilibrium constants, which may have two sets of values: 8 mM, 0.044, and 4.8 or 1.5 mM, 0.3, and 3.8, respectively for K_1, K_2, and K_3 (2). The value of k_{O2app} is the same for these two sets of values, and in the physiological tissue O_2 range, k_{O2app} is near independent of the O_2 concentration.

catalysis is shown according to Wikström (2004) (5). Oxygen binds reversibly to the reduced Fe_{a3}-Cu_B site and then undergoes a very fast reaction, which in practical terms leads to a kinetic trapping of O_2 by COX. This part of the cycle is not under control of the membrane potential across the inner membrane and vectorial proton pumping does not occur in it; therefore, the whole process can be accurately described by mass action kinetics with an apparent rate constant given by $k_{O2app} = k/(K_1K_2K_3 + K_2K_3[O_2] + K_3[O_2] + [O_2])$ (2). In the physiological range of O_2 concentrations, i.e. lower than 30 μM, the value of k_{O2app} is near constant and close to 1.4×10^8 $M^{-1}s^{-1}$ (Figure 2).

In the second part of the cycle, the steps are slower, are coupled with proton vectorial pumping, and are controlled by both the membrane potential and the availability of electrons coming from cytochrome *c*. Therefore, an accurate kinetic description is more difficult for this part of the cycle, and actually it would have to include other components of the mitochondrial respiratory chain. Even without a full kinetic description, some useful quantitative considerations can be made. If one assumes that the intermediates F, H, E_H, O, and E are in a near steady-state, a valid assumption in the time scale of seconds or slower, the transition between P_M and R can be lumped without loss of rigor in a single step thus facilitating the analysis. The apparent rate constant for this lumped process, k_{IV}, will be a function of all elementary steps lumped in it. Consequently it will depend on the membrane potential and the level of reduction of cytochrome *c* among other factors. It will be higher in state 3 respiration where ADP concentration, rate of ATP production and rate of respiration are high, as compared with state 4 mitochondrial respiration, where ADP concentration, rate of ATP production and rate of respiration are low.

Comparing Figures 1A and 1B, it can be seen that the catalytic cycle of COX fits the classic Henri-Michaelis-Menten scheme, if several intermediate forms for the substrate-enzyme complex are considered. Therefore, COX catalysis follows saturation kinetics that can be described by the following equation (6):

$$-\frac{d[O_2]}{dt} = \frac{k_{IV}[COX_{tot}]\,[O_2]}{[O_2] + k_{IV}/k_{O2app}} \quad [1]$$

By fitting the observed experimental rates of respiration to equation 1, k_{IV} can be estimated to be 7.5 s^{-1} and 0.75 s^{-1} in states 3 and 4, respectively (from (6) and accounting for the stoichiometry of four electrons per O_2 consumed). The $K_{0.5}$ values are also often referred as K_{MO2} and $[O_2]_{0.5}$. However, the apparent affinity of respiration for O_2, the $K_{0.5}$ value, is not a constant or a true K_M value, being given by the ratio of k_{IV}/k_{O2app}. Because k_{IV} increases with the rate of respiration, $K_{0.5}$ also increases with this rate, as it is observed experimentally in mitochondrial states 4 and 3 (4, 17, 18). In conclusion, the minimal model shown in Figure 1A accommodates well the dependency of mitochondrial respiration on O_2, and we will use it as a starting point to discuss the inhibition of COX by NO.

4. INHIBITION OF CYTOCHROME OXIDASE BY NO

The interaction of NO with both reduced COX (7) and oxidized COX (8) is known for a long time and NO was actually widely used as a probe to study COX, due to its similarity to O_2 and CO. But it took a long time to fully understand the biological consequences of this interaction; initially it was recognized that the NO produced by denitrifying microorganisms could inhibit COX (9) and later the inhibition of COX from bovine heart submitochondrial by NO produced by nitrite reductase was observed *in vitro* (10). In 1994-95, several independent laboratories reported the reversible inhibition of mitochondrial respiration or the selective inhibition of COX, among other respiratory complexes, by NO (11-14). Soon the basic biochemical characteristics of the reversible inhibition of the mitochondrial respiration by NO become established: (a) it is done through the inhibition of COX; (b) it is competitive with O_2, *i.e.* the degree of inhibition increases when O_2 concentrations decrease (Table 1); (c) it is very fast, within the time scale of seconds (15); and, (d) it takes place at relatively low concentrations of NO (Table 1), which are found *in vivo* (16).

4.1. Binding of NO to the reduced Fe_{a3}-Cu_B site

Concerning the detailed molecular mechanism of the inhibition of COX by NO, it was difficult to achieve a complete understanding. Spectral evidence pointed to the reversible binding of NO to the fully reduced binuclear site

Table 1. Experimental NO concentrations that inhibit mitochondrial respiration by 50 % (IC_{50}) at various levels of O_2.

Preparation	O_2 (μM)	IC_{50} (nM)	Reference
Synaptosomes	145 30	270 60	13
Brown adipose tissue mitochondria	180 119 72 49 32	364 148 69 41 11	27
Heart mitochondria	150 60	570 180	32
Rat brain submitochondrial particles	≈ 220	2000	55
Heart COX	50	1300	15
Liver mitochondria	≈150	950	26

Fe_{a3}-Cu_B in competition with O_2 (15; 17), according to the following four equations (see also Figure 1C)

$$Fe_{a3}^{2+}\text{-}Cu_B^{+} + O_2 \xrightarrow{k_{O2app}} Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}O_2 \quad k_{O2app} = 1.4\times10^{8}\ M^{-1}s^{-1}$$

$$Fe_{a3}^{2+}\text{-}Cu_B^{+} + NO \xrightarrow{k_{NOon}} Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO \quad k_{NOon} = (0.4\text{-}1)\times10^{8}\ M^{-1}s^{-1}$$

$$Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}O_2 \xrightarrow{k_{O2off}} Fe_{a3}^{2+}\text{-}Cu_B^{+} + O_2 \quad k_{O2off} = 0\ s^{-1}$$

$$Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO \xrightarrow{k_{NOoff}} Fe_{a3}^{2+}\text{-}Cu_B^{+} + NO \quad k_{NOoff} = 0.01\ s^{-1}$$

O_2 and NO binding rate constants to COX are similar, and O_2 is kinetically trapped while NO is able to dissociate, albeit slow, from COX. Therefore, *a priori* one could think that it would be necessary a ratio of O_2 to NO near 1 to achieve half-inhibition, and that the onset of inhibition would be relatively slow, exactly the opposite of what is observed experimentally (15; 17-20). In fact: a) half-inhibition of respiration is attained at O_2 to NO ratios in the range 40-500; b) the onset of COX inhibition upon exposure to NO is fast (within a few seconds); and c) the population of COX presented as the fully reduced binuclear site Fe_{a3}-Cu_B constitutes only a minor fraction of total COX, and so it seemed that NO could not inhibit COX by competing with O_2 for this form of COX.

4.2. Binding of NO to a single-electron reduced Fe_{a3}-Cu_B site

Taking into account the previous arguments, a second mechanism was proposed where NO would actually bind to a single-electron reduced Fe_{a3}-Cu_B, either reduced Cu_B (15) or reduced heme a_3 (17) for which O_2 has a low affinity (2). The experimental evidence for this hypothesis came a few years later when it was observed the binding of NO to the one-electron reduced Fe_{a3}-Cu_B center (21) in a bacteria mutant in which the reduction of COX is very slow. But whether this reaction is important from a quantitative point of view in the wild-type protein remains unknown. Nevertheless, it is widely accepted that O_2 does not bind to the single-electron reduced Fe_{a3}-Cu_B (2) and therefore this mechanism cannot account for the competitive nature between NO and O_2 for the inhibition of COX induced by NO. If the single-electron reduced Fe_{a3}-Cu_B were the site of NO inhibition, an uncompetitive inhibition, in which the degree of inhibition increases with the increase of O_2 concentration (22), would be expected for low non-saturating O_2 concentrations. At an average O_2 tissue concentration of 30 μM or higher, at which COX is expected to be saturated with O_2, uncompetitive inhibition is indistinguishable from pure non-competitive inhibition, and so the degree of inhibition would be expected to be independent of O_2 concentration.

4.3. Binding of NO to the oxidized Fe_{a3}-Cu_B site

A third possible mechanism for COX inhibition by NO is based on the reaction of NO with the fully oxidized Fe_{a3}-Cu_B site (Figure 1D). This reaction was first reported in 1979 (8), but its biological consequences are not fully understood yet. It is now known that NO can react with any of the intermediates where Cu_B is oxidized namely P, F, and O (18; 23; 24), reducing Cu_B. The rate constants for the reaction with each of these three intermediates are 8×10^4 $M^{-1}s^{-1}$ for P (24), $(1\text{-}2)\times10^4$ $M^{-1}s^{-1}$ for F (18; 24), and 2×10^5 $M^{-1}s^{-1}$ for O (24), and the stoichiometry is one with each of the three intermediates (24). After this initial reaction NO is rapidly oxidized to NO_2 in the binding site. NO_2 is released very slow from this site, with a time scale of 1 hour approximately, but under normal turnover conditions the release is much faster and the fully reduced center is rapidly regenerated (24; 25). Thus, even at very high concentrations of NO there is always a residual COX activity, which has been estimated to be approximately 10 to 20 % (24; 25). Therefore, this mechanism does not lead to a full inhibition of COX contrary to what is observed experimentally (12; 26; 27). It is also accepted that the reaction with the fully-oxidized Fe_{a3}-Cu_B site cannot lead to a COX inhibition by NO that is competitive with O_2, because O_2 does not react with this center (28), as discussed before for the single-electron reduced Fe_{a3}-Cu_B site. Actually, the predicted uncompetitive inhibition was observed experimentally in two *in vitro* systems with isolated COX, (29; 30), and its occurrence was recently associated with low electron fluxes (30).

Further experimental evidence for the relevance of the reaction of NO with oxidized Fe_{a3}-Cu_B site comes from elegant experiments in which the susceptibility of the inhibition of COX by NO to light is explored. The release of NO from the fully reduced Fe_{a3}-Cu_B site increases linearly with light intensity (31). While in the dark the "off" rate constant from the reduced site is 3.5×10^{-3} s^{-1} at 20ºC and 0.01 s^{-1} at 37ºC (31), the same rate constant under bright light conditions is 0.13 s^{-1} at 20ºC (23). So, when NO is removed from the incubation medium the rate of respiration is expected to come back to control values with a time scale of $1/3.5\times10^{-3} = 286$ s in the dark, while under light bright light conditions this time scale is $1/0.13 = 7.7$ s. In contrast, the displacement of NO_2 from COX is light independent. Taking advantage of these properties, it was

Table 2. Summary of the characteristics of the three possible mechanisms accounting for COX inhibition by NO

NO binding site	**Fully reduced Fe_{a3}-Cu_B**	**Single electron-reduced Fe_{a3}-Cu_B**	**Fully oxidized Fe_{a3}-Cu_B**
Type of inhibition	Competitive	Uncompetitive	Uncompetitive
Full inhibition	Yes	?	Partial (80-90 %)
For increasing $[O_2]$ if $[O_2] \leq 30$ μM if $[O_2] \geq 30$ μM	 Inhibition decreases Inhibition decreases	 Inhibition increases Inhibition is constant	 Inhibition increases Inhibition is constant
For increasing electron flux	Inhibition increases	?	Inhibition decreases
Reversal of inhibition depends on light	Yes	?	No
Experimental evidence	Spectroscopy evidence; dependence on $[O_2]$ and on the electron flux; full inhibition is observed experimentally; mechanism well defined; most experimental observations can be reproduced by a kinetic mathematical model assuming this mechanism and the known rate constants.	Spectroscopy evidence obtained in a mutant COX.	Dependence of inhibition reversal on light; spectroscopy evidence; production of NO_2 in mitochondria; accounts for inhibition observed at low electron-fluxes
Problems	Does not account for the independence of inhibition reversal on light observed in some experiments; underestimates degree of inhibition observed at low electron-fluxes	Was never observed with a wild-type protein; the dependence of the degree of inhibition on $[O_2]$ predicted is the opposite of what is observed experimentally.	The dependence of the degree of inhibition both on $[O_2]$ and on electron flux are the opposite of what is observed experimentally; fully inhibition is observed experimentally.

observed that under low electron flow, the reversal of NO inhibition is light-independent while under high electron flow, this reversal is light-dependent (28; 31). Concerning the biological relevance of these results, it is critical to evaluate whether the electron flow conditions used *in vitro* are observable *in vivo*.

Nevertheless, because the COX inhibition by NO observed experimentally is both competitive with O_2 and full inhibition is achieved, the inhibition through a mechanism involving NO_2 cannot account for most of the experimental observations, and therefore it is probably only relevant under very low electron fluxes through the respiratory chain (see below).

5. INHIBITION OF CYTOCHROME OXIDASE – A CRITICAL EVALUATION

The characteristics, the experimental support, and the major criticisms against each of the three mechanisms described above for the inhibition of COX by NO are indicated in Table 2. It is clear that the mechanism involving the binding to the fully reduced Fe_{a3}-Cu_B site is the one that reproduces the hallmark of the inhibition of COX by NO: its competitive character with O_2. The arguments against this mechanism are based on both thermodynamic and kinetic considerations and not on hard experimental evidence. Recently, evidence from a kinetic mathematical model showed that these arguments were misleading, and it conciliated the main experimental characteristics of the inhibition of COX by NO with a mechanism based on a simple competition of NO with O_2 for the reduced Fe_{a3}-Cu_B site, as shown in Figure 1C (6). In what follows we critically analyze each of arguments used against this simple mechanism.

5.1. Inhibition of cytochrome *c* oxidase is too sensitive to NO

One argument states that the high sensitivity of COX to NO, half-inhibition of respiration attained at O_2 to NO ratios in the range 40-500 (1), is incompatible with the known rate constants for the binding of NO and O_2 to the reduced Fe_{a3}-Cu_B site, which are similar. Equations 2 and 3 describe the inhibition of COX by NO based on a simple competition of NO with O_2 for the reduced Fe_{a3}-Cu_B (6); they indicate that the turnover of COX described by k_{IV} has a position in the reaction scheme that is formally identical to a dissociation rate constant describing the dissociation of O_2 from COX.

$$-\frac{d[O_2]}{dt} = \frac{k_{IV}[COX_{tot}]\,[O_2]}{[O_2] + \frac{k_{IV}}{k_{O2app}}\left(1 + \frac{k_{NOon}}{k_{NOoff}}[NO]\right)} \quad [2]$$

$$IC_{50} = \frac{k_{NOoff}}{k_{NOon}}\left(1 + \frac{k_{O2app}}{k_{IV}}[O_2]\right) \quad [3]$$

This can be understood if the turnover of COX is viewed as the pathway that restores the free enzyme dissociated from O_2. The true dissociation equilibrium constant of NO with COX is given by the ratio k_{NOoff}/k_{NOon} while the apparent dissociation equilibrium dissociation constant of O_2 towards COX is given by k_{IV}/k_{O2app} (this is apparent since k_{IV} is not a true dissociation rate constant). k_{NOon} and k_{O2app} have similar values, but k_{IV} (7.5 s^{-1} in state 3 and 0.75 s^{-1} in state 4) is much higher than k_{NOoff} (0.01 s^{-1} in the dark (31)). Therefore, the dissociation equilibrium constant for the binding of O_2 to COX is higher than that of NO, the affinity of NO towards COX is higher than the apparent affinity of O_2, and the competition between O_2 and NO favors NO, explaining why levels of NO much lower than O_2 are able to efficiently inhibit COX.

5.2. Inhibition of cytochrome *c* oxidase by NO is too fast

Concerning the argument that the fast onset of COX inhibition within seconds is incompatible with similar rate constants for the binding of O_2 and NO to COX, this is based on a misunderstanding of the dynamics of COX cycle. The time scale for the inhibition of COX by NO is given by two reaction times.

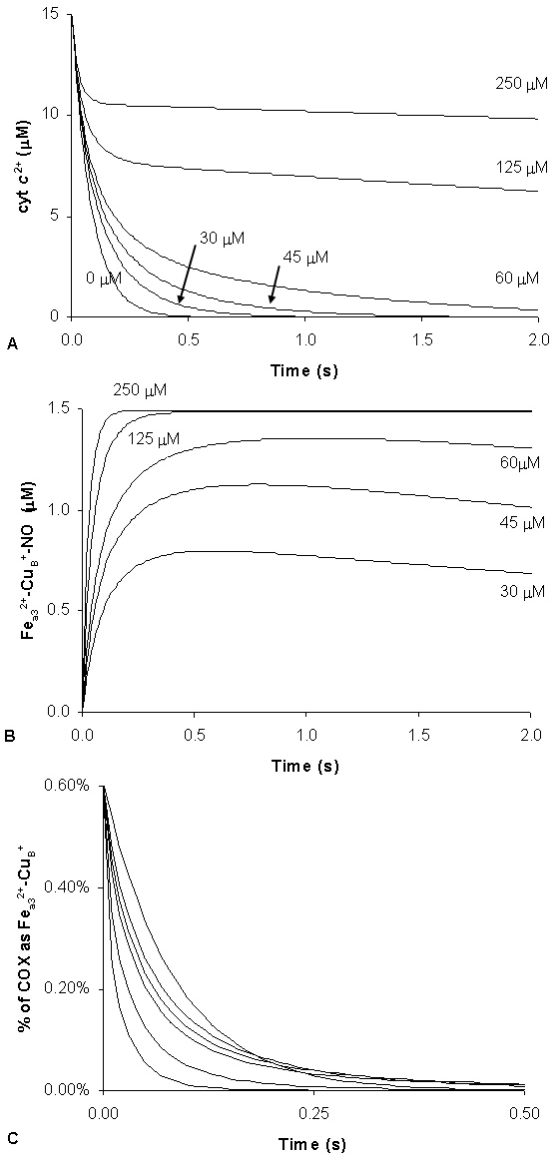

Figure 3. The onset of COX inhibition is very fast. Results were obtained from simulations of the extended minimal model (see Figure 1C) run with the software PLAS (53). A) Cytochrome *c* oxidation is followed by showing the decrease of cytochrome c^{2+}. B) the formation of the complex Fe_{a3}^{2+}-Cu_B^+-NO is shown. C) The fraction of COX populated as the fully reduced Fe_{a3}-Cu_B center is shown. Parameters used in the simulation were chosen to mimic experimental conditions in (17) and were as follows: $k_{NOon} = 4\times10^7$ $M^{-1}s^{-1}$; $k_{NOoff} = 0.13$ s^{-1}; $k_{O2app} = 1.4\times10^8$ $M^{-1}s^{-1}$; $k_{IV} = 8\times10^6$ $M^{-1}s^{-1}$; k_{III}=0 s^{-1}; $[Cyt.\ c]_{tot} = [Cyt\ c^{2+}] = 15$ μM; $[COX]_{tot} = 1.5$ μM; $[O_2] = 140$ μM; $[Fe_{a3}^{2+}\text{-}Cu_B^+] = 9.1$ nM. At time 0 s, cytochrome *c* and NO were added at the indicated concentration; in C higher trace corresponds to absence of NO, and other traces correspond to increasing NO concentrations as indicated in A and B.

- The reaction time of the interaction between NO and COX, which is independent of the interaction of O_2 with COX, even if NO and O_2 compete for the same site. The kinetics of the reaction of NO binding with COX is given by $k_{NOon}\times[NO]\times[Fe_{a3}^{2+}\text{-}Cu_B^+]$, and so the pseudo-first order rate constant that contains the information on how fast $[Fe_{a3}^{2+}\text{-}Cu_B^+]$ is removed from the catalytic cycle by NO causing COX inhibition is given by $k_{NOon}\times[NO]$; the reaction time is simple the inverse given by $1/(k_{NOon}\times[NO])$. At a $[NO] = 1\times10^{-8}$ M, a time scale of approximately 1 s is obtained, with higher NO levels giving faster inhibitions similar to what is observed experimentally (15; 17).

- The reaction of time of the catalytic COX cycle. Because only a minor fraction of COX is present as the fully reduced Fe_{a3}^{2+}-Cu_B^+ center, the onset of inhibition triggered by binding of NO to this center will be dependent on how fast the various intermediates of COX are converted into Fe_{a3}^{2+}-Cu_B^+. This value is given by the rate constant k_{IV} in Figure 1, and so a time scale of $1/7.5$ s^{-1} = 0.13 s or $1/0.75$ s^{-1} = 1.3 s is obtained, respectively in states 3 or 4, under conditions *in vivo*.

This time scale analysis constitutes an approximation because the actual time for the onset of inhibition will be slower due to the competition with O_2. But the main conclusion is confirmed by carrying out simulations with the extended minimal model described in Figure 1C; in Figure 3 the experiment carried out in reference (17) with isolated COX is simulated. The inhibition of the oxidation of cytochrome *c* by NO and the formation of the Fe_{a3}^{2+}-Cu_B^+-NO complex are shown to occur within one tenth of a second after exposure to NO, as observed experimentally. Therefore, the fast inhibition of COX by NO is compatible with a direct competition between NO and O_2 for COX.

5.3. The fully reduced Fe_{a3}-Cu_B site is not sufficiently populated during the cytochrome oxidase catalytic cycle

During the catalytic cycle of COX, only a tiny fraction of COX is present as the reduced Fe_{a3}-Cu_B site, being most of COX present either as the fully oxidized site or as a one-electron reduced site. It is concluded that NO would exert its action through the more populated forms of COX. As mentioned before, the effect of NO through these sites cannot lead to an inhibition that is competitive with O_2, since O_2 either does not bind or does it very slow to these sites, and the inhibition would be uncompetitive. Moreover, the competition of NO with O_2 for COX does not depend on the fraction of COX that is as the fully reduced Fe_{a3}-Cu_B, as can be observed from equations 2 and 3. This can be intuitively understood if one notes that both O_2 and NO compete for the same low-abundant site. In Figure 3C, the percentage of the various forms of COX obtained by simulation of the model of Figure 1C, for various NO concentrations is shown. It can be observed that in the fully reduced Fe_{a3}-Cu_B site constitutes only a minor fraction, lower than 1 %, of total COX as predicted. In spite of that, this model reproduces most aspects of COX inhibition by NO. Therefore, the argument of the low occupancy of the fully reduced Fe_{a3}-Cu_B site does not stand against a rigorous evaluation of the COX dynamical behavior.

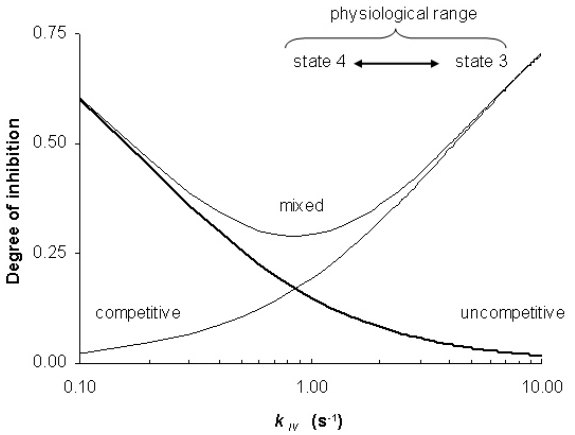

Figure 4. Comparison of the relative importance of the binding (competitive inhibition) versus reaction of NO (uncompetitive inhibition) with the Fe_{a3}-Cu_B site. Mixed inhibition simulation curve is obtained by solving with Gepasi (54) the model shown in Figure 1D with the following parameters: $k_{NOon} = 1\times10^8$ $M^{-1}s^{-1}$; $k_{NOoff} = 0.01$ s^{-1}; $k_{O2app} = 1.4\times10^8$ $M^{-1}s^{-1}$; $k_{NOreac} = 2\times10^5$ $M^{-1}s^{-1}$; $[O_2]$=30 μM; [NO]=100 nM; $[COX]_{tot}$ = 140 μM; k_{IV} as indicated and β=0.1. Competitive and uncompetitive simulation curves are obtained by setting k_{NOreac} and k_{NOon} to zero, respectively.

The simple reaction cycle constituted by four reactions shown in Figure 1C represents a paradigmatic example where an intuitive reasoning is not sufficient to achieve a complete understanding of the dynamics of the system: the cyclic topology introduces a non-linearity that is not grasped by the intuitive reasoning. The use of a mathematical model proved to be essential to completely understand the control structure of cycle, namely that the degree of inhibition of COX increases with an higher rate of electron flow through COX, a prediction that was recently confirmed experimentally (30).

5.4. Inhibition of COX by NO: a case of mixed inhibition

The picture arising by analysis of Table 2 is that at low electron flows the uncompetitive mechanism could be operative while as the electron-flux increases the competitive mechanism predominates. Recently the mathematical model by Antunes et al was extended to include the uncompetitive binding (30). Both models were confronted with experimental data obtained with isolated COX: for high-electron fluxes both models give good fittings while for low electron fluxes the extended model gives a better fitting (30). This points to a picture where the inhibition of COX by NO is mixed but where the competitive component predominates, explaining why a competitive nature of inhibition between NO and O_2 is observed experimentally (30). In Figure 4, simulations of the inhibition of COX by NO explore three possible models of NO inhibition; while for very low electron fluxes the uncompetitive component is important, at the predicted physiological electron fluxes the competitive component constitutes the major mechanism yielding NO inhibition.

6. EFFECTS OF NO IN STATE 3 AND STATE 4 RESPIRATION

One of the puzzling questions about NO effects on mitochondria is that state 3 respiration is much more sensitive to NO inhibition than state 4 respiration (32). This experimental observation was never satisfactorily explained and was tentatively attributed to a lower control exerted by COX on the mitochondrial respiration at low rates of respiration (32). Recently, this explanation found experimental support by showing that for the same level of COX inhibition the rate of mitochondrial respiration was more inhibited at state 3 than at state 4 (33). However the difference found was relatively small, and it does not explain the complete insensitivity of state 4 observed in some conditions.

In reference (6) it was shown that the higher sensitivity of state 3 towards NO inhibition is explained by the higher turnover of COX in this state. This higher turnover is expressed by an higher value of the rate constant k_{IV} yielding therefore an higher apparent dissociation rate of O_2 from COX, explaining the higher apparent affinity of NO in state 3 compared to state 4. Here we expand that study to analyse in more detail the control structure underlining the higher sensitivity in state 3. To that end we used the model shown in Figure 1C in which cytochrome c is explicitly considered. With this model, for the same level of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct, the degree of inhibition of the rate of respiration is higher in state 3 than in state 4 (Figure 5A), indicating that at state 3 respiration is more sensitive to COX inhibition than in state 4, *i.e.* COX exerts a higher control over respiration in state 3, as observed experimentally (33). Moreover, the level of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct (*i.e.* the level of COX inhibition), is higher in state 3 than in state 4 for the same concentration of NO (Figure 5B). This last prediction was not observed experimentally (33), but at the low O_2 pressures used experimentally, simulation predicts that the differences between the degree of inhibition of COX at state 3 and at state 4 would be hard to distinguish (not shown). In conclusion, both the higher formation of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct, and the higher control exerted by COX over respiration cause the higher sensitivity of state 3 over state 4 respiration.

It is of interest to quantify the relative importance of these two factors in justifying the different sensitivities observed. To achieve this aim, a rigorous control analysis is needed (34). The decomposition of the dependency of the rate of respiration on the two components identified above is given by the following equation, which accounts for the logarithmic sensitivity of the rate of respiration on the concentration of NO, *i.e.* it gives the percentile variation of the rate of respiration as a function of a percentile variation in the concentration of NO.

$$\frac{d\log(V_{O_2})}{d\log([NO])} = \frac{\partial\log(V_{O_2})}{\partial\log([Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO])} \cdot \frac{\partial\log([Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO])}{\partial\log([NO])} \Leftrightarrow$$

$$S(V_{O_2},[NO]) = S(V_{O_2},[Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO]) \cdot S([Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO],[NO])$$

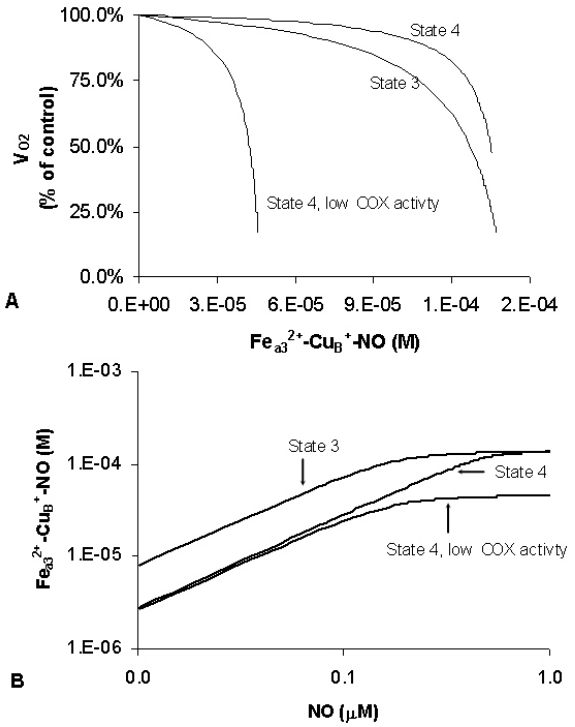

Figure 5. Relationship between rate of respiration, concentration of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct, and NO concentration at states 3 and 4 of mitochondrial respiration. The same model used in Figure 3 was run with the following parameters: $k_{NOon} = 4\times10^7$ $M^{-1}s^{-1}$; $k_{NOoff} = 0.01$ s^{-1}; $k_{O2app} = 1.4\times10^8$ $M^{-1}s^{-1}$; $k_{IV} = 1.5\times10^6$ $M^{-1}s^{-1}$; $k_{III} = 23.3$ s^{-1} (state 3); $k_{III} = 7.77$ s^{-1} (state 4); $[O_2]$=150 μM; $[\text{Cyt C}]_{tot}$ = 200 μM; normal levels of COX were 140 μM, while low COX activity was simulate by setting $[\text{COX}]_{tot}$ = 46.7 μM.

In Figure 6, it can be observed that at low NO concentrations when the dependency of V_{O2} on [NO] is low (i.e., $S(V_{O2}, [NO])\approx0$) the dependency of Fe_{a3}^{2+}-Cu_B^{+}-NO adduct on [NO] is high (i.e., $S([Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO], [NO])\approx1$), while the dependency of V_{O2} on $[Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO]$ is also low (i.e., $S(V_{O2}, [Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO])\approx0$). In addition, only when the dependency of V_{O2} on $[Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO]$ becomes significantly different from zero, is V_{O2} inhibited by NO. In other words, the component that is responsible for the higher sensitivity of state 3 respiration than that of state 4 respiration is $S(V_{O2}, [Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO])$, i.e. the control exerted by COX on the overall respiration. However, this does not mean that the different levels of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct observed in state 3 and state 4 are irrelevant for the observed pattern of inhibition. Actually, the reason why the $S(V_{O2}, [Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-}NO])$ becomes significantly different from zero at higher levels of NO at state 4 compared to state 3, is because the % of COX in the form of adduct is lower in state 4 than in state 3, which is a consequence of the lower turnover of COX at state 4. In fact, if COX activity is decreased, then the pattern of inhibition observed in state 4 with a low COX activity, becomes exactly the same as that observed in state 3 with an higher COX activity (Figures 5 and 6), showing that the critical factor for the pattern of inhibition is the % percentage of COX that is bound to NO.

In summary, a rigorous sensitivity analysis showed that while the inhibition of respiration is triggered when the overall rate of respiration becomes sensitive to COX inhibition, the cause for different thresholds values for NO in sate 4 and in state 3 is the formation of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct.

7. PHYSIOLOGICAL AND PATHOLOGICAL RELEVANCE

The biological role of NO was first identified as a second messenger and as an activator of guanylate cyclase. Guanylate cyclase is even more sensitive to NO than COX oxidase having half-maximal activation responses in the 4-20 nM range (35-37). Therefore, there are two operative concentrations ranges for NO that do not overlap or overlap moderately (6; 37): below 25-50 nM NO activates NO cyclase without significantly inhibiting COX, and for higher concentrations NO exerts its inhibitory role on the respiratory chain. It should be mentioned that the so called excess activity of COX (38) is not excessive in the presence of low *in vivo* concentrations of NO, and actually the observed high COX activity is important to avoid an excessive overlap between these two roles of NO (6). In fact when there is a decrease in COX activity, such as those observed in COX deficiency diseases (39), concentrations of NO that normally have a regulatory role may be deleterious by causing an excess inhibition of COX (40).

The sensitivity of respiration to NO is fully compatible with the levels of NO observed *in vivo*, which are in the range 10 nM to 5 μM (16). Because NO diffuses very fast across biomembranes and these do not constitute a barrier (41), it is usually assumed to be irrelevant whether NO has a mitochondrial or an extra-mitochondrial origin, as NO will equilibrate quickly among sub-cellular compartments. Nevertheless, in spite of ongoing discussions (42) there is now plenty of evidence for the existence of the mitochondrial NO synthase (43-47). Moreover, recently NO synthase was shown to co-localize and to interact with COX in the inner mitochondrial membrane, suggesting that NO could diffuse directly from NO synthase into its target inhibitory protein COX (48), thus establishing a basis for an NO sub-cellular compartmentalization overcoming the high permeability of this gas across biomembranes.

8. COMMENTS AND PERSPECTIVES

On a final comment on regulation, it has been suggested that the alosteric inhibition of COX by ATP could work as an alternative mechanism to the classic proton-motive force for the control of the respiratory chain avoiding the build up of a high membrane potential (49; 50), which triggers the onset of free radical production by the respiratory chain (51). The stimulation of the mitochondrial NO synthase by a high membrane potential (52), and the physically interaction of NO synthase with COX (48), both recently observed, constitute the basis for a feed-back loop involving the mitochondrial production of NO under control of the membrane potential, and the generation of this potential under control of NO, via inhibition of COX.

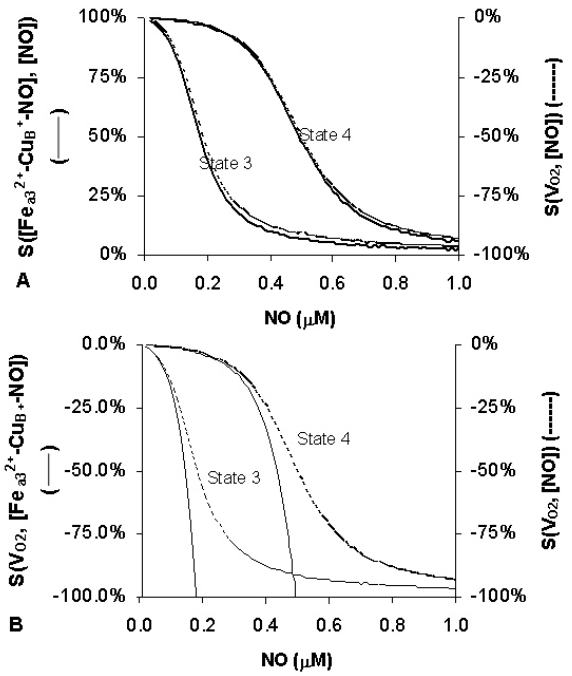

Figure 6. Relative importance of the Fe_{a3}^{2+}-Cu_B^{+}-NO adduct formation - $S([Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-NO}],[NO])$ - and of the control exerted by COX on the respiratory chain – $S(V_{O2}, [Fe_{a3}^{2+}\text{-}Cu_B^{+}\text{-NO}])$ – on the inhibition of respiration by NO - $S(V_{O2}, [NO])$. State 4 respiration at low COX activity gives curves identical to the indicated state 3 curves. Parameters as in Figure 5.

The operation of such feed-back loop would constitute an elegant way of controlling the respiratory chain and avoiding the onset of superoxide production through the build up of a high membrane potential.

9. ACKNOWLEDGEMENTS

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Abbreviations and definitions: COX, cytochrome oxidase, IC50, the concentration that reduces a specified response by half, VO_2: Rate of mitochondrial respiration

Key Words: Mitochondria, Respiration, Competitive Inhibition, Uncompetitive Inhibition, Mathematical Model, Cytochrome C Oxidase, Deficiency, Diseases, Excess, Capacity, Cytochrome C, Oxidase, Review

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