CMLS, Cell. Mol. Life Sci. 60 (2003) 1752–1759
1420-682X/03/081752-08
DOI 10.1007/s00018-003-3127-3

CMLS Cellular and Molecular Life Sciences

Research Article

Control of respiration by nitric oxide in Keilin-Hartree particles, mitochondria and SH-SY5Y neuroblastoma cells

D. Mastronicola [a], M. L. Genova [b], M. Arese [c], M. C. Barone [c], A. Giuffrè [d], C. Bianchi [b], M. Brunori [c], G. Lenaz [b] and P. Sarti [c,*]

[a] IFO, Cancer Institute Regina Elena (SSD – SAFU), 00100 Rome (Italy)
[b] Department of Biochemistry, University of Bologna, 40126, Bologna (Italy)
[c] Dipartimento di Scienze Biochimiche 'A. Rossi Fanelli', Università di Roma 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome (Italy), Fax +39 06 4440062, e-mail: paolo.sarti@uniroma1.it
[d] CNR Institute of Molecular Biology and Pathology, University of Rome 'La Sapienza', 00185 Rome (Italy)

Received 1 April 2003; received after revision 22 May 2003; accepted 3 June 2003

Abstract. The pattern of cytochrome c oxidase inhibition by nitric oxide (NO) was investigated polarographically using Keilin-Hartree particles, mitochondria and human neuroblastoma cells. NO reacts with purified cytochrome c oxidase forming either a nitrosyl- or a nitrite-inhibited derivative, displaying distinct kinetics and light sensitivity of respiration recovery in the absence of free NO. Keilin-Hartree particles or cells, respiring either on endogenous substrates alone or in the presence of ascorbate, as well as state 3 and state 4 mitochondria respiring on glutamate and malate, displayed the rapid recovery characteristic of the nitrite derivative. All systems, when respiring in the presence of tetramethyl-p-phenylenediamine, were characterised by the slower, light-sensitive recovery typical of the nitrosyl derivative. Together the results suggest that the reaction of NO with cytochrome c oxidase in situ follows two alternative inhibition pathways, depending on the electron flux through the respiratory chain.

Key words. Reaction mechanism; NO scavenging; haemoprotein; tumour cells; control of respiration.

Under turnover conditions, mitochondrial cytochrome c oxidase (CcOX) promptly reacts with nitric oxide (NO), causing a transient inhibition of respiration, which reverts upon removal of free NO on a time scale compatible with physiology (seconds or less) [1–3]. Owing to the pathophysiological relevance of this reaction [4–8], the interaction between CcOX and NO has been under active investigation since its discovery [for reviews see refs. 9–12].

NO reacts with the binuclear active site of purified reduced CcOX with the formation of a nitrosyl [a_3^{2+}–NO] complex [13, 14], or with oxidised CcOX, with the formation of a nitrite [a_3^{3+}–NO_2^-] derivative [15, 16]. The reactions and the relative kinetic parameters, obtained at 20 °C using purified beef heart CcOX, are as follows:

Nitrosylation:

$$Cu_B^+ a_3^{2+} + NO \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathbf{Cu_B^+ a_3^{2+} - NO} \quad (1)$$

where $k_1 = 0.4–1 \times 10^8$ M^{-1} s^{-1} [17] and $k_2 = 4 \times 10^{-3}$ s^{-1} [18].

Nitrite formation:

$$Cu_B^{2+} a_3^{3+} + NO \xrightarrow{k_1} Cu_B^+ NO^+ a_3^{3+} \overset{k_2}{\underset{OH^-}{\nearrow}} \mathbf{Cu_B^+ a_3^{3+} NO_2^-} + H^+ \quad (2)$$

where $k_1 = 10^4 – 10^5$ M^{-1} s^{-1} and $k_2 \gg k_1$, [15, 16].

Although with different kinetics, both inhibited states recover activity fully by releasing bulk NO and nitrite, re-

* Corresponding author.

spectively. Starting from the reduced enzyme, the nitrosyl adduct is formed very rapidly, but dissociates very slowly ($k_{off} = 1 \times 10^{-2}$ and 4×10^{-3} s^{-1}, at 37 and 20 °C, respectively [18]), the functional recovery occurring over a time scale of many seconds to minutes. On the other hand, under turnover conditions, the nitrite derivative promptly recovers activity upon reduction of the active site, with bulk nitrite release [11, 15].

From the experimental point of view, another useful difference between the two reaction products is the light sensitivity of the nitrosylated CcOX [19], a feature not shared by the nitrite adduct of the ferric haem iron [20]. Thus, in the absence of free NO, only in the former case does illumination accelerate the recovery of activity by increasing the rate of NO dissociation from the enzyme. This unequivocal difference in photochemistry allowed Sarti et al. [18] to show that purified CcOX can be inhibited by either mechanism, depending on experimental conditions. Based on these experiments and on computer simulations [16], they proposed that one inhibition pathway may prevail over the other depending on the CcOX turnover intermediates populated[1], and thus on the experimental parameters controlling their occupancy, particularly the rate of electron transfer to the enzyme.

To assess the occurrence in situ of these two inhibition mechanisms, we performed experiments on Keilin-Hartree (KH) particles, coupled mitochondria and cells. The SH-SY5Y cell strain of the human neuroblastoma was selected, because the respiratory chain of these tumour cells has been shown to be sensitive to either exogenously or endogenously provided NO [21, 22].

Materials and methods

Materials

Sodium ascorbate, tetramethyl-p-phenylenediamine (TMPD) and cell culture medium were from Sigma (St. Louis, Mo.). Stock solutions of NO (Air Liquide, Paris, France) were prepared equilibrating degassed water with the pure gas at 1 atm and 20 °C ([NO] = 2.1 ± 0.1 mM). Human neuroblastoma cells SH-SY5Y, a kind gift of Dr. T. J. J. Blanck (New York University, N. Y), were cultured at 37 °C under 5% CO_2. Culture medium was RPMI containing 12% fetal calf serum.

KH particles were obtained at early stages of CcOX purification from beef heart, according to the procedure of Soulimane and Buse [23] prior to detergent solubilisation, and suspended in 0.1 M N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), pH 7.3. The concentration of mitochondrial proteins was evaluated using the biuret method. The concentration of CcOX was evaluated spectroscopically using $\Delta\varepsilon_{(red\text{-}ox)} = 24\ mM^{-1}\ cm^{-1}$ (at 605 nm, per functional unit [24]).

Liver mitochondria were prepared from either the mouse (1 to 2 month-old male Balbc), or rat (4-month-old male Wistar albino). Animals were bred under microbiological barrier conditions and used within 2 weeks after arrival, being kept under conditions of constant temperature, humidity, and a 12-h light-12-h dark cycle. Animals were sacrificed, following veterinary and local bioethical committee control guidelines, by cervical shock and decapitation. Mitochondria were isolated from livers according to the procedure of Kun et al. [25], with minor modifications. The values of the respiratory control ratio (RCR) [26] varied between 3.0 and 6.0. The steady-state reduction level of the $c + c_1$ cytochromes was determined with a dual-wavelength spectrophotometer (Jasco V550), at the wavelength couple 550–540 nm. Mitochondria were suspended in 0.1 M KH_2PO_4, pH 7.5 at a protein concentration of 3.3 mg protein/ml and state 3 was induced by 1.1 mM ADP. Data were expressed as percentage reduction by assuming the cytochromes to be fully oxidised and fully reduced in the presence of excess ferricyanide and dithionite, respectively.

Polarographic measurements

Oxygen consumption was measured with a Clark-type electrode (YSI Model 5300, Yellow Springs Instruments Co., Yellow Springs, Ohio). The experiments were carried out either in the dark or under bright (heat-filtered) white-light illumination (150 W tungsten lamp) [18], to probe the accumulation of the nitrosyl adduct. Following Sarti et al. [18], conditions were set to allow inhibition by NO (1–6 µM, as specified in the figure legends) to occur in the dark. Afterwards, free NO was scavenged by addition of excess human oxy-haemoglobin (HbO_2), and the time course of respiration recovery followed. Whenever recovery was slow, as expected for the nitrosylated CcOX, the sample was illuminated to verify its light sensitivity, manifested as a rapid and complete restoration of the respiratory rate. Measurements were carried out at 20 °C, since at this temperature and in the dark, NO dissociation from the nitrosyl derivative can be accurately followed, being much slower than the electrode response ($k_{NO\text{–}off} = 4 \times 10^{-3}\ s^{-1} < k'_{O2\text{–}electrode} \approx 0.5\ s^{-1}$). At 20 °C, the endogenously sustained O_2 consumption rate of the SH-SY5Y cells is rather slow, about 0.2–0.5 nmol O_2 min^{-1} per 10^7 cells, but in the presence of 10 mM ascorbate, this value rises to ~1.5–2.0 nmol O_2 min^{-1} per 10^7 cells. When necessary, TMPD was added to further increase the respiratory rate. The extent of inhibition at

[1] A number of catalytic intermediates of the CcOX binuclear active site (cytochrome a_3-Cu_B) have been described. According to an oversimplified view, starting from the fully reduced site (R), these are in sequence: the (so-called) peroxy (P), the ferryl (F), and the fully oxidised (O) intermediates. R is regenerated from O by two single-electron reduction processes, and thus transient formation of a single-electron reduced binuclear site, species E, is expected.

a given time after HbO_2 addition (20–30 s) was calculated from the ratio R between the rate measured at that time, divided by the rate before inhibition. R was used to draw a plot of percent inhibition, under all conditions investigated.

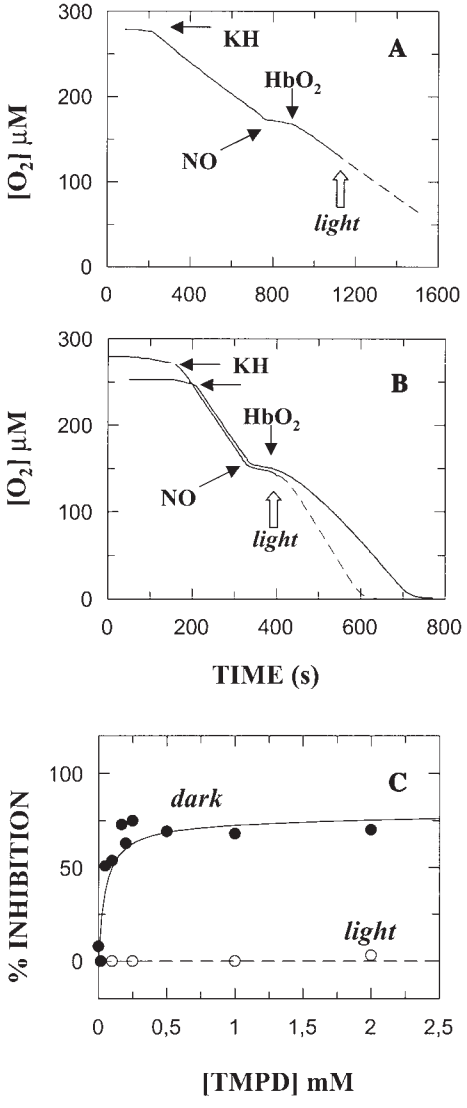

Figure 1. Respiration recovery of NO-inhibited KH particles. KH particles were allowed to respire on 10 mM ascorbate alone (*A*) or also in the presence of 2.0 mM TMPD (*B*). [CcOX] = 280 nM aa_3. Buffer: 0.1 M HEPES pH = 7.3. O_2 consumption was inhibited by addition of 2 µM NO, in the dark; afterwards, free NO was scavenged by addition of 4 µM HbO_2 (haem basis), and the kinetics of respiration recovery were followed either in the dark (solid trace) or under bright illumination (dashed trace). The light was switched on at the open arrow either during a single trace (*A*) or on an independent one (*B*). In the dark, recovery was immediate after HbO_2 addition when respiration was sustained by ascorbate alone (*A*), whereas in the presence of TMPD (*B*), it showed a pronounced lag phase (solid trace), not observed under illumination (dashed trace). Experiments were repeated at different [TMPD]; the percentage of inhibition at ~30 s after HbO_2 addition was calculated in the dark (closed circles) or under illumination (open circles) (*C*).

Results

KH particles

We have studied the KH particles respiratory response to NO inhibition using the experimental design employed with CcOX in solution [18]. As shown in figure 1 A, when respiration of KH was sustained by ascorbate alone, upon addition of 2 µM NO, respiration was promptly inhibited and, after NO scavenging by excess HbO_2, the recovery of respiration was immediate and complete in the dark, illumination being ineffective. In contrast, in the presence of 2 mM TMPD with a respiration rate accelerated five- to sevenfold, the recovery process that followed HbO_2 addition occurred with a lag time in the dark (solid trace), and more rapidly under illumination (dashed trace) (fig. 1 B). The experiments were repeated in the presence of increasing amounts of TMPD and the percentage of CcOX inhibition at ~30 s after HbO_2 addition is reported in figure 1 C. In the dark, the extent of the lag time after HbO_2 addition increased hyperbolically with [TMPD], in such a way that at ~30 s respiration was still ~75% inhibited in the presence of ~0.25 mM TMPD. The inhibition was rapidly and completely released under illumination, suggesting that in the presence of TMPD the light-sensitive nitrosylated adduct of CcOX is predominant. The same experiments repeated in the presence of 0.1% lauryl-maltoside, a mild detergent causing no harm to CcOX [23], gave identical results (not shown), excluding any interference due to a non-homogeneous CcOX distribution within KH particles.

Coupled mitochondria

We investigated the kinetics of recovery from NO inhibition of coupled mitochondria respiring on glutamate and malate. As shown in figure 2, the onset of and recovery from inhibition of state 3 and state 4 mitochondria were similar, at least at first glance (fig. 2 A, C). Upon addition of 5 µM NO, mitochondrial respiration was promptly inhibited, but also rapidly restored, even in the dark, following the addition of excess HbO_2, a behaviour fully compatible with the predominant accumulation of the nitrite derivative. Interestingly, and consistent with previous reports [27], at [NO] ≤ 1 µM, state 3 mitochondria appeared more prone to inhibition than state 4 mitochondria (data not shown). Under otherwise identical conditions, the NO concentration required to fully inhibit respiration of state 4 mitochondria was always higher than that necessary to inhibit state 3 mitochondria. Similar to KH particles, in the presence of ascorbate, the addition of TMPD to state 3 or state 4 mitochondria induced an approximately tenfold increase in the respiration rate. Under these experimental conditions, the respiration recovery time course presented a pronounced lag phase, which disappeared under illumination (see fig. 2 B, D).

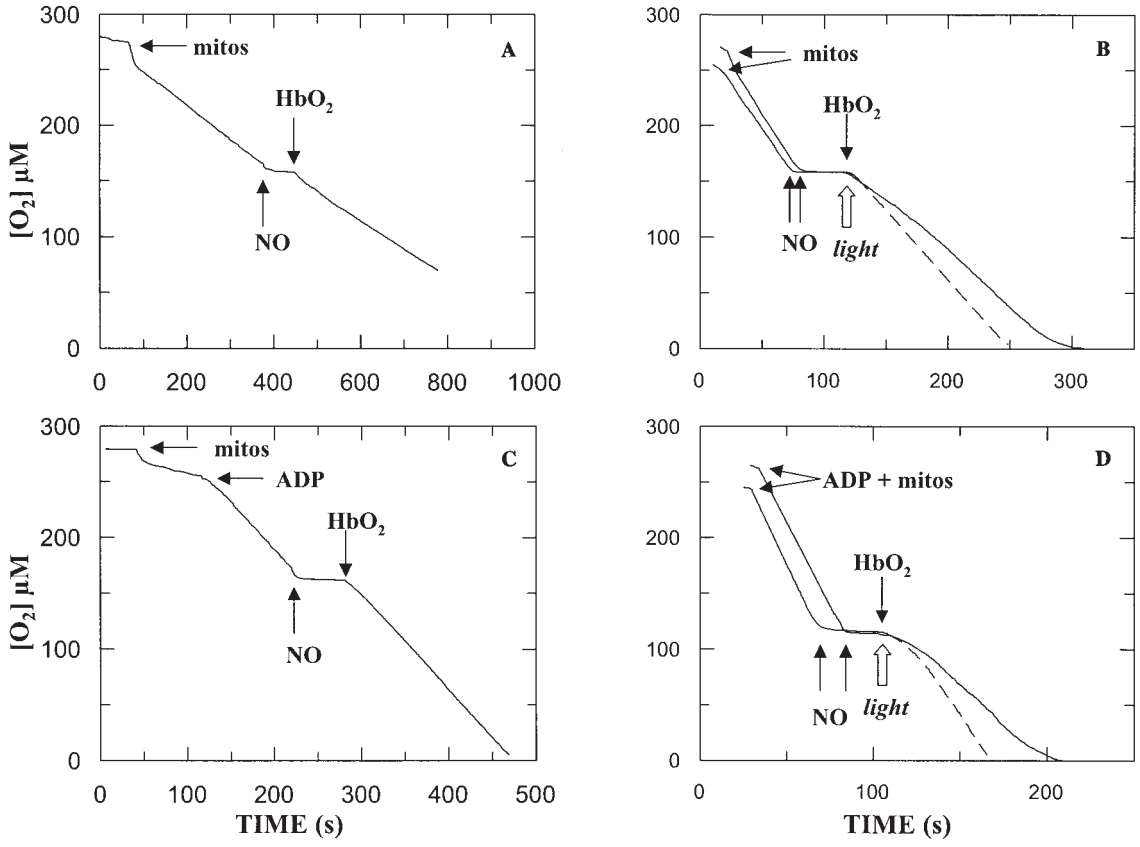

Figure 2. Respiration recovery of NO-inhibited mitochondria. State 4 (*A*) or state 3 (*C*) mitochondria were allowed to respire in a medium (10 mM KH_2PO_4, 50 mM HEPES, 4 mM $MgSO_4$, 250 mM sucrose, 0.5 mM EDTA, pH = 7.4), containing 30 mM glutamate and 30 mM malate. Total mitochondrial protein: 5 mg ml^{-1} (*A*, *C*). The state 4 to state 3 transition was achieved by addition of 2.3 mM ADP, yielding an RCR ≈ 4.0. Inhibition of respiration was induced by addition of ~ 5 µM NO and reversed by addition of 15 µM HbO_2 (haem basis). (*B*, *D*) Mitochondria in state 4 and 3, respectively, were assayed in the presence of 10 mM ascorbate and 4 mM TMPD. Since under these conditions, the rate of O_2 consumption is increased, the total mitochondrial protein concentration was lowered to 1.5 mg ml^{-1}. (*A*–*D*) As in figure 1, solid traces refer to dark, dashed to light, switched on at the open arrow.

The level of reduction of the cytochrome c and c_1 was also measured using mitochondria in different functional states (2, 3 and 4), respiring on different reducing substrates (glutamate/malate alone or plus ascorbate/TMPD); data are summarised in table 1. In the presence of glutamate and malate (even a large excess, 30 mM), the cytochrome c/c_1 couple was only ~ 30% reduced; the reduction level was not significantly affected by the state 4 to state 3 transition, whereas it increased substantially on addition of TMPD (up to ~ 90% at 6 mM TMPD).

Table 1. Reduction levels of the cytochromes c and c_1 at steady-state in respiring mitochondria.

Mitochondria functional state	Reducing substrate	Cytochromes $c+c_1$ steady-state reduction (%)
2	endogenous	11 ± 5
4	glutamate-malate	29 ± 8
3	glutamate-malate	27 ± 5
4	Asc/TMPD 1 mM	57 ± 6
4	Asc/TMPD 2 mM	68 ± 5
4	Asc/TMPD 4 mM	68 ± 5
4	Asc/TMPD 6 mM	91 ± 10

Mitochondrial respiration was sustained by different reducing substrates, in the absence and in the presence of ADP, in order to induce the indicated different functional states. The reduction level of the cytochromes was calculated as percent of the maximal Δ absorbance at 550–540 nm (dithionite-ferricyanide), determined independently at the end of each measurement. Asc, ascorbate.

Cultured cells

SH-SY5Y cells respiring on endogenous substrates and ascorbate were exposed to a pulse of NO; 1–2 µM NO promptly and fully inhibited respiration, though addition of excess HbO_2 was followed by an immediate functional recovery (even in the dark, see figure 3A). The appearance of a slower recovery and light sensitivity was also induced in SH-SY5Y cells by adding TMPD (figure 3B), which enhanced the rate of respiration by about tenfold up

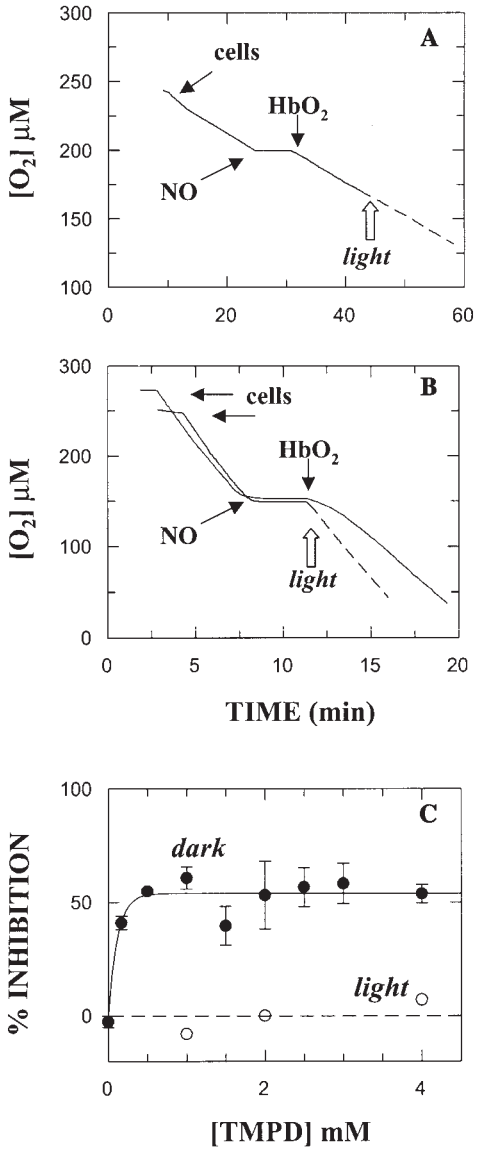

Figure 3. Respiration recovery of NO-inhibited neuroblastoma cells. Cultured human neuroblastoma cells (5×10^7 cells ml^{-1}) were allowed to respire in the presence of 30 mM ascorbate alone (*A*) or also in the presence of 4 mM TMPD (*B*). Buffer: PBS + 10 mM glucose + 1 mM $CaCl_2$. [NO] = 2 µM. [HbO_2] = 4 µM (haem basis). In the absence of TMPD (*A*), respiration recovery after HbO_2 addition was immediate and light insensitive, whereas in the presence of TMPD (*B*), recovery was much slower and light sensitive. (*C*) Percentage of CcOX inhibition at ~30 s after HbO_2 addition, as calculated at increasing [TMPD] either in the dark (closed circles) or under illumination (open circles). (*A, B*) As in figure 1, the light was switched on at the open arrow either during a single trace (*A*) or on an independent one (*B*).

to a saturation value of ~15 nmol O_2 $min^{-1} \times 10^7$ $cells^{-1}$. Thus, cells in the presence of TMPD also displayed a light-sensitive kinetics of recovery typical of the nitrosylated adduct. To evaluate the extent to which the concentration of reductant controls the mechanisms of inhibition, the concentration of TMPD was varied systematically. As shown in figure 3C, in the absence of or at low TMPD, recovery from inhibition was quite prompt and the effect of light negligible, whereas at higher [TMPD] (> 0.5 mM), the recovery was slower and the effect of light maximal.

Discussion

In addition to having multiple regulatory functions, NO has been recognised as a potential signalling molecule controlling cell respiration [9–12]. The mitochondrial respiratory chain is indeed rapidly, though reversibly, inhibited by NO in the low micromolar range and, interestingly, NO is also likely produced by a mitochondrial NO synthase [ref. 28 and references cited therein]. The fast onset of inhibition and the competition with O_2 point to CcOX as the main target of the interaction between NO and mitochondria; thus the mechanism of inhibition of CcOX by NO has been extensively investigated over the past decade [1–3, 11, 13, 14]. In a previous report [18], carried out with purified soluble CcOX, two different reaction pathways involving the formation of either a nitrosyl or a nitrite CcOX derivative were demonstrated. In the work presented here, we show that both these pathways are also operative in situ, when the enzyme is membrane integrated as in KH particles, coupled mitochondria and intact cells. Additionally, we provide evidence that, similar to the purified enzyme, the rate of electron transfer to CcOX dictates the predominance of one mechanism over the other.

Following removal of free NO by excess HbO_2, recovery of respiration in the dark can occur either immediately, i.e. the process is not time resolved in the O_2-electrode, or more slowly with a lag phase. The former behaviour is characteristic of the nitrite-inhibited CcOX, whereas the latter indicates accumulation of the nitrosylated enzyme [18]. The slow autocatalytic pattern is induced by increasing the concentration of reductants {cytochrome c or TMPD (this work)} and is affected by light, which abolishes the lag. On the other hand, an immediate recovery compatible with the oxidation of NO to nitrite is observed at low reductant concentration [18]. Thus, the different kinetic behaviour and the unique photosensitivity of one adduct [19] allow one to distinguish between the accumulation of the nitrosyl ($a_3^{2+}NO\,Cu_B^{+}$) and the nitrite ($a_3^{3+}NO_2^{-}Cu_B^{2+}$) derivative. To experimentally distinguish between the two derviatives, we monitored the kinetics of recovery from NO inhibition in KH particles, coupled mitochondria and intact cells, after scavenging free NO with HbO_2.

At every integration level (KH, mitochondria or cells), respiration in the presence of endogenous reductants was associated with a rapid recovery from NO inhibition, compatible with the nitrite reaction pathway. A similar kinetic

behaviour has already been reported for state 3 mitochondria by Shiva et al. [29], and confirmed here for state 4 mitochondria, respiring on glutamate and malate (from ≤ 1 mM up to 30 mM each). Only upon addition of TMPD, bypassing the respiratory chain at the level of complex III [30], did the slow and light-sensitive recovery appear in all systems. This finding confirms that the rate of electron transfer to CcOX, and thus the level of reduction of cytochrome c, controls the predominance of the inhibition mechanism [18]. In isolated mitochondria, the effect of TMPD on the redox level of cytochromes $c + c_1$ was directly assessed by absorption spectroscopy (see table 1) and proved compatible with this hypothesis.

Thus, our data provide clear evidence for the predominance of the inhibition mechanism likely producing nitrite in the active site, under a (relatively) low, physiological regime of electron flux at the level of complex IV. This original finding may help in the interpretation of several observations, such as the finding by Giulivi [31] that the production of nitrite/nitrate is enhanced by supplementing with succinate submitochondrial particles producing endogenous NO. Moreover, it is consistent with a more recent report [32] showing a fast production of nitrite in single cardiomyocytes electrically stimulated to produce endogenous NO. Finally, the involvement of CcOX in the stationary degradation of NO to nitrite provides a possible explanation for the observation that the NO inhibition of mitochondrial respiration is more effective and persistent at lower O_2 concentrations; even more so, when the recovery of respiration is allowed to occur in the absence of external NO scavengers [see for example fig. 4 in ref. 29 or fig. 2 in ref. 33]. One must take into consideration that at lower O_2 concentrations, NO is indeed more stable, and thus available for a longer time to the CcOX-catalysed degradation into nitrite, thereby accounting for a more persistent inhibition of the oxidase activity. In contrast to the nitrosyl enzyme, the inhibition pathway leading to the nitrite derivative is insensitive to O_2 concentration, being produced via the reaction between NO and oxidised Cu_B (which does not bind O_2 [34]). An understanding of the effect of O_2 on the NO inhibition mechanisms, particularly in the low $[O_2]$ region, is clearly needed, and further experiments are being carried out to clarify this point.

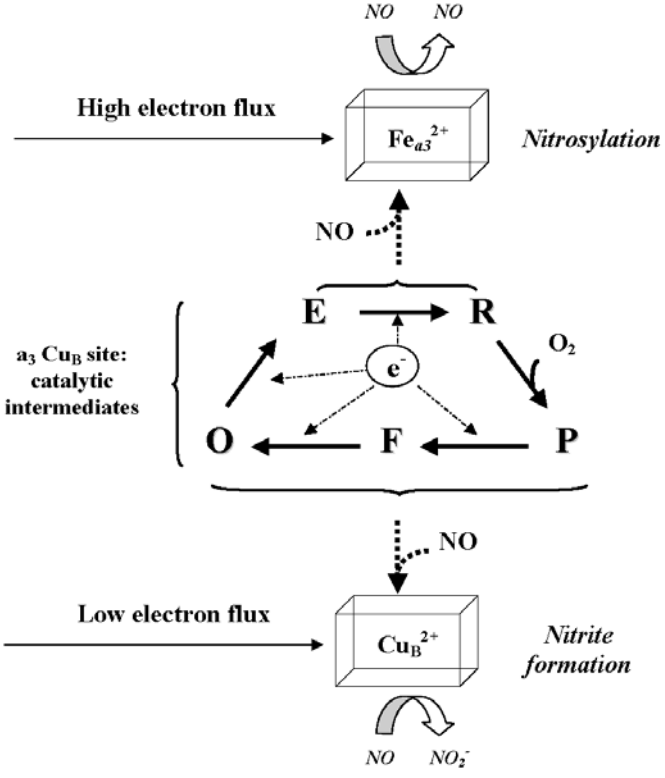

Figure 4. The two NO inhibition pathways of cytochrome oxidase. Schematic representation of the reaction of NO with the turnover intermediates of the binuclear centre of CcOX populated at different electron flow levels of the respiratory chain. Based on computer simulations [16], the relative concentration of reduced intermediates (R and E) tends to rise at higher reduced cytochrome c concentrations, whereas intermediates P and F prevail at lower concentrations. Interestingly, the half-reduced intermediate E seems to react with NO [37]. Thus at higher reductant concentration, reduced cytochrome a_3 is the primary target with formation of a stable nitrosylated adduct ($a_3^{2+}-NO$), whereas at lower reductant concentration, Cu_B^{2+} is the target and the nitrite derivative ($a_3^{3+}NO_2^-Cu_B^{2+}$) is formed. For clarity, only the putative target metal in the CcOX active site and steps of the catalytic cycle, where single-electron-donations to the site occur, are represented; protonation steps and water formation are omitted.

Borutaitè and Brown [27] reported that NO inhibition is more efficient in state 3 than in state 4 because the CcOX control coefficient over respiration was higher in state 3 than in state 4 [27, 35]. Our results may add information to this picture in so far as they suggest that under state 3 conditions, a higher fraction of complex IV might be nitrosylated, accounting for the higher degree of inhibition [ref. 11, and references cited therein].

The two reaction mechanisms between NO and CcOX and their reaction products might have different pathophysiological implications. The nitrosyl derivative releases reactive NO, whereas nitrite further degraded to nitrate can only be disposed (fig. 4). NO free in the cell environment can still perform signalling, and if it persists for too long may even induce cell bioenergetic impairment, and ultimately apoptosis and cell death [9, 36].

Acknowledgements. We wish to thank Dr. Davide Ragozzino (Department of Human Physiology and Pharmacology, the University of Rome 'La Sapienza') for performing animal surgery and Prof. V. Darley-Usmar (University of Alabama), for stimulating discussions. This work was partially supported by: Ministero dell'Istruzione, dell'Università e della Ricerca of Italy, PRIN 'Bioenergetica: aspetti genetici, biochimici e fisiopatologici' – Fondi di Ateneo (Università di Roma 'La Sapienza') – Progetto Biotecnologie 5%, L.95/95 and Ministero della Salute (2001).

1 Cleeter M. W. J., Cooper J. M., Darley-Usmar V. M., Moncada S. and Schapira A. H. V. (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. FEBS Lett. **345:** 50–54
2 Schweizer M. and Richter C. (1994) NO potently and reversibly de-energizes mitochondria at low oxygen tension. Biochem. Biophys. Res. Commun. **204:** 169–175
3 Brown G. C. and Cooper C. E. (1994) Nanomolar concentration of nitric oxide reversibly inhibit synaptosomal cytochrome oxidase respiration, by competing with oxygen at cytochrome oxidase. FEBS Lett. **356:** 295–298
4 Heales J. R., Bolaños J. P., Stewart V. C., Brookes P. S., Land J. M. and Clark J. B. (1999) NO, mitochondria and neurological disease. Biochim. Biophys. Acta **1410:** 215–228
5 Wolin M. S., Xie Y. W. and Hintze T. H. (1999) Nitric oxide as a regulator of tissue oxygen consumption. Curr. Opin. Nephrol. Hypertens **8:** 97–103
6 Nisoli E., Clementi E., Paolucci C., Cozzi V., Tonello C., Sciorati C. et al. (2003) Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science **299:** 896–899
7 Stumpe T., Decking U. K. M. and Schrader J. (2001) Nitric oxide reduces energy supply by direct action on the respiratory chain in isolated cardiomyocytes. Am. J. Physiol Heart Circ. Physiol. **280:** H2350–H2356
8 Brown G. C. (2003) NO says yes to mitochondria. Science **299:** 838–839
9 Moncada S. and Erusalimsky J. D. (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat. Rev. Mol. Cell. Biol. **3:** 214–220
10 Cooper C. E. (2002) Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? Trends. Biochem. Sci. **27:** 33–39
11 Sarti P., Giuffrè A., Barone M. C., Forte E., Mastronicola D. and Brunori M. (2003) Nitric oxide and cytochrome oxidase: reaction mechanisms from the enzyme to the cell. Free Rad. Biol. Med. **34:** 509–520
12 Brown G. C. (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochim. Biophys. Acta **1504:** 46–57
13 Torres J., Darley-Usmar V. M. and Wilson M. T. (1995) Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. Biochem. J. **312:** 169–173
14 Giuffrè A., Sarti P., D' Itri E., Buse G., Soulimane T. and Brunori M. (1996) On the mechanism of inhibition of cytochrome c oxidase by nitric oxide. J. Biol. Chem. **271:** 33404–33408
15 Cooper C. E., Torres J., Sharpe M. A. and Wilson M. T. (1997) Nitric oxide ejects electrons from the binuclear center of cytochrome c oxidase by reacting with oxidized copper: a general mechanism for the interaction of copper proteins with nitric oxide? FEBS Lett. **414:** 281–284
16 Giuffrè A., Barone M. C., Mastronicola D., D'Itri E., Sarti P. and Brunori M. (2000) Reaction of nitric oxide with the turnover intermediates of cytochrome c oxidase: reaction pathway and functional effects. Biochemistry **39:** 15446–15453
17 Blackmore R. S., Greenwood C. and Gibson Q. H. (1991) Studies of the primary oxygen intermediate in the reaction of fully reduced cytochrome oxidase. J. Biol. Chem. **266:** 19245–19249
18 Sarti P., Giuffrè A., Forte E., Mastronicola D., Barone M. C. and Brunori M. (2000) Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation. Biochem. Biophys. Res. Commun. **274:** 183–187
19 Boelens R., Wever R., Van Gelder B. F. and Rademaker H. (1983) An EPR study of the photodissociation reactions of oxidised cytochrome c oxidase-nitric oxide complexes. Biochim. Biophys. Acta. **724:** 176–183
20 Antonini E. and Brunori M. (1971) The derivatives of ferrous hemoglobin and myoglobin. In: Hemoglobin and Myoglobin in Their Reactions with ligands, pp. 29–31, Neuberger A. and Tatum E.L. (eds), North-Holland, Amsterdam
21 Sarti P., Lendaro E., Ippoliti R., Bellelli A., Benedetti P. A. and Brunori M. (1999) Modulation of mitochondrial respiration by nitric oxide: investigation by single cell fluorescence microscopy. FASEB J. **13:** 191–197
22 Brunori M., Giuffrè A., Sarti P., Stubauer G. and Wilson M. T. (1999) Nitric oxide and cellular respiration. Cell. Mol. Life Sci. **56:** 549–557
23 Soulimane T. and Buse G. (1995) Integral cytochrome-c oxidase: preparation and progress towards a three-dimensional crystallization. Eur. J. Biochem. **227:** 588–595
24 Van Gelder B. F. (1966) On cytochrome c oxidase. I. The extinction coefficients of cytochrome a and cytochrome a_3. Biochim. Biophys. Acta **118:** 36–46
25 Kun E., Kirsten E. and Piper W. N. (1979) Stabilization of mitochondrial functions with digitonin. Methods Enzymol. **55:** 115–118
26 Chance B. and Williams G. R. (1956) The respiratory chain and oxidative phosphorylation. Adv. Enzymol. **17:** 65–134
27 Borutaitè V. and Brown G. C. (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem. J. **315:** 295–299
28 Giulivi C. (2003) Characterization and function of mitochondrial nitric-oxide synthase. Free Rad. Biol. Med. **34:** 397–408
29 Shiva S., Brookes P. S., Patel R. P., Anderson P. G. and Darley-Usmar V. (2001) Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase. Proc. Natl. Acad. Sci. USA **98:** 7212–7217
30 Von Jagow G., Ljungdhal P., Graf P. A., Ohnishi T. and Trumpower B. L. (1984) An inhibitor of mitochondrial respiration which binds to cytochrome b and displaces quinone from the iron-sulfur protein of the cytochrome bc_1 complex. J. Biol. Chem. **259:** 6318–6326
31 Giulivi C. (1998) Functional implications of nitric oxide produced in mitochondrial metabolism. Biochem. J. **332:** 673–679

32 Pearce L. L., Kanai A. J., Birder L. A., Pitt B. R. and Peterson J. (2002) The catabolic fate of nitric oxide: the nitric oxide oxidase and peroxynitrite reductase activities of cytochrome oxidase. J. Biol. Chem. **277:** 13556–13562
33 Takehara Y., Kanno T., Yoshioka T., Inoue M. and Utsumi K. (1995) Oxygen-dependent regulation of mitochondrial energy metabolism by nitric oxide. Arch. Biochem. Biophys. **323:** 27–32
34 Wikström M., Krab K. and Saraste M. (1981) Kinetics and catalytic mechanism. In: Cytochrome Oxidase: a Synthesis, pp. 121–123, Academic Press, London
35 Ventura B., Genova M. L., Bovina C., Formiggini G. and Lenaz G. (2002) Control of oxidative phosphorylation by complex I in rat liver mitochondria: implications for aging. Biochim. Biophys. Acta **1553:** 249–260
36 Ghafourifar P., Schenk U., Klein S. D. and Richter C. (1999) Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria: evidence for intramitochondrial peroxynitrite formation. J. Biol. Chem. **274:** 31185–31188
37 Giuffrè A., Barone M. C., Brunori M., D'Itri E., Ludwig B., Malatesta F. et al. (2002) Nitric oxide reacts with the single-electron reduced active site of cytochrome c oxidase. J. Biol. Chem. **277:** 22402–22406