Inhibition of cytochrome *c* oxidase in turnover by nitric oxide: mechanism and implications for control of respiration

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Binding of nitric oxide (NO) to isolated cytochrome *c* oxidase in turnover was investigated by static and kinetic spectroscopic methods. These studies indicate that cytochrome *c* oxidase rapidly binds NO when the enzyme enters turnover. Our results show that NO binds to ferrocytochrome a_3, competing with oxygen for this binding site. However, the main features of the binding process, in particular the rapid onset of inhibition, cannot be fully explained on this basis. We suggest, therefore, that there is a second binding site for NO, which has lower affinity but nevertheless plays an important role in the inhibitory process. A likely possibility is that Cu_B^+ constitutes this second binding site. The fast onset of inhibition observed in the presence of NO, along with the dependence on the oxygen concentration, suggests that under physiological conditions, where the oxygen concentration is low, nanomolar concentrations of NO can effectively act as a regulator of the mitochondrial respiratory chain.

INTRODUCTION

Nitric oxide (NO) is a free radical that plays an important role in a wide range of physiological processes such as neurotransmission [1] or control of vascular tone [2]. These processes are initiated when NO binds to the haem group of guanylate cyclase thereby activating this enzyme [2,3]. At higher concentrations NO is also involved in killing tumour cells [4–8] or intracellular parasites [9,10]. Biosynthesis of NO in mammals is mediated by NO synthase. This enzyme has been found in neurons [11], endothelial cells [12] and macrophages [13]. These different isoforms have been purified, cloned and expressed [14], and their presence in other tissues has been reported also. In stimulated macrophages, NO diffuses into the surrounding tissues where it results in the inhibition of iron–sulphur centres of several important macromolecules including aconitase [5] and complexes I and II of the mitochondrial electron transport chain [5–8], probably through the secondary formation of peroxynitrite.

Recently, an NO-dependent, peroxynitrite-independent, inhibition of mitochondrial electron transport has been reported. NO was shown to inhibit oxygen utilization in rat skeletal muscle mitochondria [15], through an interaction with a form of cytochrome *c* oxidase only populated in turnover (cf. inhibition by cyanide [16]). These experiments were performed under ambient oxygen tension which is considerably higher than the oxygen concentration *in vivo*. Similar behaviour has been reported in synaptosomes, and this inhibition has been shown to be oxygen concentration-dependent. It was thus concluded that, at physiological levels of oxygen and NO, this may offer a way to modulate cellular respiration [17].

Cytochrome *c* oxidase (EC 1.9.3.1) is the final electron acceptor of the mitochondrial respiratory chain, transferring electrons from cytochrome *c* to molecular oxygen to form water. Coupled to this redox reaction, the enzyme pumps protons across the membrane, contributing to the generation of the electrochemical potential gradient ($\Delta\mu_H^+$) which drives ATP synthesis and, in turn, controls the activity of the enzyme. The enzyme contains two haem A groups which are placed in protein environments which confer on them distinctive properties giving rise to moieties termed cytochrome *a* and cytochrome a_3. The former acts as an electron transfer protein and is in rapid redox equilibrium with Cu_A, the electron entry site of the enzyme. Cytochrome a_3 together with Cu_B, forms a binuclear metal centre where oxygen is bound and reduced (see [18–20] for reviews).

NO reacts with fully oxidized or reduced cytochrome *c* oxidase exclusively at the binuclear oxygen-binding site with no apparent involvement of either cytochrome *a* or Cu_A (the electron entry sites). In the fully reduced enzyme, NO binds tightly to ferrocytochrome a_3 and Cu_B^+ acts as a second binding site with lower affinity [21,22]. On the basis of low-temperature EPR spectroscopy Stevens et al. [21] showed that, in the fully oxidized resting enzyme NO bound solely to Cu_B^{2+}. Although NO binds tightly to ferric iron in other haem proteins, this does not therefore seem to be the case for resting oxidized cytochrome *c* oxidase. It is conceivable, however, that under turnover conditions the ferric form of cytochrome a_3 may ligate NO, as the enzyme in turnover and the fully oxidized resting form are likely to have different structures at the binuclear centre. For example, in the resting enzyme ferricytochrome a_3 is reported to coordinate a weak field ligand which dissociates on reduction of the iron, i.e. when the enzyme enters turnover. NO binding to ferric haems is known to be sensitive to such ligation and to the presence/position of residues on the haem distal side. However, mechanisms involving NO binding to ferric forms of cytochrome a_3 cannot readily account for the observed competition between O_2 and NO.

The redox state of cytochrome *c* oxidase susceptible to NO in turnover is not known and this information is important to our understanding of the physiology of this process. In this paper, we report the first spectroscopic evidence which shows that in the inhibited enzyme NO is bound to ferrocytochrome a_3. We also suggest that the activity of the enzyme may be regulated directly by NO *in vivo*.

EXPERIMENTAL

Cytochrome *c* oxidase was prepared from beef heart according to Yonetani [23], and was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 1% Tween 80. The enzyme con-

Abbreviations used: Ru(II), ruthenium hexamine $Ru(NH_3)_6^{2+}$; NO, nitric oxide.

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centration is expressed in terms of (monomeric) functional units. All the experiments were carried out in 0.1 M Hepes, pH 7.4, containing 1% Tween 80. Static and kinetic experiments were performed on a Cary 5E UV–Vis–NIR spectrophotometer. To prepare solutions of NO, oxygen was removed from a vessel sealed with a vaccine cap containing distilled water and flushed with oxygen-free nitrogen. The O_2-free solution was sparged with NO gas. The possible presence of NO_2 was avoided by passing the NO gas through a low-temperature trap using solid CO_2. In a typical experiment, oxidized cytochrome *c* oxidase was incubated in a quartz cuvette with ascorbate and ruthenium hexamine [Ru(II)] (as an electron mediator between ascorbate and the enzyme) in a known volume of air-equilibrated buffer. Under these conditions the enzyme enters turnover, being oxidized by oxygen and subsequently reduced by the excess of reductants. The reduced form of the enzyme was generated once the oxygen was consumed. The gas space of the sealed cuvette was then completely filled with air-equilibrated buffer using a syringe, initiating a new steady state. The final concentrations were: cytochrome *c* oxidase, 1.1–1.8 μM; ascorbate, 3 mM; and Ru(II), 3 μM. The ascorbate and Ru(II) concentrations were chosen so that, although the enzyme was passing through its catalytic cycle, it was not operating at maximum activity. This was done to ensure a long steady state, allowing NO addition to be made and spectra recorded during the steady state. Amounts of NO in solution, approximately stoichiometric with the enzyme (1–2 μM), were added to the enzyme in turnover and the sample was mixed by repeatedly inverting the cuvette which contained two glass beads. Absorbance spectra were recorded automatically every 30 s, except after the addition of NO solution, in which case the spectrum was recorded immediately (i.e. within 10 s of NO addition).

The use of ascorbate and Ru(II) as reductants allowed us to examine the changes that take place in the Soret region of the spectrum during the experiment, as they do not contribute to the absorbance spectrum in this region.

NO was added to the enzyme in turnover over a range of oxygen concentrations.The oxygen concentration in the assay solution at any time may be accurately calculated from a knowledge of the initial oxygen concentration (120 μM in these experiments), the length of the steady state in the absence of NO, and the fact that, in steady state, oxygen is consumed at a constant rate by the enzyme. This latter fact is well documented and is a consequence of the K_m for oxygen being less than 1 μM [24]. The constant rate of oxygen uptake has been confirmed within our experiments by the fact that the steady-state level of reduction of the enzyme is constant throughout the steady state, confirming earlier studies on the pulsed enzyme [25]. This means that the rate of electron entry into the enzyme and the rate of exit, to oxygen, are the same. As the ascorbate concentration is high (relative to oxygen), the electron entry rate is constant and so, therefore, is the rate of oxygen reduction.

The oxygen concentration may therefore be calculated at any time after the initiation of the steady state and hence at the time when the NO additions were made.

The percentage of cytochrome *a* reduced in the steady state before the addition of NO was estimated from the changes in the absorbance in the Soret region when compared with the fully oxidized and fully reduced enzyme. The calculations were made assuming each haem contributes 50% to the reduced-minus-oxidized difference spectrum at 444 nm. The percentage of molecules containing the ferrocytochrome a_3–NO complex after the addition of NO to the enzyme in turnover, was calculated from the weighted sum of the spectra corresponding to the enzyme in turnover and that of the fully inhibited enzyme, assuming that only these two forms contribute significantly to the spectrum in the Soret region. The spectrum corresponding to 100% inhibition was obtained by adding aliquots of NO solution to the fully reduced enzyme under anaerobic conditions until no further spectroscopic change was detected. The spectra used in the calculation were always taken from each experiment.

Complete equilibration of NO with anaerobic buffer may not always be achieved, therefore it was important to determine independently the NO concentration in stock solutions. This was accomplished by titrating deoxymyoglobin or fully reduced cytochrome *c* oxidase with the stock solution diluted appropriately with anaerobic buffer. The method was as described below. Ferric myoglobin (Sigma Chemical Co.) was dissolved in 0.1 M Hepes buffer, pH 7, to give a protein concentration between 10 and 20 μM and a tiny amount of sodium dithionite added in order to convert it into its deoxy form and remove oxygen from the solution. After transferring a known volume of this solution to an optical cuvette (no gas space), the cuvette was sealed with a vaccine cap. The changes in absorption at 435 nm were monitored following the addition of NO solution (10 μl each addition) using a micrometre screwgauge-driven Aglar syringe. The same procedure may be employed using reduced cytochrome *c* oxidase itself, except that ascorbate (2.5 mM) and Ru(II) (0.56 μM) should be used as reductants, and the change in absorbance followed at 444 nm (for details see [26]).

The NO concentration in each turnover experiment, calculated from the addition of a known volume of the NO stock solution, is essentially unaffected by the presence of oxygen during the time period of interest, i.e. the time taken to inhibit the enzyme. Although NO and O_2 react together in solution they do so via a mechanism that is second-order in NO and first-order in O_2, the rate constant being 6×10^6 $M^{-2} \cdot s^{-1}$ [27]. At $\sim 10^{-4}$ M O_2 and $\sim 10^{-6}$ M NO the half-life of NO is many minutes.

The computer simulation of the data was carried out using the program Glint (Applied Photophysics Ltd.).

RESULTS

Figure 1 shows the results of adding air-equilibrated buffer to the reduced (anaerobic) enzyme; a steady state was generated (time period A; 0–1 min, Figure 1) which typically lasted for about 18 min in the absence of NO. If, after the steady state was initiated, NO was added to the sample ($NO_{(a)}$, Figure 1), the absorbance at 442 nm increased. This implies that the enzyme became rapidly inhibited, in agreement with previous results [15], and a new redox steady state was attained. Subsequently, the inhibited enzyme relaxed back to the turnover form (time period B; 3–15 min, Figure 1). When the oxygen in the sample was exhausted ($\sim$ 15 min, Figure 1), the enzyme became completely reduced by the small excess of reductants now present, as indicated by the further increase in the absorbance at 442 nm (time period C, Figure 1). When NO was added to this sample (time period D; $NO_{(b)}$, Figure 1), the absorbance decreased immediately, as under these conditions NO binds tightly to ferrocytochrome a_3 and the enzyme displays the known spectrum of the ferrocytochrome a_3–NO complex [21].

The spectra corresponding to the species generated during the time course described above are presented in Figure 2. The addition of buffer to the reduced anaerobic enzyme led to a steady state [Figure 1 (0–1 min) and Figure 2a] in which the average redox state of the enzyme remained unaltered until oxygen was consumed. The spectrum of this steady state is consistent with the cytochrome a_3 being oxidized and the cytochrome *a* partially reduced, as expected [28]. In these conditions, we estimated (see the Experimental section) that 40–45% of

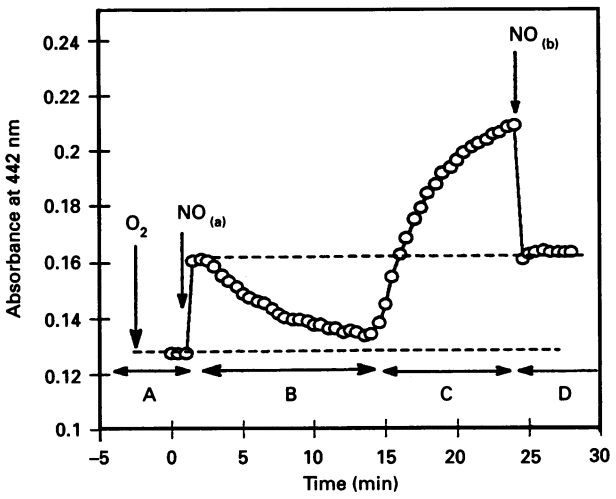


Figure 1 Time course of the spectral changes observed upon addition of NO to cytochrome *c* oxidase in turnover

The Figure shows the time course followed at 442 nm obtained using ~ 1 μM cytochrome *c* oxidase and 40 μM NO (final concentration after addition). The addition of NO ($NO_{(a)}$) was carried out when the oxygen concentration was approx. 100 μM. As shown (upper dotted line), the resulting absorbance at 442 nm after the addition of $NO_{(b)}$ to the reduced enzyme is very similar to that obtained after addition of $NO_{(a)}$ to the enzyme in turnover, indicating that under these conditions all the enzyme has been inhibited. The dotted line (bottom), indicates the absorbance corresponding to the enzyme in turnover. The four time periods in the turnover experiment are labelled A, B, C and D. See Experimental section for experimental details.

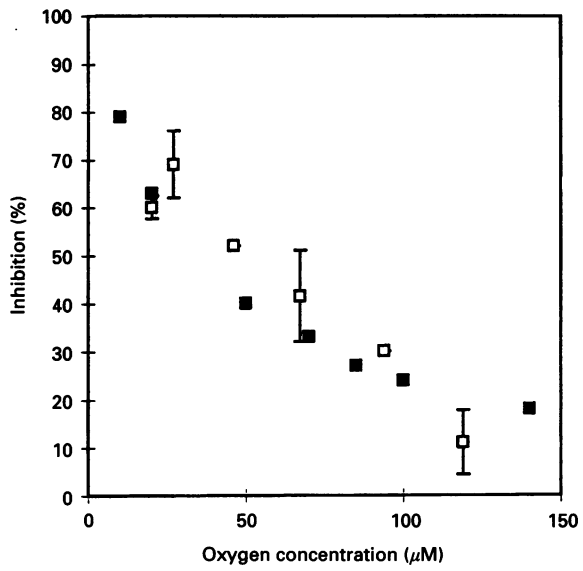


Figure 3 Competition between NO and oxygen for cytochrome *c* oxidase in turnover

Dependence on oxygen concentration of the percentage of inhibition of cytochrome *c* oxidase (1.2–1.8 μM) after addition of NO at concentrations stoichiometric with the enzyme. Each points represents a diffierent experiment or experimental set. The exact enzyme concentration was determined from the reduced spectrum before O_2 addition. The total NO concentration was, in each experiment, equal to the enzyme concentration, e.g. at 50 μM O_2 the enzyme concentration and that of the NO were 1.3 μM. The experimental points are plotted (open squares) and compared with results obtained from the model described in Scheme 1 (closed squares). Experimental points are plotted as mean $\pm$ S.D. ($n \geqslant 3$), where appropriate.

cytochrome *a* was reduced. The addition of NO to the enzyme in turnover ($NO_{(a)}$, Figure 1) gave rise to a species having a spectrum with maxima at 429 and 441 nm in the Soret region (Figure 2b) and at 604 nm in the α region (results not shown). This complex formed by the enzyme and NO relaxed back to the turnover form over a period of 10–15 min (Figure 1). This spectral transition is represented in Figure 2(b). The reduced form was generated when the oxygen became exhausted [Figure 1 (15–25 min) and Figure 2c]. The addition of NO to the fully reduced enzyme ($NO_{(b)}$, Figure 1) gave rise to the well-known NO complex of the reduced enzyme [21], which, as shown in Figure 2(d), has a spectrum virtually identical to that obtained when adding NO to the enzyme in turnover (Figures 2b and 2c). The final inhibited form of the enzyme therefore contains cytochrome a_3 reduced and coordinated to NO. By using fast kinetic techniques, we have observed that neither the initial electron transfer from cytochrome *c* to cytochrome *a* nor the internal electron transfer between the two haems is altered when the

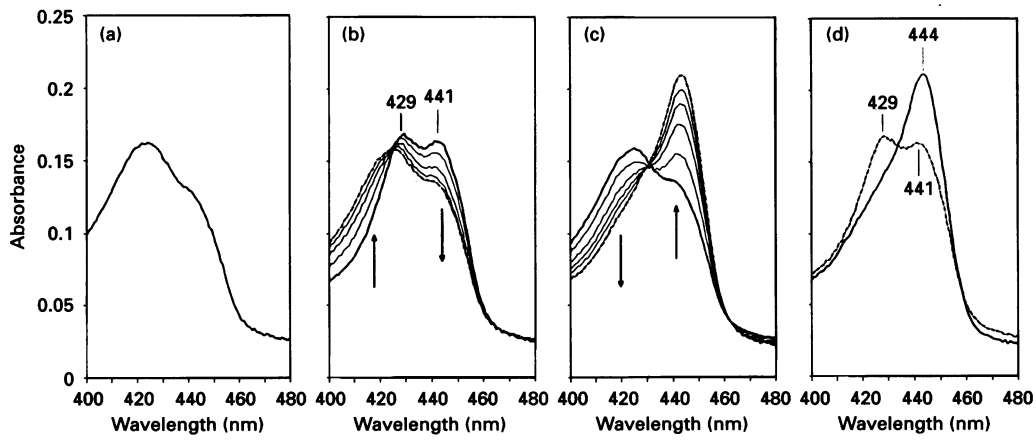


Figure 2 Spectral transitions in the Soret region corresponding to the four time periods in turnover

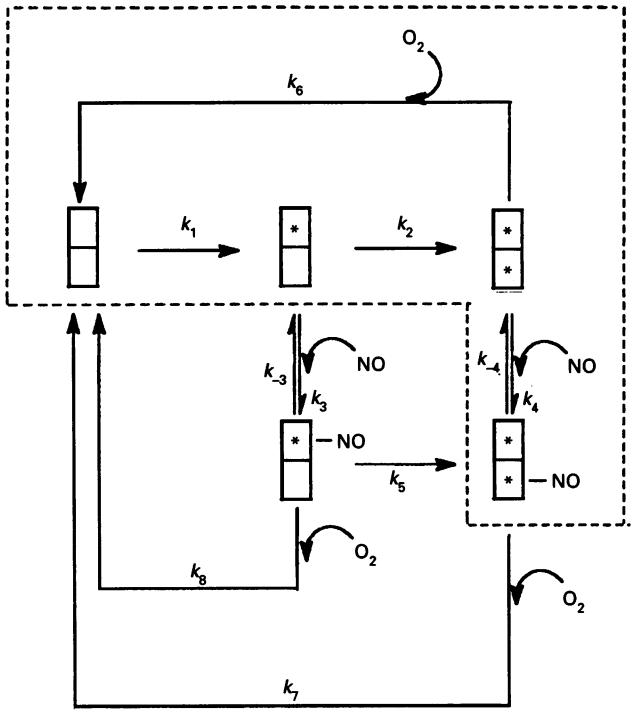
Enzyme in turnover (**a**), relaxation of the spectrum that follows $NO_{(a)}$ addition to the turnover form ($\Delta t = 3$ min) (**b**), reduction of the enzyme after the oxygen is consumed ($\Delta t = 3$ min) (**c**), and binding of NO after the addition of $NO_{(b)}$ to the reduced anaerobic enzyme (**d**). The arrows indicate the direction of the spectral changes.

oxidized enzyme has been incubated (2 min) in the presence of NO (results not shown), indicating, in agreement with others [15], that the enzyme is inhibited only when it enters turnover, i.e. NO binds to a partially reduced form of the enzyme populated in turnover.

The ability of NO to compete with oxygen for the active site was investigated by adding stoichiometric amounts of NO to the enzyme in turnover at different oxygen concentrations. The oxygen concentrations present in the sample (20–120 μM) were determined as described in the Experimental section. Figure 3 clearly shows that the percentage of inhibited enzyme (the ferrocytochrome a_3–NO complex), following the addition of NO to the enzyme in steady state ($NO_{(a)}$, Figure 1), is inversely related to the oxygen concentration.

DISCUSSION

The inhibition of cytochrome *c* oxidase in turnover in the presence of oxygen is clearly reversible (Figure 1) in agreement with previous results [15,17], but an outstanding feature is that the onset of inhibition is very fast, taking place within the dead time of our method ($\sim$ 10 s). As shown in Figure 2, the inhibited enzyme has NO bound to ferrocytochrome a_3. This fact itself strongly suggests that oxygen and NO can compete for this binding site. Indeed, the results in Figure 3 show clear competition between oxygen and NO for ferrocytochrome a_3, as the inhibition of the enzyme by NO is more effective at low oxygen concentrations. Reference to the Figure indicates that in the presence of 50 μM O_2, 1.3 μM NO converted approximately 50% of the total enzyme, also 1.3 μM, to the ferrocytochrome a_3–NO form. The $K_{i\,(apparent)}$ value for NO under these conditions is thus in the submicromolar concentration range, $K_{i(apparent)} = 0.65\ \mu$M. Similar calculations for each O_2 concentration show $K_{i(apparent)}$ decreases as $[O_2]$ decreases; extrapolation to zero O_2 yields a K_i for NO of $\sim$ 0.1 μM. These results would be expected in a type of inhibition similar to that reported for cyanide [16], in which the inhibitor binds with high affinity to some intermediate(s) formed in turnover and withdraws molecules of enzyme away from the normal cycle. What is surprising in our experiment is the velocity at which this process takes place. In fact, the onset of inhibition (formation of ferrocytochrome a_3–NO) found experimentally takes place in less than 15 s (results not shown). This implies that NO competes extremely effectively with oxygen for the ferrous a_3 site. By using the second-order rate constants for the combination of NO and oxygen with ferrocytochrome a_3 (4×10^7 $M^{-1} \cdot s^{-1}$ [29] and 1×10^8 $M^{-1} \cdot s^{-1}$[20] respectively) we have tried to simulate our data with a simple mechanism in which NO competes with oxygen for ferrocytochrome a_3 (Scheme 1, boxed steps). Using this model, at concentrations around 1 μM NO and 50 μM O_2, the ferrocytochrome a_3–NO species should be poorly populated (i.e. low level of inhibition) until oxygen is depleted. Furthermore, in order to yield a rate of inhibition comparable with that found experimentally, and assuming the formation of the complex with NO is a dead end (i.e. k_{off} taken as zero), the k_{on} for NO needed to be increased at least 10-fold. Thus, the ability of NO to compete with oxygen was much greater than can be accounted for solely on the basis of kinetic competition between NO and O_2 for ferrocytochrome a_3. In order to account for the observed rate for the onset of inhibition (Figure 1), an additional pathway must be introduced, in which NO binds to an intermediate of the enzyme for which oxygen has low affinity. We propose a reversible, low-affinity equilibrium between NO and an intermediate, which precedes the formation of the fully reduced



Scheme 1 Model for the mechanism of inhibition of cytochrome *c* oxidase by NO

The two redox sites comprising the binuclear centre are represented by the adjoining boxes, Cu_B (upper) and cytochrome a_3 (lower). Electron occupancy is denoted by an asterisk. The phenomenological rate constants are complex, in some cases comprising a number of steps; e.g. k_1 incorporates the electron entry into the enzyme from the external reductants, equilibration between Cu_A and cytochrome *a* and donation to the binuclear centre (Cu_B). The parameters used in the simulation are: enzyme concentration, 1.8 μM; NO concentration, 1.3 μM; rate constants: $k_1 = 0.2\ s^{-1}$, $k_2 = 0.2\ s^{-1}$, $k_3 = 10^6\ M^{-1} \cdot s^{-1}$, $k_{-3} = 10\ s^{-1}$, $k_4 = 4 \times 10^7\ M^{-1} \cdot s^{-1}$, $k_{-4} = 3 \times 10^{-3}\ s^{-1}$, $k_5 = 4\ s^{-1}$, $k_6 = 1 \times 10^8\ M^{-1} \cdot s^{-1}$, $k_7 = 180\ M^{-1} \cdot s^{-1}$ and $k_8 = 1 \times 10^5\ M^{-1} \cdot s^{-1}$. The first-order rate constants k_1 and k_2 were estimated from the turnover rate in these conditions and were tested in the model in the absence of NO, giving the same time for oxygen consumption as in the experiment. The second-order rate constants k_6 and k_7 were taken from the k_{on} for O_2, $10^8\ M^{-1} \cdot s^{-1}$, and NO, $4 \times 10^7\ M^{-1} \cdot s^{-1}$, respectively. The first-order rate constant k_{-4} was set arbitrarily to $3 \times 10^{-3}\ s^{-1}$, given the high affinity of NO for ferrocytochrome a_3 [17]. The rest of the rate constants were adjusted in order to fit the data, although the equilibrium constant for the binding of NO to Cu_B was restricted to $10^5\ M^{-1}$.

binuclear centre (see Scheme 1). Based on the reported ability of Cu_B to bind NO in both valence states, albeit with lower affinity than for cytochrome a_3 [21], one possibility is that NO binds to Cu_B^+ in the binuclear centre in which cytochrome a_3 is still oxidized. The reported dissociation constant (K_D) of oxygen from Cu_B^+ is $\sim$ 8 mM [30], and oxygen only reacts with the binuclear centre when both metals are reduced [19]. Thus, NO may compete effectively with oxygen for the partially reduced binuclear centre. In order to obtain the observed ferrocytochrome a_3–NO spectrum under turnover we must, in addition, propose that a further electron enters the binuclear centre reducing cytochrome a_3, and that NO is rapidly transferred to this site. Under these circumstances NO, in close proximity to cytochrome a_3 (3–4 Å) [31], competes effectively with oxygen.

These hypotheses are incorporated into the model shown in Scheme 1. The steps within the broken line depict the competition between oxygen and NO for the fully reduced binuclear centre, which alone cannot account for our data. The branch added shows the binding of NO to Cu_B^+ and eventual transfer to

ferrocytochrome a_3. As we observe the removal of NO from the system during turnover (Figures 1 and 2), we have included steps taking the NO complex back to the oxidized enzyme. At this stage it is unclear if this is accomplished by depletion of free NO in solution by oxygen or, as we suspect, by reactions within the active site holding the metal–NO complex. The reactions of NO-reduced cytochrome *c* oxidase under anaerobic conditions have been reported [21], but the reactions that take place in the presence of oxygen, if any, are not known. In fact, from experiments using ferrocytochrome *c* as an electron donor and *S*-nitrosoglutathione and dithiothreitol as a source of NO, we have detected that the inhibited enzyme may still take electrons from ferrocytochrome *c*, although at a lower rate (results not shown).

The model in Scheme 1 conforms to a wide body of experimental data regarding the nature of the binuclear centre and can simulate the main features of our experimental data, namely (a) the rate of onset of inhibition, (b) the decay of the inhibited enzyme back to the turnover form, (c) the spectral features of the inhibited enzyme, and also (d) the percentage of inhibition as a function of the oxygen concentration (see Figure 3).

In summary, our results raise the possibility that NO can modify the activity of cytochrome *c* oxidase *in vivo* by binding to ferrocytochrome a_3, facilitated by prior complexing with Cu_B. NO concentrations of up to 1 μM have been reported for human tissue in non-pathological conditions [32]. In tissues, the oxygen concentration is at most 30 μM, and the enzyme would be half inhibited at submicromolar concentrations of NO under these conditions. Clearly, the NO concentration able to modify the activity of the protein is in the range of that found *in vivo*. Furthermore, the turnover number in our experiment was very low (see Scheme 1) compared with that reported *in vivo* [33]. This difference implies that, *in vivo*, the inhibitory action of NO can be far more effective, according to our model. Whether this implies that the interactions of NO and cytochrome *c* oxidase are physiological or pathological *in vivo* would seem dependent on the relative NO and oxygen concentrations and the electron flux through the respiratory chain.

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