Nitric oxide ejects electrons from the binuclear centre of cytochrome *c* oxidase by reacting with oxidised copper: a general mechanism for the interaction of copper proteins with nitric oxide?

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Abstract Small increases in NO concentration can inhibit mitochondrial oxygen consumption by reacting at the binuclear haem a_3/Cu_B oxygen reduction site of cytochrome *c* oxidase. Here we demonstrate that under normal turnover conditions NO reacts initially with the oxidised Cu_B rather than the haem a_3. We propose that hydration of an initial Cu^+/NO^+ complex forms nitrite, a proton and Cu_B^+; the latter ejects an electron from the binuclear centre and results in the observed (100 s^{-1}) reduction of other electron transfer centres in the enzyme (haem *a* and Cu_A). These reactions may have implications for the interactions of NO with other copper proteins.

Key words: Nitric oxide; Cytochrome oxidase; Electron transfer; Mitochondria; Heme enzyme; Copper enzyme

1. Introduction

Nitric oxide reacts with mitochondrial cytochrome oxidase and this is the reaction responsible for the high (nM) affinity reversible inhibition of mitochondrial respiration by NO [1–3]. It has been reported that this inhibition may play a role in modulating the rate of oxygen consumption under normal physiological conditions [4]. It is certainly likely to be an initial event in any pathophysiological role of NO in mitochondria [5]. Although inhibited at cytochrome *c* oxidase, the mitochondrial respiratory chain (complex II) produces superoxide ions, which can subsequently react with nitric oxide to produce peroxynitrite ($ONOO^-$). This species irreversibly damages mitochondrial electron transfer complexes, a process that can lead to apoptotic cell death [6] and is implicated in neurodegenerative disorders [7].

The elucidation of the molecular mechanism for cytochrome oxidase inhibition by NO is made difficult by the large number of possible species in the enzyme with which NO may react. The enzyme contains four redox active metal centres [8]. The electron transfer sites, Cu_A and haem *a*, are unreactive to NO, but the binuclear haem a_3/Cu_B oxygen-reactive site can react with NO in a number of ways. Ferrous haem a_3 and cupric [9,10] or cuprous [11] Cu_B can all react with NO [12]. It is possible that two NO molecules may be present simultaneously in the enzyme active site [11,13]. The intermediates in the oxygen reduction process, 'P' and 'F' (probably peroxy and ferryl species respectively [14]), may also be NO reactive [15].

The kinetics of NO binding to reduced haem a_3 are fast, $k = 1 \times 10^8$ M^{-1} s^{-1} [16], but are still slower than those of reduced haem a_3 with oxygen – therefore we expect that at physiological oxygen tensions O_2 will out-compete NO. Furthermore, our previous experiments suggest that, while NO binds initially to the partially reduced binuclear center [17], the ferrous haem a_3 nitrosyl species forms later in the inhibition of enzyme turnover by nitric oxide and is therefore unlikely to be the primary site of inhibition. It has recently been reported that NO inhibition of cytochrome oxidase can be explained by binding of this ligand to the ferrous haem a_3 site [18] within a partially reduced binuclear centre (i.e. $a_3^{2+} \cdot Cu_B^{2+}$). Although this idea is appealing and very neatly explains the observed oxygen dependence of the NO inhibition [2] the surprisingly fast NO dissociation rate measured (~ 0.1 s^{-1}) means that it is difficult to explain the very low K_i observed in steady-state experiments. The observed metabolism of NO by cytochrome oxidase also suggests that simple reversible binding at haem a_3 may not be the entire story [11,13,19]. In this communication we report that under turnover conditions NO reacts rapidly with oxidised Cu_B at the binuclear centre. The resulting reaction reduces Cu_B and this makes an electron available at the binuclear centre. We suggest the reaction mechanism involves hydration of a Cu^+/NO^+ complex to form nitrite, a proton and reduced Cu_B. This site may itself bind NO leading to inhibition or donate an electron to another site(s) within the enzyme. The reactions of NO with Cu_B therefore resolve the conflicting data by (a) resulting in metabolism of NO by cytochrome oxidase and (b) generating a bound NO close to the haem a_3 centre. When this in turn becomes reduced as part of the turnover cycle of the enzyme NO migrates from Cu_B to the reduced haem in a reaction which efficiently competes with oxygen for this site.

2. Materials and methods

The reaction of oxidised cytochrome oxidase with NO has been shown to be heterogeneous with different enzyme preparations. We therefore prepared cytochrome oxidase by the method of Kuboyama [20], Yonetani [21] or Buse [22]. The enzyme was pulsed (redox-cycled) prior to use to form a 'fast' enzyme devoid of binuclear centre ligands (intrinsic or extrinsic) formed during the purification process [23]. The full decay of the oxygen intermediates ('P' and 'F') was confirmed by optical spectroscopy prior to use. As long as this procedure was performed we found no difference in the nature of the reactivity of the different enzyme preparations with nitric oxide. Optical spectroscopy was performed using a Hewlett Packard 8453 diode array spectrophotometer and stopped-flow spectroscopy using an Applied Photophysics SX18MV with diode array attachment. Nitric oxide was obtained by mixing 1 M sulphuric acid with sodium nitrite in a Kipps apparatus. Impurities were removed by passing the gas through KI, four consecutive NaOH solutions, water and finally a cold trap covered with dry ice. The gas was dissolved in degassed buffer to saturation (2 mM). Less than 300 μM nitrite was present in these solu-

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tions, a concentration that has no effect on cytochrome oxidase. NO solutions were calibrated using a NO electrode (World Precision Instruments). To obtain the enzyme in turnover, oxidised enzyme was first fully reduced by mixing with ascorbate and ruthenium hexamine in 0.1 M HEPES, 0.5% Tween 80, pH 7.4 and allowed to go anaerobic. This solution was then mixed with 0.5 ml of aerated buffer and immediately placed in the stopped-flow apparatus. Enzyme and reductant concentrations were such as to ensure slow enough turnover rates ($\sim 1\ s^{-1}$) to allow this solution to be mixed with a 2 mM solution of NO before anaerobiosis occurred. The fraction of reduced haem *a* was calculated compared to the full reduction induced by dithionite. The 605–630 nm wave pair was used, assuming an 84% contribution of haem *a* at these wavelengths upon full reduction (and 0% haem a_3 reduction in the steady state prior to NO addition). The Cu_A reduction was calculated similarly using a three wavelength method ((ΔA 730 nm+ΔA 930 nm)/2)−ΔA 830 nm.

3. Results

It has been known for some time that NO can react with fully oxidised cytochrome oxidase at the Cu_B site [9–11]. These earlier experiments were, however, carried out in the absence of oxygen and therefore are not directly relevant to inhibition in vivo. In Fig. 1 we show that NO reacts rapidly ($100\ s^{-1}$) with the fully oxidised enzyme in the presence of oxygen. No changes are seen in the haem a_3 absorbance spectrum and we therefore conclude, in agreement with the anaerobic experiments, that the reactive site is Cu_B. However, we observe, in keeping with the original report by Boelens et al. [10], that NO binding produces a species with the characteristic absorbance spectrum of ferrous haem *a*. Studies of the 830 nm Cu_A band also demonstrate that some reduction occurs at this centre. The extent of the changes in the visible (haem *a*) and near-infrared (Cu_A) regions (Fig. 2) indicate that 30% of haem *a* and 20% of Cu_A become reduced. The amplitude of these changes indicate that NO must be the source of the electron, rather than a small fraction of the

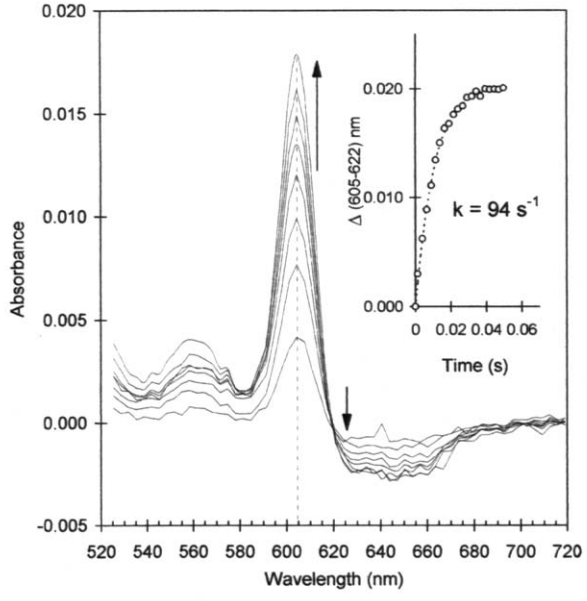

Fig. 1. Reaction of nitric oxide with oxidised cytochrome oxidase. 7.5 μM fully oxidised fast cytochrome oxidase (in 0.1 M HEPES, 0.5% Tween 80, pH 7.4) was mixed with 1 mM NO in a stopped-flow apparatus. Difference spectra with respect to the first spectrum after mixing (1.38 ms) are illustrated. The inset shows the time courses of haem *a* reduction fitted to a single exponential.

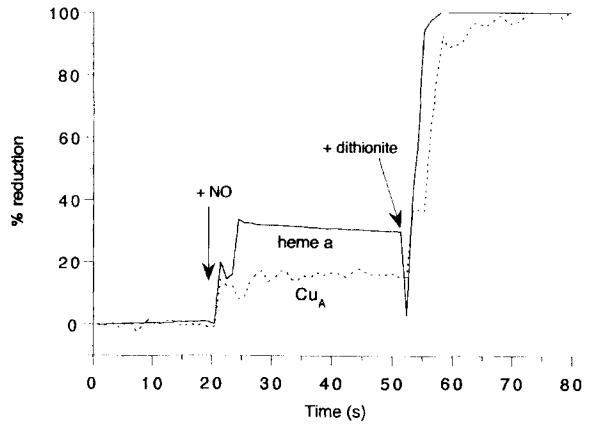

Fig. 2. Reduction of haem *a* and Cu_A following NO addition to oxidised cytochrome oxidase. 100 μM NO was added to a solution of cytochrome oxidase (2.9 μM fully oxidised fast enzyme in 0.1 M KPi, 0.5% Tween 80, pH 7.4). Full reduction was obtained by the addition of 50 mM sodium dithionite. Spikes indicate mixing artefacts as this was a manual mixing, not stopped-flow, trace.

'oxidised' enzyme containing a reduced site(s) from which an electron may be displaced by NO binding.

The electrons that we observe on haem *a* and Cu_A must be coming via a reaction of NO with the binuclear centre followed by reversed electron transfer. The alternative explanation, namely that NO directly donates electrons to Cu_A and haem *a*, can be discounted for a number of reasons. Firstly, oxidised cytochrome oxidase can be prepared in 'slow' or 'resting' forms that have sluggish reactivity to external ligands at the haem a_3/Cu_B binuclear centre and consequent slow electron transfer to this centre from haem *a* [23]. If NO directly donated electrons to haem *a* and Cu_A we would expect, if anything, enhanced reduction of these centres in slow forms of the enzyme. In fact we see significantly less reduction of haem *a* in the slow enzyme (results not shown).

Further evidence for initial NO reactivity at the binuclear centre comes from a closer inspection of the absorbance changes between 620 nm and 680 nm. These reveal changes in the binuclear centre consistent with perturbation by NO binding and/or metabolism. The absorbance change between 660 and 680 nm is superimposable on that observed in the fully reduced minus oxidised spectrum of the enzyme, indicating complete bleaching of the long wavelength tail of the broad '655 nm band'. The latter is generally attributed to ferric haem a_3 spin coupled to cupric Cu_B [24]. Thus haem *a* reduction alone is not consistent with the spectrum in this region and reduction of Cu_B or haem a_3 must also be occurring. Haem a_3 reduction can be discounted by comparing the ratio of the absorbance changes in the Soret region (444 nm) to those in the visible region (605 nm). The 444:605 nm ratio following NO addition is 3:1, as expected for haem *a* reduction alone with no contribution from the haem a_3 spectrum. Therefore we are confident that the initial site of NO reactivity at the binuclear centre is Cu_B, not haem a_3. Due to the weak absorbance of Cu_B this is hard to demonstrate directly, but indirect evidence for NO binding to Cu_B can be found in a closer inspection of the region between 620 and 660 nm. Here, unlike the fully reduced minus oxidised spectrum, the spectrum following NO addition is essentially flat, indicating that the normal trough here associated with the bleaching of the 655 nm band is absent. This suggests a new positively

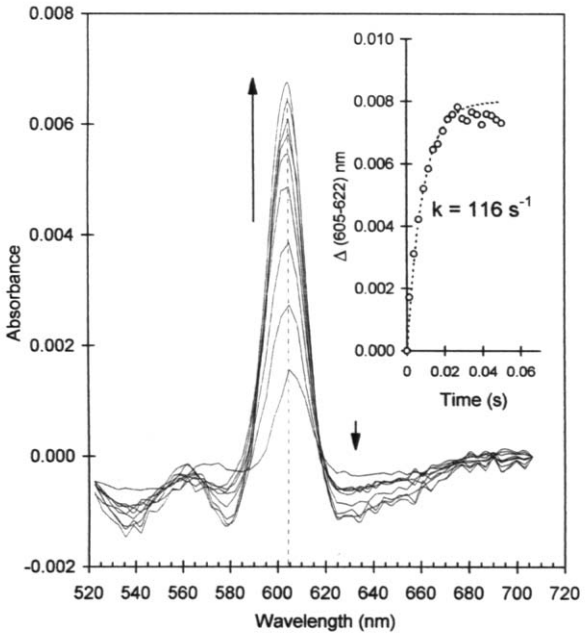

Fig. 3. Reaction of nitric oxide with cytochrome oxidase in turnover. 8.2 μM cytochrome oxidase (in 0.1 M HEPES, 0.5% Tween 80, pH 7.4) was mixed with 1.6 μM ruthenium hexamine chloride and 5 mM sodium ascorbate to initiate a slow rate of enzyme turnover (0.5 s^{-1}). Before the oxygen in the solution was removed this was then mixed with 1 mM NO in the stopped-flow apparatus. Difference spectra with respect to the first spectrum after mixing (1.38 ms) are illustrated. The inset shows the time courses of haem *a* reduction fitted to a single exponential (other slower reactions are evident at the longer time points).

absorbing species has appeared in this region and is consistent with the 640 nm absorbing species detected in photodissociation spectra of NO bound to oxidised Cu_B [10].

During its catalytic oxygen reduction cycle cytochrome oxidase passes through a series of intermediates which harbor partially reduced oxygen species within the binuclear centre. Two such intermediates, termed 'P' and 'F' (probably peroxy and ferryl species respectively [14]), may also be NO reactive [15]. These species have characteristic absorbance peaks in the difference spectrum (relative to the oxidised enzyme) at 607 nm ('P') and 580 nm ('F') that distinguish them from the normal fully oxidised enzyme ('O'). We have examined the reaction of NO with the pure 'P' and 'F' species of the enzyme. Under the conditions described in Fig. 1 the bands at 607 nm (P) and 580 nm (F) decayed on addition of NO but at a rate much slower ($\sim$10 s^{-1}) than that observed for the reaction of the fully oxidised enzyme (O). Although the reaction of NO with these oxygen intermediates exhibits some complex features [15] we suggest that reaction of NO with Cu_B is the first step in the process leading to reduction of both these oxygen intermediates.

To test our hypothesis that oxidised Cu_B is the dominant NO-reactive species we added NO to cytochrome oxidase in turnover and monitored the spectral differences by rapid scan diode array stopped-flow spectrophotometry. Fig. 3 shows that the spectral changes and rate constants we observed are essentially identical to those seen for the fully oxidised enzyme. If the reaction is followed for longer time we also see troughs appear (10 s^{-1}) at 607 nm and 580 nm characteristic of the reaction between NO and the 'P' and 'F' intermediates. Thus NO must be reacting with subpopulations of the enzyme that contain these intermediates. This confirms that these intermediates are present in the normal steady-state turnover of the enzyme. The final product of inhibition is the haem a_3 nitrosyl complex, suggesting that once NO is bound to Cu_B it can effectively out-compete the faster reaction of oxygen with ferrous haem a_3 [16].

4. Discussion

The findings in this paper have significant implications for both the observed strong inhibition [2] and the proposed role of cytochrome oxidase in NO metabolism and detoxification [4,13,19]. Our results are consistent with NO initially binding to Cu_B^{2+}, with a subsequent electron rearrangement to form Cu_B^{+}/NO^{+} [10]. By analogy with the ferrous hemoglobin/$NO^{\cdot}$ species [25], we propose Cu_B^{+}/NO^{+} is then hydrated to form HNO_2, H^{+} and Cu_B^{+} or hydroxylated to form HNO_2 and Cu_B^{+}. Reversed electron transfer would then result, as observed, in a fraction of haem *a* becoming reduced

$$Cu^{2+}-NO \rightarrow Cu^{+}-NO^{+}$$

followed by: $Cu^{+}-NO^{+} + H_2O \rightarrow Cu^{+} + H^{+} + HNO_2$ (i)

or: $Cu^{+}-NO^{+} + OH^{-} \rightarrow Cu^{+} + HNO_2$ (ii)

Similar schemes have been suggested to occur when NO reacts with copper centres in laccase [26]. Mechanism (i) has the distinct advantage of maintaining the electroneutrality of the hydrophobic oxygen-reduction site as adding or removing a charge into this centre is thermodynamically unfavoured [8,27]. The ease at which a proton could leave the binuclear centre would then control the rate of electron transfer to haem *a* and Cu_A. The first spectrum we observe in the stopped-flow spectrophotometer ($\sim$1.5 ms after mixing) is slightly different from that of the normal turnover intermediate, suggesting that the NO binding may be very rapid and the subsequent, relatively slow, electron transfer rate to haem *a*/Cu_A limited by the proton dissociation from the binuclear centre. We are currently using rapid freeze quench EPR spectroscopy and optical pH probes to investigate these possibilities further.

This reaction scheme can also explain a number of other apparently unrelated findings in the biochemistry of cytochrome oxidase/NO interactions. For example the reduction of the 'F' intermediate of cytochrome oxidase to the fully oxidised enzyme occurs at a rate $\sim$300 times faster than is observed for the direct reduction of the ferryl species in ferryl hemoglobin [28]. Reduction of Cu_B by NO and subsequent electron transfer to 'F' may provide a mechanism for such a rapid reaction. Furthermore mitochondrial metabolism of NO is sensitive to inhibitors of cytochrome oxidase [19]. The rapid production of nitrite by our proposed mechanism would help explain this finding and provide an excellent method of NO detoxification in vivo.

Finally the most interesting and confusing finding in this field is the ability of nitric oxide to compete so effectively with oxygen. For example, simultaneous oxygen electrode/NO electrode experiments reveal that cytochrome oxidase remains 50% inhibited when the NO concentration in solution is 60 nM (at an oxygen concentration as high as 30 μM). We estimate, using the data of Guiffre et al. [18], that NO is at least a 5-fold better inhibitor of cytochrome oxidase than expected if

it were a true competitive inhibitor, such as CO. We previously suggested that NO was such a strong inhibitor as it bound to reduced Cu_B formed during turnover and we explained the oxygen dependence of the inhibition by subsequent reaction of O_2 at this site [17]. Others have suggested that reduced haem a_3 is the initial site of inhibition [28]. Our suggested mechanism is able to unite these two theories and explain both the low K_i of NO for cytochrome oxidase and its oxygen dependence. The proposed initial reaction of NO with Cu_B^{2+} has the advantage that this latter species is essentially 100% populated during turnover [29]. Metabolism of NO at this site (scheme 1) would then generate a binuclear centre containing a single electron on Cu_B. As pointed out by others [30] oxygen has a very low affinity at this site and therefore NO would readily react with this Cu_B^{+}. The effective NO competition with oxygen may now be explained as NO has only to travel the much shorter (3 Å) distance from Cu_B to haem a_3, once this is reduced, whereas oxygen must gain access to haem a_3 from the external medium.

The rate of formation of the final inhibited haem a_3 nitrosyl complex is dependent on the rate of electron entry to the binuclear centre. Using the low ruthenium hexamine concentrations reported here it can take many seconds. However, with a rapid source of electron entry to the oxidase (e.g. using ferrocytochrome *c* or high ruthenium hexamine concentrations) the haem a_3 nitrosyl complex can be observed within 0.1–0.2 s, as reported by others [18]. However, even under these conditions, using the rapid scan diode array system we are still able to observe prior reactions of NO with Cu_B.

As well as explaining the mechanism for the highly effective inhibition of cytochrome oxidase by NO, our studies suggest that copper proteins may be especially sensitive to reactions with nitric oxide, a possibility that has not been generally discussed. Many copper proteins and enzymes are capable of binding NO [31], both when oxidised [32] and reduced [33]. In preliminary work we have detected strong NO inhibition of both mononuclear (galactose oxidase) and multinuclear (ascorbate oxidase) copper oxidases by NO at the μM level. We have also observed that the NO-induced reduction of copper centres in laccase [26] occurs at a rate similar to that observed for cytochrome oxidase. These latter findings are interesting, given the similarity of the copper sites in ascorbate oxidase and laccase to the serum protein, ceruloplasmin, which is also known to bind NO [34]. Of particular interest, given the relationship of nitric oxide and dopamine levels [35] is possible NO-mediated effects of biogenic (histamine and dopamine) amine levels via the copper-containing enzymes, dopamine monooxygenase and the amine oxidases.

Biological reactions of copper ions with NO are not as well characterised as those with iron. This work suggests that more attention should be paid to copper proteins as possible targets of NO modulation and toxicity in vivo.

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