Interaction of Peroxynitrite with Mitochondrial Cytochrome Oxidase

CATALYTIC PRODUCTION OF NITRIC OXIDE AND IRREVERSIBLE INHIBITION OF ENZYME ACTIVITY

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Purified mitochondrial cytochrome c oxidase catalyzes the conversion of peroxynitrite to nitric oxide (NO). This reaction is cyanide-sensitive, indicating that the binuclear heme α3/CuA center is the catalytic site. NO production causes a reversible inhibition of turnover, characterized by formation of the cytochrome α3 nitrosyl complex. In addition, peroxynitrite causes irreversible inhibition of cytochrome oxidase, characterized by a decreased Vmax and a raised Km for oxygen. Under these conditions, the redox state of cytochrome a is elevated, indicating inhibition of electron transfer and/or oxygen reduction reactions subsequent to this center. The lipid bilayer is no barrier to these peroxynitrite effects, as NO production and irreversible enzyme inhibition were also observed in cytochrome oxidase proteoliposomes. Addition of 50 μM peroxynitrite to 10 μM fully oxidized enzyme induced spectral changes characteristic of the formation of ferryl cytochrome α3, partial reduction of cytochrome α, and irreversible damage to the CuA site. Higher concentrations of peroxynitrite (250 μM) cause heme degradation. In the fully reduced enzyme, peroxynitrite causes a red shift in the optical spectrum of both cytochromes a and α, resulting in a symmetrical peak in the visible region. Therefore, peroxynitrite can both modify and degrade the metal centers of cytochrome oxidase.

The inhibition of cytochrome c oxidase by nitric oxide (NO) may play a normal role in controlling mitochondrial O2 consumption (1). Inhibition of cytochrome oxidase by NO could, for example, explain the observation that the apparent Km for O2 of whole cells is greater than that of isolated mitochondria (2). The inhibition of cytochrome oxidase by elevated levels of NO, although readily reversible, can have profound consequence for the cell in pathophysiological disease states (3). Inhibition of mitochondrial transport chain at the cytochrome oxidase level can produce superoxide (O2-) from O2. Peroxynitrite (ONOO-) can also arise via the reaction of nitroxy1 anion (NO-) and O2 (5, 6). We have recently demonstrated that both NO- and ONOO- are produced during the aerobic incubation of mitochondrial ferrocytochrome c and NO (7).

In contrast to NO, ONOO- can irreversibly damage many of the mitochondrial enzymes and complexes of oxidative phosphorylation, including aconitase, NADH/co-Q reductase, quinol/cytochrome c reductase, succinate dehydrogenase, and the ATP synthetase (8, 9). The consequent collapse of the mitochondrial membrane potential (ΔΨ) can trigger the mitochondrial permeability transition (MPT). This causes the release of cytochrome c from the inter-mitochondrial space into the cytoplasm (10–12). The movement of cytochrome c into the cytoplasm consequently triggers apoptotic cell death (13). As ONOO- has been shown to induce the MPT (14, 15), the formation of ONOO- from NO may thus be responsible for a pro-apoptotic role of NO.

Reversible inhibition of cytochrome oxidase by NO may therefore initiate a cascade of events that results in cell death. However, there is conflicting evidence as to whether ONOO- directly inhibits cytochrome oxidase function in cells and mitochondria, with some papers suggesting that there is little or no damage (9, 16) and others disagreeing (17). Here we resolve this controversy by clearly demonstrating that purified cytochrome oxidase when solubilized or in proteoliposomal form is irreversibly damaged by ONOO-. Interestingly, we also observe that cytochrome oxidase catalyzes NO production from ONOO-; this novel chemistry, converting an irreversible inhibitor into a reversible one, may play a role in ONOO- detoxification by the mitochondria.

EXPERIMENTAL PROCEDURES

Bovine heart cytochrome c oxidase was purified according to the method of Kuboyama et al. (18), substituting Tween 80 for Emsol in the latter stages of the purification. Peroxynitrite (ONOO-) was prepared from amyl nitrite and hydrogen peroxide using the method of Uppu and Pryor (19); 26 ml of amyl nitrite (washed three times with water) was added to 100 ml of 2M H2O2, 2M NaOH, and 2M diethylenetriaminepentaacetic acid (DTPA), 4°C. This solution was stirred vigorously for 3 h at 4°C. The lower aqueous layer was removed from the organic phase using a separating funnel and washed three times with an equal volume of ice-cold hexane. To remove residual H2O2, 10 g of manganese dioxide was gradually added to the ONOO- solution while it was being stirred, on ice, for approximately 1 h. The ONOO- solution was filtered and then stored at 77 K. After thawing, the ONOO- solutions were passed down a 6 x 1-cm MnO2 column equilibrated with 0.1M NaOH to remove any traces of H2O2. The ONOO- was then diluted with 0.1M NaOH to approximately 30 mM immediately before use. The concentration of ONOO- was determined spectrophotometrically using the extinction coefficient of 1670M-1cm-1 at 302 nm. Nitrite contamination of the ONOO- solution was determined to be approximately one third of the ONOO- concentration, as in (19). The addition of catalase to decomposed peroxynitrite at pH 7 resulted in no oxygen evolution, consistent with an absence of any H2O2 contamination in the solution.
Crude NO was prepared by the addition of 2 ml H₂SO₄ to solid NaN₃ in a Kipps apparatus. The NO gas was purified by passage through four NaOH (20%) traps and then through a dry ice trap, to remove NO₂. NO solutions were prepared by the addition of purified NO gas into buffer solutions that had undergone four vacuum/N₂ deoxygenation cycles. The concentration of the NO solution varied between 1.2 and 2 mM. The nitrite concentration in the NO solution was determined to be between 300 and 500 μM.

The concentrations of O₂ and NO were determined polarographically. A Perspex, water-jacketed O₂ electrode (Rank Brothers, Bottisham, Cambridge, U.K.) was modified to allow the insertion of a WPI ISO-200 NO electrode (World Precision Instruments, Sarasota, FL). The NO electrode was connected to an ISO-NO II NO meter. Data from the O₂ and NO electrodes were collected using MacLab data collection and analysis system (ADInstruments, Castle Hill, New South Wales, Australia). Nitrite concentrations were determined by the addition of aliquots of the solution to 100 mM KCl, 100 mM H₂SO₄, followed by measurement of the NO produced using the electrode (this procedure yields a 1:1 nitrite:NO stoichiometry).

Cytochrome c oxidase turnover was assayed by adding 5 mM cytochrome c to 20 mM sodium ascorbate, 300 μM N,N,N′,N′-tetramethyl-p-phenylenediamine hydrochloride (TMDP), 60 μM cytochrome c (horse heart, Sigma type C-7732) in 5 ml of 100 mM K⁺-Hepes or 100 mM K⁺-phosphate, 20 μM DTPA, 0.015% lauryl maltoside, pH 7.0, 30 °C.

In the derived plots (Fig. 4), the non-enzymatic autoxidation rate was subtracted from the overall O₂ consumption rate to give the enzymatic rate. We found that the autoxidation rate of ascorbate was directly proportional to the O₂ concentration. We calculated the autoxidation rate for ascorbate at all O₂ concentrations and subtracted this from the O₂ consumption rate.

Precise measurement of the oxidase Kₘ for O₂ of cytochrome oxidase requires high resolution respirometry (20). This was done using an Oroboros® Oxigraph (Oroboros®, Innsbruck, Austria.) The experimental conditions were as in Fig. 1, but with a smaller chamber volume, 3.4 ml.

Cytochrome c oxidase vesicles (COV) were prepared as described previously (21). 0.25 g of soybean phospholipid (Sigma type II-8 “phosphatidylcholine”) and 0.1 g of sodium cholate were dispersed in 5 ml of 100 mM K⁺-Hepes, pH 7.0, by vortex mixer. The suspension was then sonicated on ice under a N₂ stream for 8 min using a 30% duty cycle. Cytochrome oxidase was added to a final concentration of 6 μM and then sonicated for 30 s to disperse the oxidase. The mixture was centrifuged for 10 min at 20,000 × g and 4 °C to remove undispersed lipid and titanium particles. The solution was then twice dialyzed against 100 volumes of buffer and once against 200 volumes of buffer, over 3 days.

The concentration of compound D (K₁ [H₂O₂]₈) was added to anaerobic 5 nM cytochrome oxidase vesicles (COV) were prepared as described previously (21). The production of NO from peroxynitrite was inhibited by cyanide (20 mM). Addition of azide (10 mM) gave very similar results to those observed with cyanide. This suggests that the binuclear heme a₁/a₃ center is the site of NO formation in cytochrome oxidase.

Under these conditions, a small rate of NO production was seen even in the absence of the enzyme; this appears to be a reaction between peroxynitrite and ascorbate, as it did not occur when ascorbate was left out of the solution, nor did it occur if nitrite were added instead of peroxynitrite. Under certain conditions, NO donors can be formed from the addition of NOO⁻ to primary alcohols (29, 30) such as HEPES. However, we found that there was no difference in the amount of NO produced following the addition of NOO⁻ to cytochrome oxidase in phosphate buffer or in Hepes buffer, ruling out this as a significant mechanism of NO production in this system.

NO is a reversible inhibitor of cytochrome oxidase (2, 28). However, following NOO⁻ treatment, we observed an irreversible inhibition of enzyme turnover by as much as 50% (Figs. 1A and 2). The difference between the long term effects of NO and NOO⁻ on cytochrome oxidase activity can be clearly seen in Fig. 4, where we compared treatment with the same concentration of “authentic NO” (750 nM) as that formed following the addition of 100 μM NOO⁻ to the enzyme. In a control trace, in the absence of NO or NOO⁻, we see that oxidase turnover is insensitive to O₂ concentration until this drops below 10 μM (Fig. 4A). Following the addition of 750 nM NO, added at 180 μM O₂ (Fig. 4B), the enzyme was strongly inhibited.

**RESULTS**

It has been suggested that ONOO⁻ does not inhibit cytochrome oxidase activity (9, 16). Fig. 1A shows that this is not the case. Following the addition of 100 μM NOO⁻ to actively respiring cytochrome oxidase, a transient but complete inhibition of enzyme activity was observed. The onset of enzyme inhibition occurred simultaneously with the generation of NO. The addition of 100 μM ONOO⁻ induced the generation of approximately 750 nM NO, a value far above the apparent Kᵰ for NO inhibition of cytochrome oxidase turnover at the O₂ tension used. NO is metabolized by cytochrome oxidase (24) and its substrates (7, 25–27), and as the NO was consumed the oxidase began to turn over again.

However, unlike the inhibition of cytochrome oxidase by the addition of authentic NO (where enzyme turnover is fully restored following the decay of NO; Ref. 2), cytochrome oxidase turnover following the addition of ONOO⁻ was always less than the rate prior to its addition. This shows that, unlike the reversible inhibition of cytochrome oxidase by NO, ONOO⁻ is an irreversible inhibitor (2, 28). The production of NO from ONOO⁻ by the enzyme was only marginally O₂-sensitive. 100 μM ONOO⁻ added to anaerobic 5 mM cytochrome c oxidase, in the presence of reductant, still induced the formation of approximately 500 nM NO (Fig. 1B).

Fig. 2 shows the effect of ONOO⁻ concentration on the final cytochrome oxidase turnover (when all the NO generated has decayed away). It can be seen that 100 μM ONOO⁻ caused a 50% drop in the turnover of the purified enzyme. This was a much greater drop in cytochrome oxidase activity than previously observed in both intact or sonicated mitochondria, even though in the experiments with mitochondria ONOO⁻ concentrations as high as 2 mM were used (9).

Fig. 3 quantifies the amount of NO production as a function of the added ONOO⁻ concentration. Both in the presence and absence of O₂, NO production was proportional to the added ONOO⁻ concentration. NO was not produced from ONOO⁻ that had been allowed to decompose at neutral pH prior to addition. The formation of NO from peroxynitrite was inhibited by cyanide (20 mM). Addition of azide (10 mM) gave very similar results to those observed with cyanide. This suggests that the binuclear heme a₁/a₃ center is the site of NO formation in cytochrome oxidase.

Under these conditions, a small rate of NO production was seen even in the absence of the enzyme; this appears to be a reaction between peroxynitrite and ascorbate, as it did not occur when ascorbate was left out of the solution, nor did it occur if nitrite were added instead of peroxynitrite. Under certain conditions, NO donors can be formed from the addition of NOO⁻ to primary alcohols (29, 30) such as HEPES. However, we found that there was no difference in the amount of NO produced following the addition of NOO⁻ to cytochrome oxidase in phosphate buffer or in Hepes buffer, ruling out this as a significant mechanism of NO production in this system.
inhibited until the NO concentration dropped below 300 nM. Following the complete removal of NO from the solution, the enzyme returned to its pre-inhibited rate. Addition of 100 μM ONOO\(^-\) to cytochrome oxidase under steady state turnover conditions caused the release of approximately 750 nM NO. The oxidase was inhibited until almost all the NO had left the solution (Fig. 4C). However, after all the NO had gone, the final rate was still approximately half the initial rate and the enzymes sensitivity to O\(_2\) concentration was increased. ONOO\(^-\) treatment decreased the \(V_{\text{max}}\) and significantly raised the \(K_m\) for O\(_2\). Independent measurements in a dedicated high resolution respirometer demonstrate that under these conditions the \(K_m\) for O\(_2\) in the control was 0.9 μM. After the addition of 100 μM ONOO\(^-\), the \(K_m\) had risen to 14 μM (Fig. 4D).

One possible explanation for the lowering of the \(V_{\text{max}}\) and the raising of the \(K_m\) for O\(_2\) was that we were observing an artifact due to a slow degradation of the cytochrome oxidase throughout the time course of the experiment. We tested this hypothesis by allowing an oxidase solution that had been treated with ONOO\(^-\) to become anaerobic. Following re-aeration 10 min later, the cytochrome oxidase turnover was found to have the same (inhibited) \(V_{\text{max}}\) and \(K_m\) for O\(_2\) as before. This indicates that there is no continuous degradation of cytochrome oxidase following the addition of ONOO\(^-\) during the time course of the experiment, i.e. the damage occurred immediately following ONOO\(^-\) addition.

The ability of ONOO\(^-\) to decrease the \(V_{\text{max}}\) of purified cytochrome oxidase is consistent with reports that ONOO\(^-\) inhibits cytochrome oxidase activity in astrocyte cultures, macrophages (31) and in rat brain mitochondria (32). However, Radi and co-workers (9, 33) reported that ONOO\(^-\) caused no inhibition of cytochrome oxidase in intact rat liver mitochondria. One clear difference between the data in Figs. 1–4 and these in previous studies is the presence of a membrane. We therefore investigated the effects of ONOO\(^-\) on the turnover of cytochrome oxidase in a simple proteoliposomal membrane system (with no other mitochondrial components). The use of these cytochrome c oxidase-containing vesicles (COV) allows the easy manipulation of membrane potential (ΔΨ) and ΔpH, by the addition of ion selective ionophores (21, 34). In this system, enzyme turnover in the coupled state is controlled both by ΔΨ and ΔpH; addition of the K\(^+\) ionophore, valinomycin, results in a collapse of ΔΨ and addition of the K\(^+\)/H\(^+\) exchange carrier, nigericin, collapses ΔpH. Both ionophores added together completely collapse the proton electrochemical potential (maximum uncoupling).

Fig. 5 shows the effect of 100 μM ONOO\(^-\) added to uncoupled (A) and coupled (B) COV. As with the solubilized enzyme, NO is released from the uncoupled COV and inhibits enzyme turnover. When the NO concentration drops, the COV begin to consume O\(_2\) again, although the maximum rate is still lower than that prior to the addition of ONOO\(^-\). Following ONOO\(^-\) addition, the coupled COV also produce nitric oxide, which inhibits enzyme turnover; however, irreversible inhibition by peroxynitrite does not become apparent until uncouplers are added, i.e. there is little change in the coupled rate. Presumably, under these conditions, the rate limitation by the proton motive force is more important than the effect of ONOO\(^-\).

Table I shows a systematic study of the effect of ONOO\(^-\) on NO production and cytochrome oxidase turnover when ΔΨ or...
DpH are varied. In all cases the enzyme turnover is described as a percentage of the uncoupled rate in COV that were not treated with ONOO$^{-2}$. Prior to ONOO$^{-2}$ treatment, consistent with our previous findings (21, 34), the DpH is a more effective inhibitor than DC of cytochrome oxidase turnover in COV, i.e. in the presence of nigericin their rate is over 3 times higher than that in the presence of valinomycin.

The rate of NO production in COV increased as the rate of electron turnover increased, confirming that it is a catalytic function driven by electron entry into the enzyme complex. Removing the lipid permeability barrier, by solubilizing the enzyme, had little effect on NO production. Apparently peroxynitrite has little difficulty in accessing the site of nitric oxide production in cytochrome oxidase, in the presence or absence of membrane potentials and pH gradients.

Consistent with previous studies of respiration in coupled mitochondria (9, 16), ONOO$^{-}$ inhibition of enzyme turnover is not detectable in the presence of a proton electrochemical potential. However, in all cases (including the solubilized enzyme and COV “solubilized” with detergent), the maximum uncoupled turnover is inhibited by about 50% by 100 $\mu$M ONOO$^{-}$. Thus, the liposomal membrane (and presumably by implication the mitochondrial membrane) is not a permeability barrier between peroxynitrite and the site of cytochrome oxidase inhibition.

To investigate the site of the irreversible inhibition of cytochrome oxidase with ONOO$^{-}$, we examined the optical spectral changes following the addition of ONOO$^{-}$ to oxidized enzyme, enzyme in steady state turnover and the fully reduced enzyme. Fig. 6A shows the difference spectra, with respect to the oxidized enzyme, of 50 $\mu$M ONOO$^{-}$ added to 10 $\mu$M oxidized enzyme. 5 s after the addition of ONOO$^{-}$ formation of a species absorbing at 580 nm was observed. The lineshape of this species indicates that it is ferryl cytochrome $\alpha_3$, compound F (35–37). After 15 s, an increase in the absorption at both 585 and at 605 nm was observed. After 5 min, the 605 nm peak is maximal and is larger than the 580 nm peak. Addition of the oxidant ferricyanide caused a drop in the absorbance at 605 nm, suggesting that this is due to reduced cytochrome $\alpha$. After oxidation of cytochrome $\alpha$, a small feature at 607 nm remained, consistent with the presence of the cytochrome $\alpha_3$ compound P (35–37). Concurrent with these changes in cytochrome $\alpha$ and in the ligation of the binuclear center, we observed a bleaching of the “830” nm band (inset), which is due to the presence of oxidized CuA (38, 39).

Fig. 7A compares the effects of ONOO$^{-}$ on the optical spec-
trum of the reduced cytochrome oxidase (with the reduced enzyme as the baseline). As a comparison nitrite was added to the enzyme (which in the presence of dithionite generates the heme $a_3$ nitrosyl complex; Ref. 40). Consistent with the formation of the heme $a_3$ nitrosyl species, nitrite addition caused a blue shift in 50% of the absorbance in the Soret region, a small drop at 605 nm (loss of cytochrome $a_3^{2+}$), and a small increase at 598 nm (cytochrome $a_3^{2+}NO$). The addition of ONOO$^-$ to the reduced enzyme has similarities with these features, but it is clear that events are occurring other than simple heme $a_3$ nitrosyl formation.

Fig. 8 shows the effect of ONOO$^-$ on the redox and ligation state of cytochrome oxidase under steady state turnover conditions. Fig. 8A shows the redox changes of cytochrome c, cytochromes $a$ and $a_3$ and Cu$\Lambda$, measured spectroscopically, prior to and following the addition of 250 $\mu$M ONOO$^-$. Enzyme turnover was initiated by the addition of ascorbate/TMPD to cytochrome c and cytochrome oxidase, and the solution was allowed to go anaerobic. After approximately 5 min of anaerobiosis, oxygen was rapidly re-introduced to the cuvette by the addition of 5 mM H$_2$O$_2$ in the presence of catalase. During the resultant steady state turnover, 250 $\mu$M ONOO$^-$ was added. Following ONOO$^-$ addition, an increase in the steady state reduction of cytochrome $a$, cytochrome $c$ and Cu$\Lambda$ was seen, consistent with a block in enzyme turnover at the binuclear heme $a_3/Cu$$_{b\Lambda}$ oxygen reduction site. Finally, after the sample again became anaerobic, dithionite was added.

Fig. 8B shows the difference spectra of the cytochrome oxidase in the steady state 5 s after the addition of NO or ONOO$^-$ (with the contribution of cytochrome c to the optical spectrum having been subtracted). The spectra show a combination of both cytochrome $a^{2+}$ and cytochrome $a_3^{2+}NO$. It can be seen that peak height at 605 nm increases with ONOO$^-$ concentration and the spectral shape is identical to that following the addition of "authentic NO" in the steady state. Fig. 8C shows the same spectra following further deconvolution, whereby the
contribution due to cytochrome \(a\) is removed. This deconvolution shows that cytochrome \(a_3\)\textsuperscript{2+} NO has been formed following the addition of ONOO\textsuperscript{−} to oxidase in the steady state. At higher ONOO\textsuperscript{−} concentrations, heme damage occurs. This can be seen clearly by examining the total amount of cytochromes \(a\) and \(a_3\) present in the oxidase following treatment with ONOO\textsuperscript{−}. Following the addition of dithionite, there is a drop in the total absorbance of the 605–630 nm wavepair in the ONOO\textsuperscript{−} treated enzyme compared with the control enzyme. Thus, the total amount of dithionite-reducible heme decreases after ONOO\textsuperscript{−} treatment. Following the addition of ONOO\textsuperscript{−}, the decrease in the 830 nm band would represent an increase in reduction of approximately 40%. However, the increase in the cytochrome \(c\) redox state is only 21%. Under most conditions these two centers show similar changes in redox state during enzyme turnover (41), and it therefore seems likely that some of the bleaching of the \(CuA\) 830 nm band following ONOO\textsuperscript{−} treatment represents not reduction but destruction.

**DISCUSSION**

**Conversion of Peroxynitrite to Nitric Oxide**

Cytochrome \(c\) oxidase catalyzes the formation of NO from ONOO\textsuperscript{−}. This occurs when the enzyme is fully reduced and in the catalytic steady state, but not when the enzyme is fully oxidized. It is difficult to quantify exactly the rate and extent of NO formation due to the relatively slow \(t_{1/2} = 15\) s response time of the NO electrode. In addition, NO itself reacts rapidly with ONOO\textsuperscript{−} to produce \(NO_2\) and \(NO_2\) (42) and, thus, increasing the ONOO\textsuperscript{−} concentration increases both the rate of NO formation and the rate of its decay. However, it is clear that NO production by cytochrome oxidase competes with non-enzymatic ONOO\textsuperscript{−} breakdown pathways and the amount of NO produced is dependent on the rate of electron entry into the enzyme from reduced cytochrome \(c\). Furthermore, NO formation is inhibited by both cyanide and azide, indicating that the immediate source of electrons is the binuclear CuA/heme \(a_3\) center. Although electrons can convert nitrosylated thiols to yield free NO, we feel that this is an unlikely pathway here as there is no obvious mechanism to generate RS-NO species from ONOO\textsuperscript{−} (43–45). The lack of a very strong oxygen concentra-
**Peroxynitrite and Cytochrome Oxidase**

**TABLE I**

Irreversible inhibition of proteoliposomal or solubilized cytochrome c oxidase by 100 μM peroxynitrite and extent of NO release

| Conditions     | ΔΨ present | ΔpH present | Uncontrolled turnover | [NO] Released |
|----------------|------------|-------------|-----------------------|---------------|
| Uncoupled COV | –          | –           | 100                   | 48, 590       |
| 100 μM ONOO⁻  | –          | +           | 9                     | 11, 180       |
| Coupled COV   | +          | +           | 11                    | 11, 180       |
| 100 μM ONOO⁻  | –          | +           | 63                    | 11, 180       |
| Uncoupled COV | –          | –           | 36                    | 37, 54        |
| COV + 15 nm valinomycin | – | + | 11 | 11, 180 |
| 100 μM ONOO⁻  | –          | –           | 11                    | 11, 180       |
| Uncoupled COV | –          | –           | 36                    | 37, 54        |
| COV + 100 nm nigericin | – | – | 120 | 65, 445 |
| 100 μM ONOO⁻  | –          | –           | 120                   | 50, 750       |
| Uncoupled COV | –          | –           | 100                   | 50, 750       |
| Solubilized COV | – | – | 120 | 65, 445 |
| 100 μM ONOO⁻  | –          | –           | 120                   | 50, 750       |
| Solubilized oxidase | – | – | 100 | 50, 750 |

- COV were fully uncoupled by the addition of 1 μM valinomycin and 1 μM nigericin.
- Addition of 0.1% lauryl maltoside to COV.
- Purified enzyme in 0.1% lauryl maltoside.

Inhibition of Cytochrome Oxidase Turnover by ONOO⁻

Cytochrome c oxidase inhibition by ONOO⁻ occurs in two phases. Initially, there is a transient inhibition of turnover caused by the cytochrome oxidase-catalyzed production of NO. The $K_{e}^{uncoupled}$, that we observe in these studies, and the detection of the nitrosyl heme $a_3$ complex in the steady state are all consistent with previous studies on the reversible, competitive (with oxygen) inhibition of the enzyme by NO that has been previously described by ourselves and others (2, 28). The production of NO and subsequent inhibition of turnover is not restricted to the purified enzyme system. For example, when ONOO⁻ is added to mitochondria respiring on succinate, it is evident in the oxygen electrode trace that there is a complete inhibition of mitochondrial oxygen consumption (see Fig. 2 in Ref. 9). This inhibition goes undetected if oxygen consumption rates are only measured some time after ONOO⁻ addition (9).

Unlike NO, ONOO⁻ is not a reversible inhibitor of cytochrome oxidase. After NO has disappeared from the solution, cytochrome oxidase turnover does not return to the initial rate. ONOO⁻ causes concentration-dependent change in both $K_{m}$ and $V_{max}$ The $K_{m}$ for $O_{2}$ is increased, and the $V_{max}$ is lowered (Figs. 2 and 4). These changes in $V_{max}$ occur when enzyme turnover is measured either polarographically or spectrophotometrically. The spectroscopic data show that there is a loss in the cytochrome $a$ and $a_3$ content of ONOO⁻-treated oxidase.

This indicates that ONOO⁻ or one of its short-lived breakdown products are responsible for the heme degradation. However,
the steady state kinetics are not consistent with simple enzyme inactivation, as, if this were the case, there would be a $V_{\text{max}}$ increase with no change in the $K_m$ for oxygen (46).

A possible clue as to the mechanism for the raised $K_m$ can be found in the proteoliposomal studies. COV displayed a similar O$_2$ consumption profile to that of the solubilized enzyme. As in the case of the solubilized enzyme, treatment of COV with ONOO$^-$ caused a drop in the $V_{\text{max}}$ and an increase in the $K_m$ for O$_2$. These effects were only apparent when COV turnover was not under control of DC and/or DpH. Only small changes in cytochrome oxidase turnover are seen following ONOO$^-$ addition to COV under controlled conditions. Previous studies suggesting that ONOO$^-$ had no effect on cytochrome oxidase turnover (9) were performed on mitochondria in coupled (state 3) conditions and therefore would have missed the inhibition observed in this paper.

The fact that peroxynitrite inhibition is primarily observed in the uncoupled system suggests that it may inhibit in a similar manner to the mitochondrial membrane potential. The presence of a mitochondrial membrane potential increases the $K_m$ for O$_2$ of cytochrome oxidase (47), presumably via effects on the electron transfer rates to and from the binuclear center. Slowing the rate of electron transfer to heme $a_3$ has also recently been shown to raise the $K_m$ for O$_2$ in homologous bacterial cytochrome oxidases (48). Therefore, we suggest that one possibility is that the main inhibitory effect of ONOO$^-$ is due to a decrease in the intramolecular electron transfer rates in the enzyme, with a consequent raising of $K_m$ for O$_2$. In coupled proteoliposomes or mitochondria, intramolecular electron transfer rates already limit enzyme turnover and therefore the effects of ONOO$^-$ are less dramatic. Direct effects of ONOO$^-$ on the oxygen reaction site cannot, however, be ruled out.

An alternative explanation of the proteoliposomal data is that ONOO$^-$ damages the lipid membrane and therefore, si-

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**FIG. 6.** Effect of peroxynitrite on the optical spectrum of oxidized cytochrome c oxidase. 10 μM cytochrome c oxidase was incubated in 100 mM K$^+$-phosphate, 20 μM DTPA, 0.015% lauryl maltoside, 30 °C. 50 μM ONOO$^-$ was added and the changes in the optical spectrum were recorded (A). After 12 min, 20 μM ferricyanide was added. The inset shows the changes in the 830 nm band. The spectrum was normalized to 730 nm, and a linear base-line correction was used to make the 930 nm absorbance equal to zero. B, changes in the cytochrome a redox state, the loss of Cu$^{2+}$, and the production and loss of compound F.
multaneous with enzyme inhibition (decreasing turnover) there is uncoupling (increasing turnover). However, it would seem fortuitous that these effects would balance out evenly, resulting in the observed lack of effect on enzyme turnover.

In summary, the irreversible effects on the $V_{\text{max}}$ and $K_{\text{m}}$ for cytochrome oxidase by peroxynitrite can be best explained by a combination of enzyme inactivation (decreased $V_{\text{max}}$) and enzyme modification (raised $K_{\text{m}}$).

Spectroscopic Effects of ONOO$^-$ on Cytochrome Oxidase

Oxidized Enzyme—The reason for the complex inhibitory pattern described above becomes apparent when one observes the effect of ONOO$^-$ on the optical spectrum of cytochrome oxidase. Peroxidases and catalases form two intermediates following peroxide addition, termed compound I and II. Compound I is a ferryl iron and a cation free radical and is two electrons more oxidized than the ferric state. Compound II is ferryl iron alone and therefore only one electron more oxidized than the ferric enzyme. In cytochrome oxidase the equivalent oxidation state to compound I is termed the P intermediate and has been described (49) as a stable heme-peroxide adduct or a ferryl intermediate (with CuB in the $3^+$ state). There is general agreement that the equivalent of compound II, the F intermediate, is a ferryl iron species.

ONOO$^-$ causes the formation of Compound II in catalase (50), myeloperoxidase, and lactoperoxidase (42). Compound I is generated in horseradish peroxidase (42). Thus ONOO$^-$ can act as a one-electron or two-electron oxidant of ferric heme proteins. Both events occur in cytochrome oxidase. The addition of ONOO$^-$ to oxidized cytochrome oxidase causes the formation of

![Figure 7](image_url)

**Fig. 7.** Effect of peroxynitrite and NO on the spectrum of reduced cytochrome c oxidase. 10 μM cytochrome c oxidase was incubated in 100 mM K$^+$-phosphate, 20 μM DTPA, 0.015% lauryl maltoside, 30 °C. To this was added 30 μM dithionite, and the oxidase was allowed to become fully reduced by incubation for 15 min. ONOO$^-$ or 1 mM nitrite (to generate NO) was then added and changes in the optical spectra were recorded. Panel A compares the dithionite reduced difference spectra generated with 100 and 500 μM ONOO$^-$, compared with that generated by nitrite (resulting in the NO complex). Panel B shows the absolute spectra.
ferryl cytochrome \(a_3\), compound \(F\). The most likely reaction of ONOO\(^-\) with oxidized cytochrome oxidase is therefore the direct one-electron oxidation of cytochrome \(a_3\), leading to the formation of compound \(F\), as shown below in Reaction 3.

\[
\text{ONOO}^- + a_3^{3+} \rightarrow a_3^{4+} = \text{O}^{2-} + \text{NO}_3^-
\]

**REACTION 3**

Additionally, the slow time course of the formation of compound \(F\) suggests that \(F\) is still being formed when ONOO\(^-\) is no longer present in solution. Control studies demonstrated that \(\text{H}_2\text{O}_2\) is not present in our ONOO\(^-\) solutions, nor is it a breakdown product of its decomposition. Therefore, there must be an intermediate species formed that is spectrally indistinguishable from the oxidized enzyme, *e.g.*, a high spin heme adduct.
In addition, a small amount of compound \( P \) is also observed when \( \text{ONOO}^- \) is added to the oxidized enzyme. This is probably due to the two-electron oxidation of cytochrome \( a_3^{3+} \), shown in Reaction 4.

\[
\text{H}_2\text{O} + \text{ONOO}^- + a_3^{3+} \rightarrow a_3^{2+} = \text{O}_2 + \text{H}^+ + \text{HNO}_2
\]

**REACTION 4**

We consider Reactions 3 and 4 as the simplest explanation of the data. However, it is not possible to exclude completely a mechanism where one of the species, \( P \) or \( F \), is produced from \( \text{ONOO}^- \) and a side reaction with another species in the solution performs the requisite one-electron oxidation or reduction leading to their interconversion.

Although the optical spectra we observe are consistent with the known oxygenated intermediates described above, we cannot entirely rule out the possibility that there is some contribution from novel species, perhaps due to the \( \text{NO}_2 \) produced via Reaction 3 binding to the enzyme. However, a more likely role for \( \text{NO}_2 \) is to catalyze the slow reduction of a fraction of heme \( a \) (Fig. 6) that occurs long after the added \( \text{ONOO}^- \) has decayed (the half-life is approximately 1 s at pH 7). This is consistent with oxidation of \( \text{NO}_2 \) by cytochrome oxidase to produce \( \text{NO}_3^- \) (Reaction 5).

\[
\text{H}_2\text{O} + \text{NO}_2 + a^{3+} \rightarrow a^{2+} + 2\text{H}^+ + \text{NO}_3^-
\]

**REACTION 5**

None of the species described above is likely to be inhibitory, as they are all intermediates present in the normal enzyme cycle and would be rapidly reduced or oxidized once \( \text{ONOO}^- \) was removed from the solution. However, the loss of the \( \text{Cu}^{2+}_A \) near-infrared absorbance band following the addition of \( \text{ONOO}^- \) is more serious, as it is not restored by the addition of the oxidant ferricyanide. Therefore, it represents destruction, not reduction, of this center. This suggests that \( \text{ONOO}^- \) reacts directly with \( \text{Cu}^{2+}_A \), presumably causing oxidation of the thiol ligands. Higher concentrations of \( \text{ONOO}^- \) added to the oxidized enzyme cause more profound optical changes in the spectra of cytochromes \( a \) and \( a'_p \) indicating heme degradation. However, the loss of the \( \text{Cu}^{2+}_A \) occurs at relatively low \( \text{ONOO}^- \) levels and may therefore be a more important inhibitory pathway.

**Reduced Enzyme**—Addition of \( \text{ONOO}^- \) to the dithionite reduced enzyme showed nitrosyl heme \( a'_p \) formation, as expected by the formation of NO in the solution. However, somewhat surprisingly modification of cytochrome \( a \) by relatively low concentrations of \( \text{ONOO}^- \) was observed. A 10:1 excess of
ONOO\textsuperscript{−} to cytochrome oxidase induced the total loss of reduced cytochrome \(a\). The resulting spectrum indicated the formation of a new species with an absorbance peak at 596 nm. This spectrum may be a new heme derivative of cytochrome \(a\) (perhaps due to the formation of the formyl group), or it may be due to ligand exchange (perhaps NO is replacing histidine). We favor the latter hypothesis, as the symmetrical peak in the visible region suggests that heme \(a\) and heme \(a\) have similar ligation states.

**Enzyme in Steady State Turnover**—The optical spectral changes in the steady state confirm that peroxynitrite is an inhibitor of cytochrome oxidase. Initially following the addition of ONOO\textsuperscript{−}, the cytochrome \(a_2\) NO\textsuperscript{−} complex is observed. This disappears as NO disappears from the solution. However, the redox levels of cytochrome \(a\) and cytochrome \(c\) do not return to their previous level. Both centers are more reduced, demonstrating that inhibition of cytochrome oxidase by ONOO\textsuperscript{−} is occurring downstream of cytochrome \(a\). This is consistent with our interpretation of the kinetic data and suggests that ONOO\textsuperscript{−} damage results in a decrease in the electron transfer rate to the binuclear center, either due to modification of the enzyme between heme \(a\) and the binuclear center, or due to damage at the binuclear center itself.

At higher levels of peroxynitrite, more severe effects on the enzyme in turnover are clearly observed. Heme degradation is apparent, as is the loss of the 830 nm band due to Cu\(A\). Again, this is consistent with the notion that enzyme degradation results in the decrease in \(V_{\text{max}}\).

**Relevance to the Cell**

In this paper we demonstrate that ONOO\textsuperscript{−} is capable of irreversibly inhibiting purified cytochrome oxidase. The effect on the \(K_m\) for O\(_2\) is especially relevant as much of the ONOO\textsuperscript{−} inhibition observed in this paper would have been missed in the previous studies using cell/tissue homogenates or mitochondria, where unphysiologically high oxygen tensions were used to assay the enzyme’s activity. Our results have important implications for the mechanism of irreversible damage to mitochondrial respiration induced by NO addition. Damage to cytochrome oxidase activity following prolonged NO treatment of cells (51) and mitochondria (52) has been previously reported (reviewed in Ref. 53). As NO is a reversible inhibitor of cytochrome oxidase (2), we suggest that the formation of the irreversible inhibitor ONOO\textsuperscript{−} is the most likely explanation of these findings. ONOO\textsuperscript{−} is generated when NO is added to respiring mitochondria, due to the production of superoxide from the inhibited respiratory chain (4). In addition, ONOO\textsuperscript{−} can be generated from the reaction of O\(_2\) and NO\textsuperscript{−} when the latter is formed via reduction of NO by ferrocytochrome \(c\) (7). Formation of ONOO\textsuperscript{−} from NO may therefore lead to long term irreversible effects of NO on mitochondrial oxygen consumption by cytochrome oxidase with possible pro-apoptotic consequences (54). Given the ability of ONOO\textsuperscript{−} to increase the enzyme’s \(K_m\) for O\(_2\), these damaging consequences may become more apparent when the oxygen tension is also reduced in the cell (e.g., due to hypoxia or ischemia).

However, we feel that of equal importance to cellular respiration is our finding that cytochrome oxidase can catalyze the rapid reduction of ONOO\textsuperscript{−} to NO. Thus, cytochrome oxidase can play a role in ONOO\textsuperscript{−} detoxification in vivo, converting ONOO\textsuperscript{−} to the less toxic NO; the latter can in turn react with another molecule of ONOO\textsuperscript{−} to produce nitrite and NO\textsuperscript{2−}. Low concentrations of ONOO\textsuperscript{−} can therefore be safely detoxified by cytochrome oxidase.

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