The Peroxynitrite Reductase Activity of Cytochrome c Oxidase Involves a Two-electron Redox Reaction at the Heme $a_3$-Cu$_B$ Site*

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FULLY AND PARTIALLY REDUCED FORMS OF ISOLATED BOVINE CYTOCHROME C OXIDASE UNDERGO A TWO-ELECTRON OXIDATION-REDUCTION PROCESS WITH ADDED PEROXYNITRITE, LEADING TO CATALYTIC OXIDATION OF FERROCYTOCHROME C TO FERRICYTOCHROME C. THE OTHER MAJOR REACTION PRODUCT IS NITRITE, 86% OF THE ADDED PEROXYNITRITE BEING MEASURABLY CONVERTED TO THIS SUBSTANCE. THE REACTION IS INHIBITED IN THE PRESENCE OF CYANIDE, IMPLICATING THE HEME $a_3$-Cu$_B$ BINUCLEAR PAIR AS THE ACTIVE SITE. Moreover, provided peroxynitrite is not added to excess, the reductase activity of the enzyme toward this oxidant efficiently protects other protein and detergent molecules IN VITRO FROM NITRATION OF TYROSINE RESIDUES AND OXIDATIVE DAMAGE. If the enzyme is exposed to ~10$^2$-fold excesses of peroxynitrite, then significant irreversible loss of electron transfer activity results, and the heme $a_3$-Cu$_B$ binuclear pair no longer undergo a characteristic carbon monoxide-driven reduction. The accompanying rather small changes in the observed electronic absorption spectrum are suggestive of a modification in the vicinity of one or both hemes but probably not to the cofactors themselves. This is not exclusive of undesirable cytotoxic events will occur (15–19). In an effort to begin addressing this problem, we have undertaken an investigation of the functional and electronic spectral changes observable for air-oxidized and dithionite-reduced forms of cytochrome c and cytochrome c oxidase following their reaction with ONOO$^-$, although it is apparent from a recent study by Sharpe and Cooper (20) that very high levels of ONOO$^-$ irreversibly inhibit cytochrome c oxidase and may degrade the cofactors present, it is less clear how stoichiometric and substrate-specific amounts of ONOO$^-$, i.e. more physiologically relevant levels, affect the enzyme. In this investigation we have examined the products of the reactions involving low levels of ONOO$^-$ with ferrocytochrome c and reduced cytochrome c oxidase.

EXPERIMENTAL PROCEDURES

Cytochrome c oxidase (respiratory complex IV) was prepared from beef heart as described previously (method 2) (21). Crystalline, bovine cytochrome c was obtained from Sigma (type III) and used without further purification. Lauryl maltoside was obtained from Anachema. Stable, hydrogen peroxide-free, alkaline (pH ~12) solutions of sodium peroxynitrite were prepared following the recommendations of Beckman et al. (22) using ACS Certified reagents supplied by Aldrich and/or Fisher. Sodium dithionite was obtained from EM Science and used under anaerobic conditions to make up solutions volumetrically, employing the manufacturer’s assay (93%) to calculate dithionite ion concentration. Concentrations of cytochrome c oxidase, ferrocytochrome c, and peroxynitrite were determined using, respectively, the extinction coefficients $\Delta$ext$^{280}$ = 24 mm$^{-1}$ cm$^{-1}$ (reduced minus oxidized, total heme) (23), $\epsilon$280 = 28 mm$^{-1}$ cm$^{-1}$ (24), and $\epsilon$252 = 1.67 mm$^{-1}$ cm$^{-1}$ (25). Determinations of nitrite ion concentration were performed by a modification of the method of Greiss (26). First, protein was precipitated by heating aqueous solutions to near boiling and removed by centrifugation. Next, 100-μl aliquots of the supernatant were mixed with 100 μl of the Greiss reagent (0.1% N$^+$-1-naphthylethenediamine hydrochloride, freshly mixed with an equal volume of 1.0% sulfanilamide in 5% phosphoric acid). Nitrite concentrations were then determined colorimetrically at 550 nm by comparison with a sodium nitrite standard curve. Ferrocytochrome c:O$_2$ oxidoreductase activity was determined employing the high ionic strength conditions of Sinjorgo et al. (27). SDS-polyacrylamide gel electrophoresis separation of cytochrome c and the subunits of its oxidase was performed according to the method of Schagger and von Jagow (28). Following either slot or Western blotting onto nitrocellulose, formation of 3-nitrotyrosine was observed by an immunochromatography staining procedure employing a nitrotyrosine polyclonal antibody purchased from Upstate Biotechnology, Inc. (29). Anaerobic conditions were achieved by gently blowing argon over the top of solutions at room temperature for 10–15 min, with mild agitation. In the case of experiments where cytochrome c oxidase is initially reduced, the solution can be thought of as containing an intrinsic scrubbing system, able to further scavenge any oxygen that might otherwise be present (see “Results”). Anaerobic samples were maintained in glass vessels closed with rubber septa. Quantitative transfers of sodium dithionite and sodium peroxynitrite solutions were made with Hamilton gas-tight syringes fitted with new stainless steel needles that were used for one transfer or a single titration procedure, then discarded. At no time were solutions of these reagents left standing in contact with syringe needles. The pH of all protein samples was verified at the end of experiments to ensure that conditions had not become significantly more alkaline by additions of peroxynitrite solution.

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RESULTS

The position of the Soret band maximum observed in samples of beef heart cytochrome c oxidase (as prepared) is variable and depends upon a number of factors that are incompletely understood (30, 31). The present maximum at 422 nm (Fig. 1, blue trace) is representative of the fully oxidized, resting (“slow” cyanide binding), monodispersed, 13-subunit “monomer” (21, 32). After the addition of a 50-fold excess of ONO$_2$ over total heme ($a + a_3$) at pH 7–8.5, the Soret band sharpened, increased in intensity, and exhibited a new maximum at 426–427 nm (black trace). This species was unstable, however, persisting just long enough to permit the recording of its absorption spectrum. After a couple of minutes, the Soret band shifted to a new position at 418 nm (red trace), and the new derivative was stable for hours at room temperature. Although the difference between the spectrum of the resting enzyme and that of the second species obtained following ONO$_2$ addition is clearly small, the result was entirely reproducible. The spectra of the peroxynitrite-treated derivatives were found to be the same if the spectrum of the starting enzyme exhibited a 424-nm Soret band maximum observed in samples following ONO$_2$ additions at pH 7.4 to all samples in exactly the same manner, quickly, with agitation. Subsequently, where necessary, the pH was readjusted at the start of the activity assay procedure.

The quoted excesses are ONO$_2$ added over total heme ($a + a_3$). For example, a typical experiment involved taking a solution of the enzyme 12 μM in total heme and adding peroxynitrite to a concentration of 60 μM, thereby achieving a 50-fold excess.

![Fig. 1. Electronic absorption spectra at 22 °C, 1.0-cm path length. Cytochrome c oxidase in 100 mM sodium phosphate buffer, pH 7.4, 0.05% in lauryl maltoside. Blue, 7.6 μM resting enzyme; black, immediately after the addition of sodium peroxynitrite to 0.38 mM; red, 2 min after peroxynitrite addition.](image)

![Fig. 2. Time courses for CO-driven reduction of 5.5 μM cytochrome c oxidase samples in 100 mM sodium phosphate buffer, 1.0 mM in EDTA, 0.05% lauryl maltoside. △, resting enzyme at pH 7.4; ▲, resting enzyme at pH 8.5; ●, peroxynitrite-inhibited enzyme maintained at pH 7–8.5. The inhibited enzyme was prepared with a 100-fold excess of peroxynitrite as described in footnotes b and c of Table I. Reactions were followed spectrophotometrically at 430 nm, the Soret maximum of the partially reduced CO adduct; 1.0-cm path lengths. The ordinate corresponds to the mole fraction of total oxidized metal centers (Cu$_\alpha$ + heme $a$ + heme $a_3$ + Cu$_\mu$).](image)

Table I. Activity assays of cytochrome c oxidase samples at 20 °C

| pH$^c$ | As isolated | 50-fold$^d$ ONO$_2$ | 200-fold$^d$ ONO$_2$ |
|-------|-------------|---------------------|---------------------|
| 8.5   | 20          | 240                 | 80                  |
| 7.4   | 280         | 690                 | 150                 |
| 6.0   | 690         |                     |                     |

$^a$ Determined at 20 °C in 0.1 M sodium phosphate buffer, 0.05% lauryl maltoside, as recommended by Sinjorgo et al. (27). Values indicate $k_{cat}$ (s$^{-1}$).

$^b$ The ONO$_2$ additions were made at pH 7.4 to all samples in exactly the same manner, quickly, with agitation. Subsequently, where necessary, the pH was readjusted at the start of the activity assay procedure.

$^c$ The quoted excesses are ONO$_2$ added over total heme ($a + a_3$). For example, a typical experiment involved taking a solution of the enzyme 12 μM in total heme and adding peroxynitrite to a concentration of 60 μM, thereby achieving a 50-fold excess.

One well documented reaction of proteins with peroxynitrite is the covalent modification of tyrosine residues to form nitrotyrosine (22). The results of a nitrotyrosine-sensitive immunostaining analysis showed clear evidence for the formation of nitrotyrosine in cytochrome c samples exposed to both substoichiometric and excess amounts of peroxynitrite (Fig. 3, lanes C to E). Samples containing cytochrome c oxidase, on the other hand, showed no evidence for the formation of nitrotyrosine (lane B) unless the oxidant was added in excess over the available reduced metal centers (lane A). SDS-polycrylamide gel electrophoresis resolution of peroxynitrite-treated cytochrome c oxidase into its subunits followed by Western blotting before the immunostaining (not shown) revealed that faint staining was mostly associated with subunits that do not contain the catalytic metal centers.

The direct reaction between cytochrome c oxidase and sodium dithionite is quite slow. Consequently, it is impractical to attempt controlled reduction of the oxidase by dithionite without added cytochrome c to catalyze the electron transfer. Under anaerobic conditions, a 1:1 mixture of cytochrome c:cytochrome c oxidase can be quantitatively reduced by a small excess (<5%) of sodium dithionite. The absorption spectrum of the solution (Fig. 4, broken line) is the simple sum of the individual spectra of the reduced heme proteins; that is, there is no indication of any interaction between the cytochrome c and the oxidase hemes. Maintaining anaerobic conditions, the addition of a small excess (5–10%) of sodium peroxynitrite initially resulted in the formation of a metastable species exhibiting an absorption spectrum with a distinct double Soret feature (solid line). Interestingly, the lower energy feature appeared at ~426 nm, very like the maximum observed after the addition of ONO$_2$ to the oxidized enzyme. After a period of 1–2 min, still...
maintaining anaerobic conditions, the intermediate had disappeared, and the final spectrum (dotted line) was that of the air-stable enzyme, not like the final form ($\lambda_{\text{max}} = 418$ nm) of Fig. 1. It should be noted that, provided anaerobic conditions were suitably maintained, it was routinely possible to quantitatively cycle between reduced and oxidized derivatives several times by alternate additions of dithionite, then ONO$_2^-$, with no visible indication (i.e., neither precipitation nor turbidity) of the proteins becoming denatured. The addition of ONO$_2^-$ to ferrocytochrome $c$ in the absence of its oxidoase also led to oxidation of the hemoprotein to ferriytochrome $c$ (Fig. 5), but a larger excess of the oxidant was required. The spectrum of the ONO$_2^-$-oxidized derivative (solid trace) is identical to the published spectrum of the ferricyanide- or air-oxidized protein (24), including the unchanged appearance of the 695-nm band that is sensitive to changes in conformation and axial ligands to the heme (37).

Fig. 6 shows the results of an anaerobic titration of dithionite-reduced cytochrome $c$ oxidase with ONO$_2^-$ in the presence of ferrocytochrome $c$ ( ). One molar equivalent of cytochrome $c$ was added to a molar equivalent of the oxidase (i.e., 2 molar equivalents of heme $a + a_3$). Next, dilute sodium dithionite solution was titrated into the solution until the cytochromes were just fully reduced, as determined by no further increases being observed in the measured absorbances at 550 and 604 nm. Subsequent oxidation of the cytochromes was monitored by following the decrease in absorbance at these same wavelengths. The mole fraction of ferrocytochrome $c$ was calculated as $1 - (A_{\text{final}} - A_{\text{initial}})/(A_{\text{max}} - A_{\text{initial}})$, taking the absorption measurements at 550 nm. The mole fraction of oxidized cytochrome $c$ oxidase was calculated employing the same general expression but using absorption changes measured at 604 nm. In the axis legends, “redox centers” refers to the complete set of metalloprotein oxidation-reduction centers present in the mixture of cytochromes (i.e., heme $c + Cu_A +$ heme $a + a_3 + Cu_B$). The solid line represents the theoretical relationship predicted for ONO$_2^-$ behaving as a two-electron acceptor (e.g., ONO$_2^-$ + 2H$^+$ + 2e$^-$ → NO$_2^-$ + H$_2$O). In experiments where substoichiometric additions of the oxidant were made to reduced cytochrome $c$ oxidase, subsequent nitrite determinations established that 0.86 equivalents (S.D., 0.05; $n = 3$) of the added ONO$_2^-$ was measurably converted to nitrite ion. For comparison, the results of control titrations of ferriytochrome $c$ (alone) with ONO$_2^-$ under aerobic ( ) and anaerobic ( ) conditions are also shown. The broken line represents the theoretical relationship predicted for ONO$_2^-$ behaving as a one-electron acceptor (e.g., ONO$_2^-$ + 2H$^+$ + e$^-$ → NO$_2^-$ + H$_2$O). It was possible to perform an experiment under aerobic conditions, because the direct reaction between ferriytochrome $c$ and molecular oxygen is slow. The pH at the beginning of these titrations was 7.4 and at the end 7.8. It was verified that the spectra of none of the species present exhibited any significant pH-dependent behavior in the relevant range. Clearly, ONO$_2^-$ behaves as a two-electron acceptor toward ferriytochrome $c$ and reduced cytochrome $c$ oxidase when they are present together. Moreover,
the reaction is reasonably facile, at least faster than the uncatalyzed decomposition rate of ONO$_2^{-}$, the half-life of which is estimated to be $\leq 1$ s at physiological pH, depending upon the particular buffer composition (16, 22). It is also clear that ONO$_2$ does not behave as a two-electron acceptor toward ferrocyanochrome c in the absence of its oxidase. The negative divergence of the ferrocyanochrome c data from the theoretical line apparent in Fig. 6 shows an increased tendency of added ONO$_2$ to isomerize to nitrate in solution as the available ferrocyanochrome c becomes depleted and, therefore, indicates a slow reaction with the hemoprotein. Consequently, taken together, the titration data of Fig. 6 demonstrate that cytochrome c oxidase actually catalyzes the oxidation of ferrocyanochrome c by ONO$_2$.

**DISCUSSION**

Reaction of ONO$_2$ with Oxidized Cytochrome Oxidase—Our current data concerning the consequences of the reaction between excess ONO$_2$ and air-stable preparations of cytochrome c oxidase seem to be generally in accord with the findings of Sharpe and Cooper (20). In both studies the addition of excess oxidant has been found to lead to distinct spectral changes and irreversible inhibition of electron-transfer activity. The rather subtle change observed in the electronic absorption spectrum following exposure to ONO$_2$ (Fig. 1) is indicative of a structural modification in the vicinity of one or both hemes but probably not heme degradation, as the intensity of the band is maintained. The immunostaining results show that nitrination of a tyrosine residue is apparently not the modification in question because subunit I, where the hemes are located, does not retain any immunoreactivity (data not shown).

Complete inhibition of the CO-driven reduction reaction following exposure of the enzyme to $\sim 10^{3}$-fold excesses of ONO$_2$ (Fig. 2) clearly identifies the heme $a_4$-Cu$_{a_4}$ pair as a modified site. Interestingly, however, treatment with this excess of oxidant merely results in partial loss of electron-transfer activity (Table I). The observation that these two functional characteristics exhibit distinguishable patterns of inhibition by ONO$_2$ indicates that they are mediated by different structural factors. It follows that the reaction of the oxidized enzyme with ONO$_2$ involves at least two structural modifications to subunit I. Only one of these need be a modification to the polypeptide chain; the other could be an ensuing conformational change. The marked pH dependence of the CO-driven reduction of cytochrome c oxidase as isolated has not previously been reported. At pH 8.4, a pronounced lag phase precedes any reduction, which then occurs significantly faster than at pH 7.4 (Fig. 2). A plausible explanation for the lag phase is that the reaction must involve an activation step (e.g., a conformational change in the active site structure) before electron transfer from CO. The activation step is seemingly more readily achieved at pH 7.4 than 8.4. The half-reaction involving CO generates two protons (34): CO + H$_2$O $\rightarrow$ CO$_2$ + 2H$^+$ + 2e$^-$. Consequently, it is reasonable to suggest that the accelerated CO-driven reduction rate observed under the more alkaline conditions is due to the presence of one or more basic groups able to assist in the abstraction of protons. Derivatization of any such group would then facilitate an explanation of the loss of CO-driven reduction activity following exposure to ONO$_2$. However, the present data is ambiguous in this matter, and the possibility that the effect of ONO$_2$ modification is to produce a more stable, nonactivated conformation (i.e., the resulting lag phase is infinitely long) cannot be excluded.

Reaction of ONO$_2$ with Reduced Cytochrome Oxidase—From the previous study of Sharpe and Cooper (20), it is clear that some limited NO production is observed when large amounts of ONO$_2$ are presented to cytochrome c oxidase under turnover conditions. Only 0.75% of the total ONO$_2$ added was detected as NO, and despite the addition of ONO$_2$ to greater than $10^4$-fold excess over the enzyme, there was still very substantial oxidase activity following the disappearance of NO (and ONO$_2$) from the assay solutions. Although it is certainly possible that cytochrome c oxidase could catalytically convert ONO$_2$ to NO, there are other possible explanations for the production of this species. Adventitiously bound copper ions that are inevitably found in cytochrome c oxidase preparations (40) generate NO-donor species when exposed to ONO$_2$ and HEPES buffer (41). Alternatively, in agreement with Sharpe and Cooper (20), we have shown that greater than $10^2$-fold excesses of ONO$_2$ over the enzyme leads to a modification of the active site of cytochrome c oxidase. This modified form of the enzyme could be responsible for the observed NO production.

The titration data of Fig. 6 show that the reaction between ONO$_2$ and reduced cytochrome c oxidase in the presence of one equivalent of ferrocyanochrome c is a two-electron process. This statement applies equally to the fully reduced enzyme and the various partly reduced majority species present at different stages of the titration. The observed increase in nitrite concentration (equivalent to 86% of the added ONO$_2$) is in good agreement with the electron count determined by the titration and suggests the half reaction involving ONO$_2$ to be: ONO$_2$ + 2H$^+$ + 2e$^- \rightarrow NO_2^- + H_2O$.

The half-reaction involving ONO$_2$ to be: ONO$_2$ + 2H$^+$ + 2e$^- \rightarrow NO_2^- + H_2O$. Given the nature of the active site, namely, a binuclear pair, this appears to be a very reasonable result indeed. If there are additional minor side reactions involving one-electron processes, for example, the titration data requires that these account for less than about 5% of the total ONO$_2$ consumed.

During normal aerobic respiration, the electron-accepting substrate for cytochrome c oxidase is molecular oxygen. The question then arises as to the rate of the reaction of cytochrome c oxidase with added ONO$_2$, how this reaction rate compares to that of the enzyme with its primary substrate, oxygen. Using available data from current as well as other studies enables us to calculate a reasonable estimate of the magnitude of the reaction velocity. At physiological pH, ONO$_2$ has a half-life of $\leq 1$ s; therefore, the first-order rate constant for its decomposition is given by $k_1 \geq \ln(2)/1 = 0.69$ s$^{-1}$. At approximately 10 $\mu$M cytochrome c oxidase concentrations and equimolar added ONO$_2$, all oxidant appeared to react with the protein cofactors. Assuming the reaction to be of second-order molecularity, this implies a lower limit on the catalyzed decomposition rate of $10 \times 10^{-6} M \times 0.69 \ s^{-1} = 6.9 \times 10^{-6} M s^{-1}$. Therefore, the catalyzed decomposition is described by the rate-law inequality $k_2 \times [\text{enzyme}] \times [\text{ONO}_2] > 6.9 \times 10^{-6} M s^{-1}$. Typically, in the titration experiments, the enzyme concentration was $\sim 10 \mu M$, and ONO$_2$ additions were made to achieve instantaneous concentrations of $\sim 2 \mu M$. It follows that the second-order rate constant for the reaction between reduced cytochrome c oxidase and ONO$_2$ is given by $k_2 > 6.9 \times 10^{-9}/(10^{-5} \times 2 \times 10^{-4}) = 3.4 \times 10^{5} M^{-1} s^{-1}$. In another experiment, a solution equimolar in cyanide and ONO$_2$ (verified to be stable for several minutes by monitoring the absorption spectrum of the ONO$_2^{-}$) was added to a reduced cytochrome c oxidase solution to achieve a 10-fold excess of both cyanide and ONO$_2$ over heme $a_4$. Oxidation of about 30% of the total cytochromes present was observed, indicating the rate of cyanide inhibition to be comparable to, but faster than, the reaction of the enzyme with ONO$_2$. The reported rate constants for the reaction of cyanide (as HCN) with the fully reduced and turnover enzyme are $\sim 10^{2}$ M$^{-1}$ s$^{-1}$ and $>10^{6}$ M$^{-1}$ s$^{-1}$, respectively (38). The former of these is clearly too slow to be relevant to the experiment in question. If the latter constant is taken to be applicable, it follows that the second-order rate constant for the reaction between partly reduced cytochrome c oxidase and ONO$_2$ is given by $k_2 > (30/70) \times 10^{6} = 4.3 \times 10^{5} M^{-1} s^{-1}$. Based on this
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information, it is reasonable to argue that the rate constants for the reaction of ONO₂ with fully and partly reduced cytochrome c oxidase are probably $\approx 10^6$ M⁻¹ s⁻¹ at ambient temperature and physiological pH.

Although the reaction appears to proceed with moderate velocity, the second-order rate constant we estimate is still about 2 orders of magnitude slower than that governing the reaction between the enzyme and oxygen (39). Thus, in the absence of unambiguous evidence to the contrary, one should expect oxygen to be an effective inhibitor of the reaction between ONO₂ and reduced cytochrome c oxidase.

Relevance to the In Situ Enzyme—Although some questions may persist regarding the products of the reaction, it is clear that cytochrome c oxidase can function as a peroxynitrite reductase in vitro. There are several interrelated issues that must be addressed before a firm conclusion can be drawn as to the possible physiological significance of this reaction. The kinetics of the reaction need to be investigated by rapid-mixing experiments, the inhibitory effects of oxygen more completely delineated, and the relevant rate constants for these processes measured. The results of such studies should facilitate a reasonable assessment of the likely relevance of the demonstrated peroxynitrite reductase activity to normal mitochondrial function in vitro.

Radi et al. (42) and Cassina and Radi (43) previously argued that, in situ, mitochondrial cytochrome c oxidase is not affected by ONO₂, and the present data are in accord with this position. Provided that reduced forms remain in excess, the micellar enzyme protects itself, other protein, and detergent molecules from ONO₂ efficiently converting the oxidant to nitrite. It thus seems quite reasonable to suggest that, in the mitochondrion, cytochrome c oxidase is probably able to protect other inner membrane-associated components from ONO₂-induced damage. This would be a much less tenable hypothesis if NO, rather than NO₂, were the reaction product, because one would then be in the position of proposing the enzyme to be a detoxifying system displaying product inhibition.

As ONO₂ is rapidly formed in the uncatalyzed reaction between NO and the superoxide anion, it is conceivable that the reaction between cytochrome c oxidase could constitute a major pathway for the removal of both ONO₂ and excess NO. Also, since the proposed "peroxynitrite reductase" activity of the enzyme catalyzes electron transfer from ferrocytochrome c, the reaction sustains the electron transport chain and, presumably, drives proton translocation. This latter notion is also supported by the observations of Radi et al. (42) and Cassina and Radi (43), who found no evidence for uncoupling in mitochondria exposed to ONO₂, even in situations where there clearly was evidence of some damage to the electron transport capabilities of respiratory complexes I and II.

Finally, the present results may be of some relevance to the interpretation of experiments involving cultured cells and analysis of samples derived from clinical studies. For example, explanations of the reported synergistic cytotoxicity exhibited by NO and hyperoxia toward pulmonary cells (44) should now take into account both elevated mitochondrial production of ONO₂ and possible increased inhibition of its detoxification pathway in the presence of excess molecular oxygen. Recently, cells undergoing apoptosis have been shown to exhibit an increased rate of superoxide production following release of cytochrome c from the mitochondria (45). This is a consequence of the supply of electrons to cytochrome c oxidase having been interrupted, facilitating the direct reaction of oxygen with other species such as NADH dehydrogenase and reduced ubiquinone. Also, a nitric-oxide synthase located at the inner mitochondrial membrane has now been identified (46, 47). Consequently, if NO synthesis is not suppressed during the burst of superoxide associated with apoptotic-like cell death, elevated ONO₂ levels will result, because the oxidant cannot be reduced by cytochrome c oxidase trapped in the fully oxidized state. Therefore, an explanation of the intriguing observation that increased ONO₂ production is evident in a wide range of clinical conditions (15, 17) may involve a chain of events following apoptosis.

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