Reversible inhibition of cytochrome c oxidase (CcOX) by nitric oxide (NO) has potential physiological roles in the regulation of mitochondrial respiration, redox signaling, and apoptosis. However peroxynitrite (ONOO−), an oxidant formed from the reaction of NO and superoxide, appears mostly detrimental to cell function. This occurs through direct oxidant reactions and by decreasing the availability of NO for interactions with CeOX. When isolated CeOX respires with ascorbate as a reducing substrate, the conversion of ONOO− to NO− is observed. It is not known whether this can be ascribed to a direct interaction of the enzyme with ONOO−. In this investigation, the role of ascorbate in this system was examined using polarographic methods to measure NO production and CeOX activity simultaneously in both the purified enzyme and isolated mitochondria. It was found that ascorbate alone accounts for >90% of the NO− yield from ONOO− in the presence or absence of purified CeOX in turnover. The yield of NO was CeOX-independent but was dependent on ascorbate and ONOO− concentrations and was not affected by metal chelators. Consistent with this, the interaction of ONOO− with CeOX in respiring isolated mitochondria only yielded NO− when ascorbate was also present in the incubation. These observations are discussed in the context of ONOO−/ascorbate reactivity and the interaction of CeOX with reactive nitrogen species.

Nitric oxide (NO−),1 endogenously produced by the NO−-synthase family of enzymes, is thought to modulate mitochondrial respiration by reversibly inhibiting complex IV, cytochrome c oxidase (CeOX) (1, 2). This interaction has been suggested to play a key role in both mitochondrial physiology and intracellular redox signaling (2). However, prolonged exposure of mitochondria to NO− results in irreversible damage to several mitochondrial proteins (complexes I, II, IV, V, aconitase, manganese superoxide dismutase) and these changes are likely to contribute to its cytotoxicity (3). These deleterious effects have been attributed to peroxynitrite (ONOO−), which is produced by the reaction between NO− and superoxide (O2−), the latter reported to be generated in substantial amounts within mitochondria (3). Indeed, several studies have shown that direct addition of ONOO− to mitochondria results in inhibition of the same mitochondrial proteins as inhibited by long term NO− exposure (3, 4). Direct or indirect scavenging of ONOO− in the mitochondrion by intracellular antioxidants such as glutathione is, therefore, of critical importance to NO− biology and could control the balance between physiological and pathological effects of reactive nitrogen species in the organelle (5).

Besides the ubiquitous cellular defenses against oxidative stress such as superoxide dismutase and glutathione (4, 5), CeOX has recently been proposed to contribute to the antioxidant defenses of mitochondria by directly scavenging ONOO− (6–8). Studies performed on purified CeOX have shown that the enzyme in turnover may represent a significant sink for ONOO− (6–8). The detailed mechanisms are not entirely clear with one study indicating a catalytic production of NO− from the interaction of ONOO− with the enzyme (6) and other studies suggesting a peroxynitrite reductase activity in CeOX with the product being nitrite (7, 8). Furthermore, it has been suggested that NO− can be oxidized to nitrite by CeOX directly (9, 10) or by oxygen in a reaction that is accelerated in the lipid bilayer of mitochondrial membranes (11, 12). The suggestion that CeOX can metabolize ONOO− is in agreement with other studies showing that some hemeproteins and synthetic iron-porphyrin complexes can scavenge this highly reactive species (see Refs. 13 and 14 for recent reports).

Despite these observations, the potential interaction of ONOO− with high concentrations of reductants used to assess purified CeOX activity has not been defined (6–8). Specifically, ascorbate, a ubiquitous antioxidant, is used in some of these studies at millimolar concentrations. This is potentially important because ascorbate has been shown to increase NO− bioavailability in blood vessels, an effect which may be mediated by enhanced synthesis of NO− and/or by prevention of its breakdown (15). In the mitochondrion the role of ascorbate is unknown, but its concentration in the mitochondrial matrix has been estimated in the low millimolar range.2 Moreover, Li et al. (16) have recently proposed that the mitochondrial respiratory chain can recycle ascorbate from its oxidized form (DHA) to the reduced form. In this study, we investigated the possible contribution of ascorbate to the release of NO− from ONOO− in the presence and absence of CeOX using both the purified enzyme and isolated mitochondria.

1 The abbreviations used are: NO−, nitric oxide; ONOO−, peroxynitrite; Asch2, ascorbyl radical; CeOX, cytochrome c oxidase; DHA, dehydroascorbate; DTPA, diethylenetriaminepentaacetic acid; O2−, superoxide anion; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine.

2 T. M. Hagen, personal communication.
POKSNITRITE AND CYTOCHROME C OXIDASE

MATERIALS AND METHODS

All biochemicals were from Sigma unless otherwise specified. Solutions of ONOO· were prepared in 0.5 M NaOH according to Reed et al. (17) and stored at −80 °C, and their concentrations were spectrophotometrically determined just before use (εONOO· = 1670 M⁻¹ cm⁻¹ (18)). Concentrated stock solutions (typically ~150 mM) were diluted to 10 mM in water on the day of the experiment. Solutions of (+)-sodium l-ascorbate were freshly prepared daily. Bovine heart CcOX was purified according to Yonetani (19) and stored in 0.1 M K⁺ phosphate buffer, 0.7% (w/v) lauryl maltoside, pH 7.3, at −80 °C. Concentration is expressed in functional units (cytochrome-a₃, Δε₄₅₄ nm = 156 mM⁻¹ cm⁻¹). Rat liver mitochondria were prepared as described by Rickwood et al. (20). Nitric oxide solutions were obtained by equilibrating degassed water with pure NO gas (21), and their concentrations were determined with an NO electrode (see next paragraph).

Polarographic measurements (21) were performed in a thermostated chamber equipped with electrodes for oxygen and NO (Instech, Plymouth Meeting, PA and WPI, Sarasota, FL, respectively). The NO electrode was calibrated using the acidified nitrite/potassium iodide method as recommended by the supplier (World Precision Instruments, Inc., Sarasota, FL). In experimental traces, a known amount of authentic NO (from the calibrated stock solution) was always added at the end to quantify the amount of NO generated in the experiment. Data were digitally recorded and analyzed with Windaq software (Dataq Instruments, Inc., Akron, OH). Unless otherwise stated, all isolated CcOX incubations were performed at 20 °C in 25 mM K⁺ phosphate buffer, pH 7.3, with 0.1% w/v lauryl maltoside. Isolated mitochondrial incubations were performed in respiration medium comprising KCl (100 mM), sucrose (25 mM), HEPES (10 mM), MgCl₂ (5 mM), KH₂PO₄ (5 mM), EGTA (1 mM), pH 7.3, at 37 °C. Errors are expressed as standard deviations of at least three independent experiments unless otherwise specified. Statistical significance was assessed with an unpaired Student’s t test and a p value < 0.05.

RESULTS

The ability of CcOX to catalyze the conversion of ONOO⁻ to NO was investigated using both the purified enzyme and isolated mitochondria. As shown in Fig. 1A, purified CcOX was incubated in the presence of ascorbate, TMPD, and cytochrome c in air-equilibrated buffer at 20 °C. This temperature was selected to maintain the activity of the purified enzyme during the course of the experiment. Both O₂ consumption and NO were recorded. Upon addition of ONOO⁻, release of NO and inhibition of CcOX activity were observed as being consistent with previous observations (6). Following aerobic decomposition of NO from the solution, the enzymatic activity recovered almost completely. For comparison, Fig. 1A also shows the effects of authentic NO on CcOX in turnover under the same conditions. In both cases CcOX inhibition occurred almost instantaneously upon appearance of NO in the solution, although recovery of activity did not occur until well after disappearance of NO from the solution consistent with the slow dissociation rate of NO from CcOX at this temperature (9). The percentage recovery from inhibition was similar for ONOO⁻ and NO with an ~10% irreversible component for ONOO⁻, although this was not statistically significant (p = 0.186; Fig. 1B). The observation that NO produced from ONOO⁻ leads to the same inhibition of CcOX as seen with authentic NO provides further functional evidence that NO is produced from ONOO⁻.

To assess the role of CcOX in the production of NO from ONOO⁻, we added ONOO⁻ to ascorbate, TMPD, or cytochrome c²⁺ alone under the same conditions as described previously. As Fig. 1C shows, addition of ONOO⁻ to ascorbate produced a significant amount of NO comparable with that observed when NOO⁻ was added to CcOX in turnover (2.0 ± 0.02 versus 2.4 ± 0.13 μM; quantified in Fig. 1D). This suggests that ascorbate, not CcOX, is mainly responsible for the NO generation seen in Fig. 1A. The concentration of ascorbate in the cytosol (22, 23) and the reported ascorbate levels in the mitochondrial matrix (see the Introduction) suggest that this mechanism of NO generation from ONOO⁻ may be relevant to mitochondrial physiology.

However, these data do not preclude the possibility that CcOX alone may contribute to the NO generation from ONOO⁻. To test this, we examined isolated mitochondria respiring in the presence of glutamate, malate, and ADP (Fig. 1E). In this system, electrons are delivered to CcOX through the respiratory chain (complex I → ubiquinone → complex III → cytochrome c → complex IV), thus reducing substrates such as ascorbate are not required. Addition of ONOO⁻ to respiring mitochondria resulted in neither NO generation nor inhibition of respiration. However, in the presence of 1 mM ascorbate NOO⁻ was produced and consequently respiration was inhibited. The effect of authentic NO on mitochondrial respiration is also shown further suggesting that the inhibition of respiration caused by ONOO⁻ is due to NO.

Taken together, the data in Fig. 1 show that at 50–100 μM ONOO⁻, CcOX does not catalyze the reduction of ONOO⁻ to NO, whereas ascorbate does. However, the concentrations of
ascorbate used in the experiments described above are either nonphysiological (20 mM) or in the high intracellular physiological range (1 mM) (22, 23). To further investigate the ability of ascorbate to release NO from ONOO⁻, we measured NO production at different concentrations of ascorbate and ONOO⁻. Fig. 2A shows that ONOO⁻ addition to phosphate buffer at neutral pH did not result in significant release of NO. Even upon subsequent addition of ascorbate, NO was not produced, thus ruling out the possibility of NO generation from a reaction between ascorbate and the products of ONOO⁻ decomposition, NO₃⁻ and NO₂⁻ (24). However, when ONOO⁻ was added again to the chamber in the presence of ascorbate (50 μM), a transient production of NO was observed. The height of the NO peak was proportional to the concentration of ONOO⁻ in the range 10–100 μM and to the concentration of ascorbate up to 1 mM (Fig. 2, B and C). However, above 1 mM ascorbate, the generation of NO reaches a plateau. The saturation kinetics in the supra-millimolar range suggest that the production of NO does not occur through a direct reaction between ONOO⁻ and ascorbate but through a more complex mechanism.

To provide further insight to the NO production by ONOO⁻ and ascorbate, we studied the yield of NO from ascorbate plus ONOO⁻ requires the reduced form of ascorbate, we studied the interaction of ONOO⁻ with DHA, the oxidized form where one of the hydroxyl groups of ascorbate is replaced by a carbonyl. Fig. 3C shows that although a small amount of NO is still generated (~5% of control), the reaction yield is significantly reduced suggesting that ascorbate must act as a reductant for this reaction to occur.

The effects of NaHCO₃ and superoxide dismutase are also shown in Fig. 3C. These data show that generation of NO from ascorbate plus ONOO⁻ is abrogated by the presence of NaHCO₃. In contrast, superoxide dismutase enhanced the generation of NO by a small but significant amount (p = 0.043). Because the stability of ONOO⁻ is affected by several factors, including the equilibrium with its protonated form (ONOOH, pKᵦ = 6.8), we also studied the effect of pH on the amount of NO produced (Fig. 3D). A decrease in the yield of the reaction was observed with decreasing pH.
DISCUSSION

In this study, it has been shown that physiological concentrations of ascorbate can mediate the release of NO\(^{-}\) from ONOO\(^{-}\). This result has implications not only for the physiologic scavenging of ONOO\(^{-}\) but also for interpretation of the interactions of this reactive nitrogen species with CeOX (6–8). The peroxynitrite-reductase activity of CeOX results in the formation of nitrite as shown in a recent study (7), and this pathway is likely to be contributing to the metabolism of ONOO\(^{-}\) in this system. However, an additional process is required to explain the production of NO\(^{-}\) observed in the present study. The data presented herein suggest that the majority of NO\(^{-}\) release from CeOX in turnover is accounted for by the presence of ascorbate in the reaction mixture. This is in contrast to the results of Sharpe and Cooper (6) who report that CeOX in turnover is required for NO\(^{-}\) generation from ONOO\(^{-}\) and that little or no NO\(^{-}\) is generated in the absence of the enzyme. However, several differences between the experimental systems may account for this discrepancy. For example, the temperatures are different (20 °C here versus 30 °C in Sharpe and Cooper (6)), and HEPES was a constituent of the buffer in Sharpe and Cooper (6). The use of HEPES was avoided in this study because it has been shown that it can react with ONOO\(^{-}\) to form a labile NO\(^{-}\) donor (5, 25, and acknowledged in Ref. 7).

In addition, it is possible that the reaction that yields NO\(^{-}\) from ONOO\(^{-}\) is enhanced by the ascorbyl radical (AscH\(^{+}\)), which in turn is produced by auto-oxidation of ascorbate under aerobic conditions. In this case, the formation of NO\(^{-}\) from ascorbate plus ONOO\(^{-}\) would be accelerated in the presence of any system that enhances ascorbate oxidation including the TMPD → cytochrome c → CeOX system. This may explain the slightly greater generation of NO\(^{-}\) observed with CeOX versus ascorbate alone (Fig. 1F). In addition, this may underlie the dependence of NO\(^{-}\) generation on CeOX turnover rate as reported for uncoupled versus coupled CeOX vesicles (6), because a greater CeOX turnover would equate to greater oxidation of ascorbate and, thus, more AscH\(^{+}\).

In considering a possible mechanism for the formation of NO\(^{-}\) from ascorbate plus ONOO\(^{-}\), the data in Fig. 3 are of particular importance. First, the observation that less NO\(^{-}\) is made at acidic pH suggests that the source of NO\(^{-}\) is ONOO\(^{-}\) rather than ONOOH (pK\(_{a}\) ~ 6.8). This is consistent with the observation that NaHCO\(_{3}\) abolished NO\(^{-}\) formation in this system, because it is known that CO\(_{2}\) can react with ONOO\(^{-}\) but not ONOOH (26).

Several mechanisms may exist for the generation of NO\(^{-}\) from ONOO\(^{-}\). For example, it is possible that ascorbate or one of its derivatives (e.g. AscH\(^{+}\)) may react with ONOO\(^{-}\) to form an intermediate that is an NO\(^{-}\) donor, as previously shown, for low-molecular weight compounds such as HEPES and glutathione (5, 25). However, the rapid appearance of NO\(^{-}\) in the medium following ONOO\(^{-}\) addition (see Figs. 1–3) suggests that any such intermediate would have a very short half-life so that both are made and degraded within the response time of the NO\(^{-}\) electrode. In addition, because the decomposition of certain NO\(^{-}\) donors is accelerated in the presence of metals, the lack of an effect of metal chelators on NO\(^{-}\) generation (Fig. 3A) suggests this is not a significant pathway.

Another possible mechanism for NO\(^{-}\) generation from ONOO\(^{-}\) could be the scavenging of O\(_{2}^{\bullet}\) by the ascorbate system (ascorbate plus AscH\(^{+}\)). This would serve to drive the equilibrium (NO\(^{-}\) + O\(_{2}^{\bullet}\) ↔ ONOO\(^{-}\)) to the left. Literature evidence to date has concentrated on the diffusion-limited forward reaction between NO\(^{-}\) and O\(_{2}^{\bullet}\) to yield ONOO\(^{-}\). However, a significant back-reaction does exist (k = 0.02 s\(^{-1}\) at 25 °C) and could be enhanced in the presence of a sink for O\(_{2}^{\bullet}\) (27). In this regard, Jackson et al. (28) have shown that ascorbate can effectively scavenge O\(_{2}^{\bullet}\) and compete with NO\(^{-}\) for reaction with O\(_{2}^{\bullet}\) although only at concentrations in the high millimolar range (28). Notably, the reaction between AscH\(^{+}\) and O\(_{2}^{\bullet}\) is ~1000× faster than that between ascorbate and O\(_{2}^{\bullet}\) (k = 2.3 × 10\(^{7}\) M\(^{-1}\) s\(^{-1}\) versus 3 × 10\(^{5}\) M\(^{-1}\) s\(^{-1}\), respectively) (29, 30). Thus, in a system where ascorbate oxidation is enhanced (e.g. CeOX in turnover), O\(_{2}^{\bullet}\) scavenging may be enabled because of greater formation of AscH\(^{+}\) (see above). Such enhancement may decrease into the physiologic range the effective concentration of ascorbate required for such O\(_{2}^{\bullet}\) scavenging. In support of a role for O\(_{2}^{\bullet}\) scavenging by ascorbate, Figs. 3 shows that superoxide dismutase can mildly enhance NO\(^{-}\) formation in this system. However this effect appears small, because this experiment was performed at 5 mM ascorbate at which point O\(_{2}^{\bullet}\) scavenging may have already been saturated (see concentration dependence, Fig. 2B).

Additional mechanisms for the ascorbate-driven generation of NO\(^{-}\) from ONOO\(^{-}\) may be hypothesized including reactions between ascorbate/AscH\(^{+}\) and other known decomposition products of ONOO\(^{-}\) (e.g. NO\(_{2}^{-}\), NO\(_{3}^{-}\), OH, NO\(_{2}^{-}\) (27, 31)). However, it is not yet clear whether any of these reactions would yield NO\(^{-}\).

Figs. 1–3 suggest an ~2% yield of NO\(^{-}\) from ONOO\(^{-}\). In a physiologic context, where ONOO\(^{-}\) must have come from NO\(^{-}\) originally, this raises the question whether this reaction is a significant pathway for the regeneration of NO\(^{-}\). However, given that soluble guanylate cyclase and cytochrome c oxidase are sensitive to low nanomolar concentrations of NO\(^{-}\) and that cell signaling is amplified at these enzymes, biological responses are plausible. Moreover, it does appear that ascorbate can act as an antioxidant for the prevention of ONOO\(^{-}\) formation (28), thus increasing NO\(^{-}\) availability.

In the context of the mitochondrion, the organelle has been proposed as a potential source of ONOO\(^{-}\) (32). A significant store of ~1 mM ascorbate is proposed to exist in the matrix (see Introduction), and thus ascorbate-driven ONOO\(^{-}\) decomposition may occur in this organelle. Whereas the data in Fig. 1 do not show NO\(^{-}\) generation from ONOO\(^{-}\) in mitochondria, it is unclear whether ascorbate survived the mitochondrial isolation procedure and, if so, what its redox state was, because DHA does not catalyze NO\(^{-}\) release from ONOO\(^{-}\) (Fig. 3C). Overall, water-soluble antioxidants such as glutathione (5) and ascorbate may comprise the primary mitochondrial defense against ONOO\(^{-}\). This raises the possibility that only ONOO\(^{-}\) made in situ may be damaging to the organelle. Furthermore, the ability of CO\(_{2}\) to modulate ascorbate-driven ONOO\(^{-}\) degradation (Fig. 3) is unique from a mitochondrial perspective, because the organelle is a major cellular source of CO\(_{2}\). Protein tyrosine nitration mediated by ONOO\(^{-}\) is enhanced by CO\(_{2}\), thus raising the possibility of modulation of this post-translational modification by ascorbate of mitochondrial proteins.

Whereas it is unlikely that the ascorbate plus ONOO\(^{-}\) reaction represents a significant source of NO\(^{-}\), it is the balance between these reactive nitrogen species that is important for cellular function. Therefore, these results have implications for the role of mitochondria and reactive nitrogen species in processes such as apoptosis, because it has been shown that NO\(^{-}\) can prevent cytochrome c release from mitochondria, whereas ONOO\(^{-}\) enhances it (33, 34). Thus, ascorbate may act as a modulator of mitochondrial apoptotic signaling as has previously been proposed for glutathione (31).

In summary, we have shown that the generation of NO\(^{-}\) from ONOO\(^{-}\) by CeOX in turnover is most likely because of ascorbate, and it appears that, whereas CeOX does possess a ONOO\(^{-}\) reductase activity (7), this is not the primary source of...
Acknowledgments—We thank Paolo Sarti (Rome, Italy), Jack R. Lancaster, Jr., and Rakesh Patel (University of Alabama at Birmingham) for insightful discussions.

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Reversible Inhibition of Cytochrome c Oxidase by Peroxynitrite Proceeds through Ascorbate-dependent Generation of Nitric Oxide
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J. Biol. Chem. 2003, 278:27520-27524.
doi: 10.1074/jbc.M304129200 originally published online May 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304129200

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