Nitrite Reductase Activity of Cytochrome c*

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Small increases in physiological nitrite concentrations have now been shown to mediate a number of biological responses, including hypoxic vasodilation, cytoprotection after ischemia/reperfusion, and regulation of gene and protein expression. Thus, while nitrite was until recently believed to be biologically inert, it is now recognized as a potentially important hypoxic signaling molecule and therapeutic agent. Nitrite mediates signaling through its reduction to nitric oxide, via reactions with several heme-containing proteins. In this report, we show for the first time that the mitochondrial electron carrier cytochrome c can also effectively reduce nitrite to NO. This nitrite reductase activity is highly regulated as it is dependent on pentacoordination of the heme iron in the protein and occurs under anoxic and acidic conditions. Further, we demonstrate that in the presence of nitrite, pentacoordinate cytochrome c generates bioavailable NO that is able to inhibit mitochondrial respiration. These data suggest an additional role for cytochrome c as a nitrite reductase that may play an important role in regulating mitochondrial function and contributing to hypoxic, redox, and apoptotic signaling within the cell.

Accumulating data demonstrates that nitrite is likely to function as a storage pool for nitric oxide (NO) (1) and act as a physiological hypoxic signaling molecule (2). Sodium nitrite increases blood flow when infused at slightly supraphysiological levels (3), and in tissues nitrite has been shown to protect against ischemia/reperfusion injury and regulate gene and protein expression. Because of its potential role in physiology, pathophysiology, and therapeutics, there has been a surge in interest in characterizing the mechanisms of cellular nitrite reduction in mammals (4). Several proteins have been shown to be capable of facilitating nitrite bioactivation by reducing it to NO, including hemoglobin (3, 5), myoglobin (6, 7), xanthine oxidoreductase (8, 9), nitric-oxide synthase (10), and cytochrome c oxidase (11, 12). Reaction 1 involving hemoglobin (which is likely to be analogous to that of the other heme proteins) is described by Ref. 13,

\[
\text{NO}_2^- + \text{Fe}^{II} + \text{H}^+ \rightarrow \text{NO} + \text{Fe}^{III} + \text{OH}^- \quad \text{REACTION 1}
\]

where the \(\text{Fe}^{II}\) and \(\text{Fe}^{III}\) are the ferrous and ferric hemes of (in the case of hemoglobin) deoxyhemoglobin and methemoglobin. Reaction 1 predicts faster nitrite reduction at lower pH. The NO formed in Reaction 1 will rapidly bind to a vacant ferrous heme iron forming an iron nitrosyl species (Reaction 2).

\[
\text{NO} + \text{Fe}^{II} \rightarrow \text{Fe}^\text{II} \text{NO} \quad \text{REACTION 2}
\]

Cytochrome c (cyt c)\(^3\) is a peripheral membrane protein that is normally found in the intermembrane space of the mitochondrion, where it shuttles electrons from complex III to cytochrome c oxidase (complex IV). In addition, the release of cyt c initiates cellular apoptosis (14, 15). Cytochrome c is also involved in oxidative and nitrosative stress responses (16, 17). The heme iron of cyt c is six-coordinate, with histidine 18 and methionine 80 as the heme iron ligands, and would not be expected to easily react with nitrite. However, the iron-methionine bond is prone to rupture when the methionine is oxidized (18, 19), tyrosines are nitrated (17, 20), or the cytochrome interacts with anionic phospholipids such as those present in the inner membrane of the mitochondria (21–23). The weakening or breakage of the iron-methionine bond is thought to influence its role in apoptosis (22, 24–28) and potentially has other roles in non-apoptotic cells (29).

Nitric oxide is an integral regulator of mitochondrial function. At low concentrations, NO reversibly inhibits mitochondrial respiration by binding to cytochrome c oxidase (30, 31), regulates mitochondrial reactive oxygen species generation, and prevents cytochrome c release. At high concentrations NO can irreversibly inhibit respiration and ATP generation, and nitrosylate cyt c, leading to cyt c release and apoptosis (32–37).

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3 The abbreviations used are: cyt c, cytochrome c; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; TOCL, 1,1′,2′,2′-tetraeoylcardiolipin; EPR, electron paramagnetic resonance; MES, 2-(N-morpholinol)ethanesulfonic acid; SMP, submitochondrial particles; MOPS, 4-morpholinepropanesulfonic acid.
While NO can diffuse into the mitochondrion from other parts of the cell, it is likely that some of this NO-dependent signaling may be mediated by nitrite reduction by cytochrome c oxidase (11, 12).

In this study, we show that under low pH and low oxygen conditions, pentacoordinate cyt c reduces nitrite to produce NO. This discovery could have important implications regarding hypoxia-induced redox signaling and apoptosis.

**EXPERIMENTAL PROCEDURES**

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,1’,2,2’-tetraoleoyl cardiolipin (sodium salt, TOCL) were purchased from Avanti Polar Lipids (Albaster, AL). Bovine heart cyt c and all other chemicals were purchased from Sigma-Aldrich. Liposomes were prepared by sonication of a 1:1 molar ratio of DOPC:TOCL under nitrogen (38).

Absorption spectroscopy was performed on a Perkin Elmer Lambda 9 spectrometer that has been updated with Lambda 90 software and coupled to an integrating sphere detector so that light scattered from the liposomes was collected and did not contribute to measured absorption. Determination of iron nitrosyl species of cyt c was performed using electron paramagnetic resonance (EPR) spectroscopy using an EMX 10/12 spectrometer cooled to 110 K using liquid helium. The spectrum was operated at 9.4 GHz, 5-G modulation, 10.1-milliwatt power, 81.92-ms time constant, and 83.8-s scan over 1000 G. The concentrations of nitrosyl cyt c were determined by performing the double integral of the spectra and comparing this to a standard. As iron nitrosyl species from different heme proteins should produce similar sized signals, we initially fit the nitrosyl cyt c to basis spectra for iron nitrosyl hemoglobin as described previously (39). Briefly, high quality basis spectra of betanitrosyl, pentacoordinate alphanitrosyl, and hexacoordinate alphanitrosyl spectra were made and normalized to concentration. Linearity in concentration determination has been conducted in our laboratory many times in micromolar to millimolar ranges and specifically confirmed for this experiment. A nitrosyl cyt c basis spectrum was also made by reaction of 5 μM nitrite with cyt c and confirmation that 5 μM nitrosyl cyt c was made using Hb basis spectra. Subsequent fitting for concentration determination with either cyt c or Hb basis spectra gave similar results with 10% or less difference.

Cyt c was reduced using either 2.5 mM sodium dithionite or a 10-fold molar excess ascorbate. In many experiments, the reducing agent was removed by passing the sample through two consecutive G-25 Sephadex columns. In some experiments, sodium dithionite was not removed as it has been shown recently that dithionite does not effectively reduce nitrite (40, 41). To make sure that dithionite would not effectively reduce nitrite under experimental conditions used in this study, 2.5 mM sodium dithionite was placed into the reaction reservoir of a Sievers 280i Nitric Oxide Analyzer (GE Analytical Instruments, Boulder, CO), and nitrite was injected. No significant signal corresponding to NO formation was measured for nitrite injected at 100 μM, 500 μM, or 1 mM concentrations (data not shown), whereas the assay is sensitive to formation of submicromolar concentrations of NO.

Nitrite reductase activity was measured by mixing reduced cyt c (100 μM) with lipids (4 mM) and then adding nitrite (5 μM to 5 mM). All solutions were purged with argon to remove oxygen. Cyt c nitrosylation was monitored by either absorption spectroscopy or EPR. For EPR, aliquots of the samples were frozen as a function of time after mixing. All data are reported in the text as averages with standard deviations from three separate trials.

Submitochondrial particles (SMP) were isolated by sonication of liver mitochondria in SMP buffer (sucrose (300 mM), MOPS (5 mM), EGTA (1 mM), KH2PO4 (5 mM), pH 7.4, 4 °C) followed by centrifugation at 20,000 × g and one wash of the pellet. Protein concentration was determined using the BCA protein assay kit (Pierce). The SMPs were >99% pure, as determined by measuring citrate synthase activity as a marker of mitochondrial matrix contamination.

NO and oxygen concentrations were measured simultaneously using Clark type electrodes (WPI, Sarasota, FL and Instech, Plymouth Landing, MA, respectively). All measurements were made in a sealed, stirred chamber at 37 °C. Data were collected using a digital recording device (DATAQ Instruments, Akron, OH). The amount of NO generated (in electrode experiments) was quantified by integrating the measured NO curve over the initial 10-min period.

In respiration experiments, SMPs were incubated in respiration buffer (KCI (120 mM), Hepes (3 mM), EGTA (1 mM), sucrose (25 mM), MgCl2 (5 mM), KH2PO4 (5 mM) with NADH (1 mM)) to stimulate respiration. To measure the inhibition of respiration, SMPs were allowed to consume oxygen until the respiratory chamber became anaerobic. Once all the oxygen was consumed, the lid of the chamber was opened to allow equilibration of the SMP suspension with room air. The oxygen trace did not immediately increase upon removal of the lid due to the high rate of oxygen consumption by the respiring SMPs. However, as soon as substrate was exhausted or respiration was inhibited, the oxygen concentration increased in the chamber. To determine the extent of inhibition, the time from when the lid of the chamber was opened to the time that the oxygen trace deviated from zero was measured and called “time to inhibition.” This time was compared with the time to inhibition of substrate exhaustion (0% inhibited) and the time to inhibition in the presence of cyanide (100% inhibition) as described previously (6).

**RESULTS**

Initial studies examined whether cyt c could reduce nitrite to NO by examining the formation of iron-nitrosyl heme. Cyt c (96 μM) was incubated with liposomes that promote heme iron pentacoordination, in an anaerobic environment at pH 5.4 and monitored using absorption spectroscopy (Fig. 1A). Addition of liposomes to the reduced cyt c (in which the reducing agent was removed using G-25 columns) blue-shifted the Soret bands and partially converted the two peaks around 520 nm and 549 nm to a single one, consistent with partial pentacoordination of the heme. Addition of the NO donor ProlinONOate to the mixture of cyt c and liposomes red shifted the spectrum giving a peak at 416 nm in the Soret and giving two peaks at 530 and 564 nm that were similar in intensity. This spectrum is similar to that of
Nitrosylated cyt c measured at pH 6.8 (which has peaks at 417, 529, and 562 nm (42)). The spectrum obtained upon addition of 5 mM nitrite to the cyt c is consistent with a mixture of oxidized (Fe$^{III}$) cyt c and iron nitrosyl cyt c as predicted by Reactions 1 and 2. Formation of iron nitrosyl cyt c is confirmed by EPR (Fig. 1B). No signal is observed with cytochrome c in the absence of liposomes with or without the addition of nitrite. In the sample containing liposomes, addition of ProliNONOate resulted in a triplet hyperfine signal that is characteristic of a pentacoordinate nitrosyl heme. Likewise, a similar (although smaller) signal is observed upon addition of nitrite. The nitrosyl signals observed here are similar to those in previous reports in which another NO donor (DEANOate) was added to a similar liposome and cyt c mixture and the observed nitrosyl signal was attributed to about 70% pentacoordinate (iron bound to the heme and NO) and 30% hexacoordinate (iron bound to heme, histidine 18, and NO) nitrosyl heme (38). Similar results were obtained when ascorbate rather than dithionite was used to reduce the cytochrome c, and no signal was seen in the absence of liposomes (data not shown).

Nitrosyl cytochrome c nitrite reductase activity might be relevant to certain physiological or pathophysiological conditions, the oxygen and pH dependence was examined (Fig. 2). Cyt c was reduced with sodium dithionite and passed through two consecutive G-25 columns. When the cyt c was mixed with 4 mM liposomes for 10 min and diluted into deoxygenated buffer (pH 5.4), 4.3 μM iron nitrosyl cyt c was measured after 60 min following the addition of 5 mM nitrite. No nitrosyl cyt c was detected when this experiment was repeated in the absence of liposomes or if the cyt c/liposome mixture was diluted into air-equilibrated buffer. 8, pH dependence of nitrite reductase activity was measured by reducing cyt c with 2.5 mM dithionite, incubating with 4 mM liposomes, and diluting into buffers at various pH to final concentration of cyt c of 100 μM. In this experiment sodium dithionite was not removed. As discussed under “Experimental Procedures,” sodium dithionite does not reduce nitrite to NO under the conditions studied. Nitrite reductase activity was assessed by examining nitrosyl cyt c formation 60 min after nitrite (5 mM) addition. The amount of nitrosyl cyt c detected was 6.0, 2.0, 0.6, and 0.3 μM at pH 5.4, 6.0, 6.4, and 7.4.

To determine whether the nitrite reductase activity of cyt c might be relevant to certain physiological or pathophysiological conditions, the oxygen and pH dependence of nitrite reductase activity was examined (Fig. 2). Cyt c was reduced with sodium dithionite and passed through two G-25 columns under aerobic conditions. Incubation of cyt c and nitrite in the absence of liposomes did not produce any nitrosyl cyt c. When the reduced cyt c was incubated with 5 mM nitrite in the presence of liposomes under anaerobic conditions at pH 5.4, iron nitrosyl cyt c was formed (Fig. 2A). However, when the measurement was repeated in air-equilibrated buffer, no signal was observed (Fig. 2A). When this experiment (using 5 mM nitrite) was repeated at 1% oxygen 5 ± 1 μM nitrosyl cyt c was made after 16 min; however, when the nitrite concentration was decreased to 5 μM nitrite at 1% oxygen, no nitrosyl cyt c was detected (data not shown). These results are consistent with those described in the figure.

FIGURE 1. Nitrite reduction by cyt c with formation of iron nitrosyl cyt c. A, cyt c (96 μM final concentration) was reduced with 2.5 mM sodium dithionite and passed through two consecutive G-25 columns to remove the dithionite. The cyt c was diluted into anaerobic MES buffer at pH 5.4. A, absorption spectra were collected before and after addition of 4 mM liposomes. Additional spectra were collected after 30-min incubations of the cyt c/liposomes with either nitrite or ProliNONOate. B, EPR spectra were taken on the samples after freezing in liquid nitrogen. The characteristic triplet hyperfine structure of pentacoordinate nitrosyl hemes is seen for the samples mixed with liposomes for which either nitrite or proliNONOate are added. These data were found to be reproducible for three separate trials.

FIGURE 2. Effectors of nitrite reductase activity of cyt c. A, cyt c was reduced by dithionite and passed through two consecutive G-25 columns. When the cyt c was mixed with 4 mM liposomes for 10 min and diluted into deoxygenated buffer (pH 5.4), 4.3 μM iron nitrosyl cyt c was measured after 60 min following the addition of 5 mM nitrite. No nitrosyl cyt c was detected when this experiment was repeated in the absence of liposomes or if the cyt c/liposome mixture was diluted into air-equilibrated buffer. B, pH dependence of nitrite reductase activity was measured by reducing cyt c with 2.5 mM dithionite, incubating with 4 mM liposomes, and diluting into buffers at various pH to final concentration of cyt c of 100 μM. In this experiment sodium dithionite was not removed. As discussed under “Experimental Procedures,” sodium dithionite does not reduce nitrite to NO under the conditions studied. Nitrite reductase activity was assessed by examining nitrosyl cyt c formation 60 min after nitrite (5 mM) addition. The amount of nitrosyl cyt c detected was 6.0, 2.0, 0.6, and 0.3 μM at pH 5.4, 6.0, 6.4, and 7.4.
below using an NO electrode showing NO generation from cyt c is dramatically inhibited by oxygen.

The pH dependence of the nitrite reductase activity of cyt c was studied under anaerobic conditions in the presence of dithionite (which does not reduce nitrite under the conditions studied). The reductase activity as measured by iron nitrosyl cyt c formation, was greatest at the lowest pH studied (5.4) where an average of 5.6 ± 0.4 μM nitrosyl cyt c was made (n = 3) and became smaller approaching neutral pH (Fig. 2B). At pH 6 substantial nitrosyl cyt c was formed (1.89 ± 0.12 μM), but much less was made at pH 6.4 and 7.4 (0.63 ± 0.01 and 0.28 ± 0.08 μM, respectively, n = 3).

The time course of cyt c-dependent nitrite reduction was assessed by measuring iron-nitrosyl cyt c formation upon addition of nitrite to cyt c under anaerobic conditions. Fig. 3A shows EPR spectra of nitrosyl cyt c growing as a function of time after the addition of 5 μM nitrite. Even after only 1 min, substantial nitrosyl cyt c is made indicating that, under these low pH (5.4) conditions, nitrite is reduced rapidly by cyt c (Fig. 3A). The kinetics of nitrosyl cyt c formation when 100 μM cyt c is mixed with 4 mm liposomes, 2.5 mm sodium dithionite, and varying amounts of nitrite is shown in Fig. 3B. Importantly, it is seen that substantial nitrosyl cyt c is made by the time the first aliquot is collected for EPR (1 min after the reaction is initiated by nitrite addition). The yield of nitrosyl cyt c made after 1 min is dependent on nitrite concentration (Fig. 3C). Nitrosyl cyt c yield increases as the concentration of added nitrite increases from 5 μM to 500 μM, but is not further increased at this point. At high concentrations of nitrite (500 μM and 5 mM) the nitrosyl cyt c yield continues to increase slowly after the initial minute of fast growth (Fig. 3B).

To further characterize the nitrite reductase activity of cytochrome c, NO formation and oxygen tension were measured simultaneously at pH 6.5 and 37 °C using conventional electrodes. Reduced cytochrome c (200 μM) was incubated with liposomes and nitrite (5 mM) and sodium dithionite was added to decrease the oxygen concentration. NO generation of NO was observed until the chamber became completely anoxic, after which NO was generated and remained at a steady state for at least 15 min. The addition of oxyhemoglobin (200 μM) caused a rapid and immediate drop in the NO trace, confirming that it was indeed authentic NO that was being measured (Fig. 4A). Nitric oxide could not be detected in the absence of liposomes, in the absence of cyt c, or at a pH greater than 6.9 (data not shown).

To determine whether cyt c could contribute to NO generation in its physiological setting within the mitochondrial electron transport chain, SMP, which are devoid of cytochrome c and mitochondrial matrix proteins, were used as a model of the electron transport chain. Because cytochrome c oxidase has been shown to be a nitrite reductase, NO generation by the respiratory chain in the absence of pentacoordinate cyt c was first measured. In the presence of nitrite (5 mM), cyt c (200 μM) and NADH (to stimulate respiration), the SMPs (2 mg/ml) generated 3.61 ± 0.14 μM NO in 10 min, similar to previously published levels of NO generation by mitochondria and isolated cytochrome c oxidase (Fig. 4B). When liposomes (4:1 lipid to cyt c ratio) were added to generate pentacoordinate cyt c, the NO generated in 10 min (4.38 ± 0.23 μM) was significantly greater than NO generation by SMPs or pentacoordinate cyt c alone (1.7 ± 0.21 μM) (Fig. 4, C and D).

To determine whether the nitrite reductase activity of cyt c could play a role in cellular respiration, we tested whether NO generated by cyt c could inhibit oxygen consumption by cytochrome c oxidase at pH 6.5 (Fig. 5). To measure respiration at low oxygen tensions, we used a conventional oxygen electrode in a sealed chamber and allowed the respiring SMPs to make the chamber anoxic. Once anoxic, the lid of the chamber was
opened to allow equilibration of the solution with ambient air.

In this experiment, the time from the removal of the lid to the time that the oxygen trace deviates from zero correlates inversely to the extent of inhibition, with a shorter time to inhibition indicative of a greater extent of inhibition (Fig. 5A, see “Experimental Procedures” for details).

In the absence of liposomes, the addition of nitrite (30 μM) significantly inhibited respiration (28 ± 9% inhibition) due to the NO generated by cytochrome c oxidase itself. To increase pentacoordination of cyt c, increasing concentrations of liposomes were added. Increasing lipid decreased respiratory rate consistent with the decreased ability of pentacoordinated cyt c to transfer electrons to cytochrome c oxidase (38). However at each lipid concentration, respiration in the presence of nitrite was inhibited significantly compared with the respective control (Fig. 5B). Moreover, as pentacoordinated cyt c was increased, the extent of inhibition was also increased (29.6 ± 11% and 48.5 ± 16% inhibition at 4:1 and 25:1 lipid: cyt c ratios, respectively), presumably due to greater NO generation by cyt c (Fig. 5C). Here, the percentage inhibition is calculated as the amount of inhibition with nitrite to that without nitrite for each lipid concentration. *, p < 0.01 versus 0 lipid condition. All experiments are means ± S.E. for at least three independent experiments.

**DISCUSSION**

We have shown that when cyt c is placed under conditions where the heme becomes pentacoordinate, it functions as a nitrite reductase. This activity is most effective at low pH and in the absence of oxygen. Further, we show that this novel function for cyt c can generate bioavailable NO that can regulate mitochondrial respiration.
Kinetic analysis of nitrite reduction by cytochrome c shows that the formation of nitrosyl cyt c is fast (Fig. 3). The rate of formation of nitrosyl cyt c is related to the rate of nitrite reduction. The high equilibrium constant \(2.9 \times 10^6 \text{ M}^{-1}\) for hexacoordinate and about 100 times higher for pentacoordinate), and slow dissociation constant \(2.9 \times 10^{-5} \text{ s}^{-1}\) (43, 44) insures that once NO binds to cyt c, it is not likely to come off on the time scale of our experiments. The association constant of NO to hexacoordinate cyt c is only 8.3 M\(^{-1}\) s\(^{-1}\) (43), but is likely to be at least 100 times faster for pentacoordinate cyt c (44). There are, however, additional factors that mitigate cyt c NO yield. Only the pentacoordinate form of cyt c (which is induced by the liposomes but there is always still substantial hexacoordinate cyt c present) can easily bind NO and easily react with nitrite. However, when NO binds to one heme, that heme is no longer capable of further reactions with nitrite. This contributes to an element of auto-inhibition. While the final yield of nitrosyl cyt c for low nitrite concentrations (e.g. 5 \text{ \mu M}, Fig. 3B) is limited due to the amount of nitrite available, at higher nitrite concentrations it is limited by the amount of pentacoordinate cyt c present. We suggest that the slow kinetics seen with higher concentrations of nitrite in Fig. 3B is due to slow additional formation of pentacoordinate cyt c.

The amount of nitrosyl cyt c made within 1 min (Fig. 3C) can be viewed as a minimum initial rate of nitrite reduction by cyt c per minute. The actual rate may be higher as the reaction may have already slowed down within 1 min due to low substrate availability (in either nitrite or pentacoordinate cyt c). Due to the rapid reaction rate, we were unable to accurately determine the linearity of the reaction rates between zero and 1 min, and attempts to slow the reaction down by lowering the temperature were not helpful (the reaction was still too fast, data not shown). With these considerations in mind, one observes that the average minimum initial rates of NO production presented (32) can easily bind NO and easily react with nitrite. However, when NO binds to one heme, that heme is no longer capable of further reactions with nitrite. This contributes to an element of auto-inhibition. While the final yield of nitrosyl cyt c for low nitrite concentrations (e.g. 5 \text{ \mu M}, Fig. 3B) is limited due to the amount of nitrite available, at higher nitrite concentrations it is limited by the amount of pentacoordinate cyt c present. We suggest that the slow kinetics seen with higher concentrations of nitrite in Fig. 3B is due to slow additional formation of pentacoordinate cyt c.

The amount of nitrosyl cyt c made within 1 min (Fig. 3C) can be viewed as a minimum initial rate of nitrite reduction by cyt c per minute. The actual rate may be higher as the reaction may have already slowed down within 1 min due to low substrate availability (in either nitrite or pentacoordinate cyt c). Due to the rapid reaction rate, we were unable to accurately determine the linearity of the reaction rates between zero and 1 min, and attempts to slow the reaction down by lowering the temperature were not helpful (the reaction was still too fast, data not shown). With these considerations in mind, one observes that the average minimum initial rates of NO production presented in Fig. 3C are 13, 14, 71, 130, and 140 nmol/sec for added nitrite concentrations of 5, 10, 50, 500, and 5000 \text{ \mu M}, respectively. Further work is required to determine whether actual initial rates are (at least in some cases) faster than this. In addition, additional experiments are required to determine if the dependence of these values on the concentration of nitrite stems from sub-state saturation of a bimolecular reaction or maximum formation of the enzyme/substrate complex so that the maximum catalytic rate is achieved at high nitrite concentrations.

To assess the extent that NO may be generated via cytochrome c mediated nitrite reduction, it is best to examine the experiments using lower concentrations of nitrite, that is 5 \text{ \mu M} or 10 \text{ \mu M}. Levels of nitrite vary in vivo from one to a few hundred nanomolar in the plasma to about 20 \text{ \mu M} in the aorta (45). Cytosolic levels of cytochrome c have been measured to be as high as 0.4 to 0.5 \text{ \mu M} following release from the mitochondria (46) and the concentration in the intermembrane space are between 0.5 \text{ \mu M} and 5 \text{ \mu M} (47). Thus, although oxygen tension and percentage of pentacoordination are major factors, our experiments using 100 \text{ \mu M} cyt c are within physiological ranges. Our data show that 100 \text{ \mu M} cyt c produces NO at a minimum rate of 13 or 14 nmol/s when the concentration of nitrite is 5 or 10 \text{ \mu M}. This rate is quite substantial when considering that steady state tissue NO is in the nm range.

Recent studies utilizing isolated mitochondria and purified enzyme have shown that cytochrome c oxidase is also a nitrite reductase that is most active at low pH and low oxygen (11, 12). These reports provided evidence in a yeast system that NO produced by cytochrome c oxidase contributes to hypoxic signaling (11). In these studies, cyt c was used as an electron donor for cytochrome c oxidase. The possibility that the NO produced from nitrite was due to cyt c rather than the cytochrome c oxidase is diminished by the fact that in those studies there was no evidence that the heme of the cyt c was pentacoordinate, and our data show that hexacoordinate cyt c does not effectively reduce nitrite. Furthermore, in the present study, we have observed separate NO generating activities of cytochrome c oxidase and pentacoordinate cyt c.

In speculation of the relative contribution of each protein to mitochondrial nitrite reduction and NO generation it is important to note that while the concentration of cytochrome c oxidase remains constant, the concentration of pentacoordinate cyt c within the cell may fluctuate depending on lipid binding and redox state of the cell. It is also interesting to consider that the localization of cytochrome c is dynamic within the mitochondrion during normal respiration and in the cytosol once apoptosis is initiated. These unique characteristics of cyt c may lend themselves to the regulation of NO generation within the cell.

Our finding that cyt c does effectively reduce nitrite under conditions likely to be found in hypoxia and ischemia, where the heme of cyt c may become pentacoordinate due to protein nitration or methionine oxidation, is likely to be relevant to mitochondrial hypoxic/ischemic signaling. Indeed, here we show that at low pH and in hypoxia, micromolar concentrations of nitrite can significantly inhibit mitochondrial respiration through the inhibition of cytochrome c oxidase. This transient inhibition of respiration may be a mechanism by which oxygen is conserved in an ischemic situation. Once oxygen concentration increases, the inhibition is reversed and respiration is restored (30, 31, 48, 49). In addition to inhibition of cytochrome c oxidase, nitrite-dependent NO generation may also contribute to S-nitrosation of complex I leading to a more permanent inhibition of respiration (50–52). This type of inhibition has been associated with cytoprotection after ischemia/reperfusion (53, 54).

The ability of cyt c to reduce nitrite is potentially of particular significance in apoptosis. Cardiolipin has been shown to increase during apoptosis leading pentacoordination of the cyt c heme (22, 24–27). In addition, pentacoordination may occur due to hypoxia-associated oxidative and nitrosative stress. Low levels of nitrite reduction and NO generation by cyt c may prevent cyt c release from the mitochondria just as is observed with low concentrations of authentic NO. Indeed other studies have found that nitrite prevents cyt c release and permeability pore opening in isolated mitochondria after I/R (54). At a high rate of nitrite reduction, NO formed by cyt c may bind to cytochrome c oxidase, leading to membrane depolarization and contributing to cyt c release in the cytosol. In addition, NO may bind cyt c (as is observed here) which has itself been shown to enhance cyt c release and subsequent formation of the apoptosome (37).
**Cytochrome c Nitrite Reductase Activity**

Generally, cyt c has been shown to play a significant role in redox signaling and its ability to produce NO under certain conditions would have profound implications in this area. For example, our results expand the role of cyt c in nitration reactions in which cyt c has been found to be a target and a catalyst (16). Nitration is known to impact cell signaling and function in many ways (55–59).

It has also previously been shown that cyt c gains substantial peroxidase activity when the heme becomes pentacoordinate and this activity contributes to the peroxidation of mitochondrial cardiolipin, promoting the release of cyt c (22, 38). Nitrite reduction by cyt c may lead to the generation of NO that can act as an antioxidant to prevent lipid peroxidation (60).

Our results indicate that under hypoxic conditions and those in which the cyt c heme becomes pentacoordinate, cyt c will reduce nitrite to NO. Precisely how this will affect signaling in apoptotic and non-apoptotic cells remains to be determined and deserves further study.

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