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## A Novel Role for Cytochrome c: Efficient Catalysis of S-Nitrosothiol Formation

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### Abstract

While S-nitrosothiols are regarded as important elements of many NO-dependent signal transduction pathways, the physiological mechanism of their formation remains elusive. Here, we demonstrate a novel mechanism by which cytochrome c may represent an efficient catalyst of S-nitrosation in vivo. In this mechanism, initial binding of GSH to ferric cytochrome c is followed by reaction of NO with this complex, yielding ferrous cytochrome c and GSNO. We show that when submitochondrial particles or cell lysates are exposed to NO in the presence of cytochrome c, there is a robust formation of protein S-nitrosothiols. In the case of submitochondrial particles protein S-nitrosation is paralleled with an inhibition of mitochondrial complex I. These observations raise the possibility that cytochrome c is a mediator of S-nitrosation in biological systems, particularly during hypoxia, and that release of cytochrome c in to the cytosol during apoptosis potentially releases a GSNO synthase activity which could modulate apoptotic signaling.

### Keywords

nitric oxide; nitrosation; nitrosylation; cytochrome c; mitochondria

### Introduction

S-Nitrosation, also referred to as S-nitrosylation, is thought to be a major mechanism of NO-mediated signaling in pathology and physiology [1]. S-Nitrosated proteins have been found in many tissues [2], and proteins reported to be S-nitrosated are involved in a wide range of physiological functions including transcription, channel activity, response to hypoxia, and cell death [1]. It has been proposed that dysregulation of S-nitrosation is involved in many

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pathological states and that control of S-nitrosation could lead to new therapeutic strategies [1].

Although it is widely accepted that S-nitrosation occurs in vivo, mechanistic details have been elusive. NO is synthesized from nitric oxide synthase, but does not directly react with thiols to form S-nitrosothiols (RSNO) [3]. Several mechanisms of S-nitrosation have been proposed that fall into three main categories: (i) formation of nitrosating species from NO oxidation by a molecular oxygen-dependent process [4,5] (ii) thiol oxidation to form a thiyl radical, which can combine with NO to form RSNO [5,6] and (iii) direct addition of NO to RSH to form the radical intermediate RSNOH followed by oxidation of this radical by oxygen or some other one-electron acceptor [7]. While the first two mechanisms have been shown to be viable in vitro, evidence for the third reaction in vitro is equivocal. Redox-active metal ions [8], metalloproteins [9] and hydrophobic environments [10,11] have been implicated as elements that enhance S-nitrosation via the above mechanisms. However, no specific intracellular S-nitrosothiol synthetic pathway has been established. Although S-nitrosation has been thought to be largely oxygen dependent, recent evidence points to hypoxia as being a stimulus of S-nitrosothiol synthesis [12,13].

In this study we show that ferric cytochrome c (cyt c) is an efficient S-nitrosoglutathione (GSNO) synthase in both the presence and absence of oxygen. Ferric cyt c is reduced by NO. At pH below 7, NO reversibly binds ferric cytochrome c, but at higher pH, it will slowly reduce ferric cytochrome c to the ferrous form [14]. This process is believed to involve nucleophilic attack by OH<sup>-</sup> on the ferric nitrosyl which is expected to have considerable Fe<sup>II</sup>-NO<sup>+</sup> character, forming nitrite and the ferrous heme. In the case of other heme proteins like hemoglobin and myoglobin, excess NO in solution rapidly binds to the reduced heme so that the process is often referred to as reductive nitrosylation [14]. In the case of cytochrome c, NO doesn't avidly bind the ferrous heme and, therefore, mainly ferrous cyt c rather than the ferrous nitrosyl form is made [14]. It has been known for many years that ferric cyt c can also be reduced by thiols to the ferrous form [15–20]. The reaction is thought to involve both metal-catalyzed and metal-independent pathways [15]. The metal-independent pathway involves a glutathione-cyt c intermediate where GSH is loosely bound to the heme [20]. The end products reported in these reactions are ferrous cyt c and glutathione disulfide (GSSG) [15,19]. We show here that the rate of reduction of cyt c by GSH is increased at least 10 fold by NO and that the major product of this reaction is GSNO. The reaction is most efficient at low NO concentrations and at higher concentrations a secondary GSSG-forming reaction occurs that lowers efficiency. This represents the first report of a specific intracellular S-nitrosothiol synthetic mechanism.

## Experimental Procedures

PROLI/NO was purchased from Cayman Chemicals (Ann Arbor, MI) and 1,2-Dioleoyl-sn-glycero-3 phosphocholine and 1,1',2,2'-tetraoleoyl cardiolipin (sodium salt) were purchased from Avanti Polar Lipids (Albaster, AL). We conducted experiments using three different cyt c preparations from Sigma (bovine heart C-2037, horse heart C-7752, and horse heart C-2506) with no qualitative differences in observed phenomenon. To insure that the purity of our samples was not likely to contribute to our results we included experiments using horse heart cytochrome c (C-7752) which has been shown to be substantially devoid of impurities [1,2]. Experiments were performed using this preparation following centrifugation at 16,000 g to sediment any aggregates and further purification using G-25 columns. Similar results were obtained with all cyt c preparations. All other chemicals were purchased from Sigma Chemicals unless otherwise noted. Liposomes were prepared by sonication of a 1:1 molar ratio of the two lipids as described [21]. All experiments were performed in either 0.1 M phosphate buffer or 2-(N-morpholino)ethanesulfonic acid buffer (MES) adjusted to the appropriate pH with NaOH

or HCl. Metal chelating agents diethylenetriamine penta-acetic acid (DTPA, 100  $\mu$ M) or ethylenediaminetetraacetic acid (EDTA, 100  $\mu$ M) or both were added to all reaction mixtures.

Absorption spectroscopy was conducted either on a Cary 50, Perkin Elmer Lambda 9, or an Agilent 8452 diode array UV-Vis spectrophotometer. The Perkin Elmer instrument is equipped with an integrating sphere detector so that scattered light from turbid samples, such as those containing liposomes, is collected. Time-resolved absorption was analyzed by global analysis using Specfit (Spectrum Software Associates, Chapel Hill, NC) where singular value decomposition is applied and the absorption data at all wavelengths and times are fit to a kinetic model, such as a single exponential process or double exponential process in order to obtain observed rate constants.

Anaerobic conditions were achieved by dilution of reagents into deoxygenated buffer following purging of the buffer and reagents with an inert gas (argon or nitrogen). The samples were transferred using Hamilton air-tight syringes to septum-capped absorption cuvettes and a positive pressure of inert gas was maintained during the measurements. For some experiments anaerobiosis was achieved using a Coy anaerobic chamber. Samples were prepared within the chamber and rapidly transferred to the spectrophotometer for kinetic analysis.

Low oxygen (1%) conditions were achieved by adding air to samples that had been prepared anaerobically while monitoring the oxygen pressure using a Unisense (Aarhus, Denmark) Oxy-meter with a needle electrode.

The concentration of nitrosothiols was determined by either chemiluminescence or HPLC. To determine the concentration of nitrosothiols using chemiluminescence measurements at a particular time point in the reaction, 5 mM N-ethylmaleimide (NEM) was added to the reaction mixture to prevent any subsequent nitrosation reactions during analysis. After a forty minute incubation, low molecular weight thiols were separated from the protein using Microcon centrifugal filter devices with a molecular weight cutoff of 10,000 Da (Millipore, Billerica, MA). The concentration of low molecular weight S-nitrosothiols was then determined using a Sievers 280i nitric oxide analyzer employing the copper/cysteine assay described previously [22]. The authenticity of the nitrosothiols was confirmed by verification that addition of 5 mM mercury chloride eliminated the chemiluminescent signal.

All HPLC analyses were performed on a Kromasil C-18 column (25 cm length, 0.46 cm internal diameter, 0.5  $\mu$ m particle size). GSNO was eluted isocratically with a mobile phase consisting of methanol and 0.05% trifluoro-acetic acid (TFA) in a ratio of 6:94. GSSG was analyzed by an ion-pair method, using 3% acetonitrile and 97% 10 mM  $K_2HPO_4$  and 10 mM tetra-butyl-ammoniumhydrogensulfite (TBAHS) as eluent. The effluents were monitored with a diode array spectrophotometer at 336 and 210 nm for GSNO and GSSG, respectively [23].

Submitochondrial particles (prepared from rat liver by sonication and centrifugation [24]) were incubated anaerobically with ferric cyt c, GSH, and PROLI/NO for 0–120 minutes, after which the samples were centrifuged (3 min. at 10,000 g) to separate GSH from SMP protein. Successful separation was confirmed by measuring the absence of complex I activity in the supernatant, but not in the pellet, as well as the presence of reduced thiol in the supernatant (measured using Ellman's reagent). Both fractions were subjected to copper cysteine based reductive chemiluminescence to measure GSNO and protein SNO levels. Complex I activity was also measured in the SMP fraction by monitoring the rotenone sensitive oxidation of NADH at 340 nm as previously described [25].

RAW 264.7 cells were lysed under anaerobic conditions in lysis buffer (50 mM Tris, pH 7.4, 1 mM DTPA, 0.5 % Tween-20, protease inhibitors) and incubated with PROLI/NO in the presence or absence of ferric cyt c for 30 minutes. To prevent any subsequent S-nitrosation

reactions free thiols were blocked with 50 mM NEM in the presence of 6.5 M urea and 3 % CHAPS. Samples were precipitated with trichloroacetic acid to separate proteins from low molecular weight compounds and S-nitrosothiol levels in protein fraction of cell lysate were assessed by tri-iodide-based chemiluminescence.

All data are reported as a mean  $\pm$  one standard deviation unless otherwise designated.

## Results

Both NO and glutathione alone reduce cyt c. The reduction of cyt c (50  $\mu$ M) by GSH (1 mM) at pH 7.4, followed by absorption spectroscopy, is shown in Fig. 1a. The spectrum of ferric cyt c consists of a single broad absorption centered at 530 nm. Reduction to the ferrous hexacoordinate heme-protein results in the evolution of two absorption peaks at 525 and 550 nm. The time course of absorbance changes at 550 nm is shown in the inset to Fig. 1a. A global fit to the data (fitting all wavelengths simultaneously) using a single exponential model (inset of Fig. 1a) exhibits a relatively poor fit with a  $k_{\text{obs}}$  of  $0.25 \pm 0.16 \times 10^{-3} \text{ s}^{-1}$  ( $n=6$ ). Previous studies have demonstrated that the reduction of ferric cyt c by GSH is complex and may be autocatalyzed by the formation of GSSG resulting in non exponential kinetics [15–20].

Cyt c reduction by NO at pH 7.4, under anaerobic conditions, was followed by adding the rapid-release NO donor proliNONOate (PROLI/NO, 25  $\mu$ M,  $t_{1/2}$  of approximately 2 s) to ferric cyt c (50  $\mu$ M, Fig. 1b). The first spectrum, taken immediately after NO addition, indicates a mixture of the ferric and ferric nitrosyl forms of the heme protein. The time course of reduction fits well to a single exponential process (inset of Fig. 1b) with an observed rate constant of  $0.27 \pm 0.16 \times 10^{-3} \text{ s}^{-1}$  ( $n=3$ ).

The synergistic enhancement of cyt c reduction by the combination of NO and GSH is shown in Fig. 1c and inset. The time-course again fits well to a single exponential process with an observed rate constant of  $4.2 \pm 1.1 \times 10^{-3} \text{ s}^{-1}$  ( $n=7$ ). The additive rate constant under these conditions is  $0.52 \times 10^{-3} \text{ s}^{-1}$ , indicating an almost 10-fold increase in rate constant over the expected value.

Full analysis of the spectral changes that occur during the reduction of cyt c by GSH/NO indicates that at least four spectrally distinct species are involved. These four spectra are shown in Fig. 2a and are used as basis spectra for multiple linear regression analysis of the data. The spectra of the ferric and ferrous forms of cytochrome c are well established and spectral subtraction revealed the presence of the two distinct NO-bound species that have been previously observed and referred to as compound 1 and compound 2. Compound 1 is formed upon addition of NO to ferric cyt c and is either ferric nitrosyl cyt c or a derivative thereof, and compound 2 is formed from treating compound 1 with sodium dithionite and is likely ferrous nitrosyl cyt c [26]. Deconvolution of the spectral changes that occur during reduction of cyt c by 20  $\mu$ M, 100  $\mu$ M and 500  $\mu$ M PROLI/NO are shown in Figs. 2b to d respectively. At low NO concentration there is an initial formation of ferric nitrosyl cyt c, which then decays in parallel with the remaining ferric cyt c to generate ferrous cyt c as the only heme product. Under these conditions the reaction is almost complete within 3 minutes. At intermediate and high NO levels, there is a much greater initial formation of ferric nitrosyl cyt c, and ferrous nitrosyl cyt c is generated as a product. Importantly, at high levels of NO the rate of cyt c reduction decreases. The initial rate of cyt c reduction (from the sum of both ferrous and ferrous nitrosyl cyt c), as a function of PROLI/NO concentration, is shown in Fig. 3a. The maximal initial rate of cyt c reduction occurs at an NO concentration approximately stoichiometric with cyt c. This unexpected behavior indicates underlying mechanistic complexity at higher non-physiological levels of NO. The fact that high initial levels of ferric nitrosyl cyt c, as occurs at high PROLI/NO concentrations, result in a slower reaction suggests that ferric nitrosyl cyt c

is not an intermediate in the reduction of cyt c, but rather represents a complex that inhibits the reaction by reducing the amount of available ferric cyt c and NO. This would suggest a mechanism whereby GSH initially binds to cyt c, and NO reacts with this complex to result in reduction of the heme iron. In favor of this mechanism the initial rate of cyt c reduction appears to follow a hyperbolic dependence as a function of GSH concentration (Fig. 3b) indicative of saturable binding. The apparent  $K_m$  for GSH derived from a hyperbolic fit to these data (shown on Fig. 3b) is  $181 \pm 68 \mu\text{M}$ . This behavior also favors reaction of NO with GSH in an active site of cyt c, rather than the mechanism similar to that proposed by Gow et al [7] where GSH and NO would combine in solution to form a radical intermediate which would then reduce an available oxidant.

In agreement with published data,[3] the anaerobic incubation of GSH with NO in the absence of cyt c did not result in GSNO formation (data not shown). However, the inclusion of cyt c resulted in robust glutathione S-nitrosation that occurs on the same time scale as cyt c reduction (Fig 4a). To examine if glutathionyl radical is an intermediate in GSNO formation experiments were performed in the presence and absence of the radical scavenger 5,5-dimethyl-1-pyrroline N-oxide (DMPO). No significant difference in GSNO formation or the observed rate constant for cyt c reduction were observed ( $21 \pm 3 \mu\text{M}$  GSNO vs  $20 \pm 3 \mu\text{M}$  final GSNO yield and observed rate constant of  $3.3 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$  vs  $3.3 \pm 0.5 \times 10^{-3} \text{ s}^{-1}$  when  $50 \mu\text{M}$  PROLI/NO is added to  $1 \text{ mM}$  GSH and  $50 \mu\text{M}$  cyt c without and with  $10 \text{ mM}$  DMPO,  $n=3$ ). The dependence of GSNO yield on NO concentration is shown in Fig. 4b. It is clear from these data that the formation of GSNO is not catalytic and that the amount of GSNO formed is limited by either the concentration of NO or the concentration of cyt c, whichever is the smaller. The efficiency of GSNO formation from NO decreases as NO concentration is increased (Fig 4b), suggesting a competing reaction that involves GSH-dependent NO consumption but that does not generate GSNO or reduce cyt c, and this is likely related to the slower kinetics of cyt c reduction observed at higher NO concentrations (Fig. 3). Efficiency values were calculated based on the measured release of NO from PROLI/NO. While it is usually assumed that PROLI/NO generates 2 NO/molecule, our measurements, conducted independently in two laboratories (using oxy hemoglobin oxidation and direct NO-dependent chemiluminescence as observables), indicated that the yield of NO from PROLI/NO is close to 1:1 (see Supplementary Figure 1) and this value was used for efficiency calculations. The yield of GSNO formation as a function of GSH concentration is shown in Fig 4c. This somewhat mirrors the GSH-dependence of cyt c reduction shown in Fig 3b, and again indicates that the GSNO-generating reaction is a saturable phenomenon. The diminished efficiency of GSNO formation at higher NO levels suggests that alternative glutathione-derived products are being formed. An ion-pair HPLC method (TBAHS method) was employed to assess GSH oxidation products. It was observed that at low NO, GSNO was the only detectable product of GSH (data not shown), but at high NO the major product was GSSG (Fig. 4d). In fact NO results in a large increase in GSSG formation over that observed in the absence of NO. It is of interest to note that at higher NO concentrations, although the rate of cyt c reduction is diminished (Fig 3a), there is a higher initial level of ferric nitrosyl cyt c (Figure 2d) and GSSG is additionally formed (figure 4d), and the yield of GSNO is not decreased (Figure 4b). This suggests that at high NO, the yield of GSNO depends largely on the initial level of ferric cyt c, and that other side reactions may interfere kinetically with the formation of GSNO, but do not ultimately change the yield of S-nitrosation.

The anionic phospholipid cardiolipin has been shown to interact with cyt c, leading to pentacoordination of the cyt c heme iron and subsequent alterations in its chemical reactivity. [27–31] To examine if cardiolipin affects the GSNO synthase activity of cyt c, the reaction was examined in the presence of cardiolipin-containing liposomes. As shown in Fig. 5a, the GSNO synthase activity was apparent in the presence of cardiolipin albeit with a slightly lower  $k_{\text{obs}}$ . We found that, as is the case for when liposomes are absent, the combination of GSH and NO greatly enhanced the rate of cyt c reduction compared to when only one of these reagents

is present (observed rate constants of  $1.8 \pm 1.0 \times 10^{-3} \text{ s}^{-1}$  with both NO and GSH present compared to  $0.18 \pm 0.07 \times 10^{-3} \text{ s}^{-1}$  with only GSH and too slow to fit with only NO,  $n=3$ ). Interestingly, the amount of GSNO generated is greater when anionic phospholipids are present compared to when they are absent (Fig. 5a, inset), suggesting that pentacoordination may modulate GSNO synthesis.

The effect of local acidity may also be an important effector of cyt c activity in mitochondria, especially during hypoxia. It was observed that GSNO synthesis is slower at pH 6.5, but yields are not substantially affected. (Fig. 5b and 5c).

To examine if oxygen affects GSNO formation the reaction was performed in a 1% oxygen atmosphere. The time course of cyt c reduction under these conditions fitted well to a bi-exponential model (Fig. 6a). The majority of the cytochrome is reduced during the fast phase which occurs with an observed rate constant of  $7.5 \pm 2.1 \times 10^{-3} \text{ s}^{-1}$  while reduction in the slow phase is described by an observed rate constant of  $0.53 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$  ( $n=3$ ). Importantly, substantial GSNO is formed even when oxygen is present (Fig. 6b).

To determine whether the GSH/NO/cyt c system could lead to protein S-nitrosation in a model of a biological system, submitochondrial particles (SMPs), which are devoid of cyt c and mitochondrial matrix proteins, were incubated with NO, GSH and ferric cyt c, under anaerobic conditions, and both total S-nitrosation and complex I activity were determined. Robust S-nitrosation of SMP proteins was only observed in the complete system, whereas much lower levels were observed when individual components were omitted (Fig. 7a). Parallel measurements of complex I activity, which has been shown to be inhibited by S-nitrosation, demonstrated that all reaction components were necessary for an inhibition of activity (Fig. 7b), and that the magnitude of inhibition of activity was temporally correlated to S-nitrosothiol formation (Fig. 7c). The yield for protein S-nitrosation is slightly lower than that for GSNO formation in the absence of SMPs (compare Fig. 7c and Fig. 4a), and the rate of S-nitrosation is considerably slower. This is consistent with cyt c-mediated GSNO formation followed by slower GSNO-mediated transnitrosation of SMP protein thiols.

The ability of cyt c to generate protein S-nitrosothiols in cell lysate under strictly anaerobic conditions was determined by incubating PROLI/NO (100  $\mu\text{M}$ ) and ferric cyt c (100  $\mu\text{M}$ ) with lysate from RAW 264.7 cells (1.5 mg/ml). Proteins were precipitated and S-nitrosothiol content determined by chemiluminescence. As shown in Fig 7d, in the absence of cyt c, a small but measureable level of S-nitrosation was observed. In the presence of added cyt c, the level of S-nitrosation was greatly enhanced approaching 1nmol/mg protein. Filtration of cell lysate using a centricon 10 KDa cut-off filter revealed about 40% of total RSNO associated with the high molecular weight fraction and 60% associated with low molecular weight component (data not shown).

## Discussion

In this study we have demonstrated that ferric cyt c can act as an electron acceptor allowing the efficient formation of GSNO from a mixture of GSH and NO. Coupled with cyt c oxidation, this observation suggests that cyt c may represent an important catalyst of S-nitrosation *in vivo*. The yield in terms of GSNO formed compared to NO added is the largest of any proposed mechanism that is likely to occur under physiological conditions. While protein S-nitrosation has been proposed as an important intracellular signaling paradigm, and there are many examples of proteins that have been reported to be S-nitrosated under various conditions and stresses, no concerted and efficient S-nitrosothiol synthetic machinery has been described in cells. Most discussion of S-nitrosothiol formation has focused on its intrinsic chemistry with oxygen. This reaction is known to generate S-nitrosothiols *in vitro* through the intermediacy

of nitrogen dioxide. However there is significant doubt, based mainly on kinetic arguments, of the validity of this mechanism at physiological oxygen and NO levels. Recent reports have suggested S-nitrosation can occur and may even be enhanced under anaerobic conditions [12,13]. Proposed mechanisms for anaerobic S-nitrosation include the reductive bioactivation of nitrite by heme proteins [32], and the intermediacy of dinitrosyl iron complexes [12]. Since NO has a short biological half-life, its chemical conversion to a nitrosothiol must compete with its other reactions and we suggest the GSH/NO/cyt c reactions demonstrated in this paper may constitute such a mechanism.

The proposed mechanism of GSNO formation is shown in Fig 8. The characteristics of GSNO formation from ferric cyt c, NO and GSH suggests that the mechanism involves initial binding of GSH to the heme protein followed by reaction of NO with this complex. The rate of cyt c reduction saturates at high GSH and is diminished at high NO. The loose association of GSH with the cyt c heme has been previously described [20], and our data indicates an apparent  $K_m$  of about 200  $\mu\text{M}$  for GSH. We propose that the reaction of NO with GSH-bound cyt c generates a ternary complex that can be thought of as a hydroxyl amino radical (or a related tautomer as described previously [33]) bound in the vicinity of the cyt c heme. This bound radical then reduces the heme to the ferrous state, resulting in the generation and release of GSNO (Fig. 8 red arrows). Alternatively, and more effectively at higher NO concentrations, NO can react with the bound radical leading to GSSG formation and presumably nitrous oxide, and regenerate ferric cyt c (step g, green arrows, in Fig. 8). As the concentration of NO increases, the unproductive pathway via step g becomes more favorable compared to step f, slowing down cyt c reduction.

Since many ferric heme nitrosyl species have  $\text{Fe}^{\text{II}}\text{-NO}^+$  character, direct transfer of the  $\text{NO}^+$  to a reduced thiol has been considered as a viable nitrosation pathway [34,35]. This pathway is shown in steps b and d of Fig. 8, but is not consistent with the observed dependence of cyt c reduction on NO concentration. One would expect that as the concentration of NO increases, more ferric nitrosyl cyt c would form (Fig. 3) and the reaction pathway depicted in steps b and d would result in an increase, as opposed to a decrease, in the rate of reaction. Thus, NO binding to the ferric cyt c (step b in Fig. 8) actually represents a non-productive competitive pathway in GSH/NO-dependent cytochrome c reduction and GSNO production. One could consider an electron transfer as depicted in step c, yielding the reduced heme and a thiyl radical that could form GSNO upon reaction with NO (step c in Fig. 8). However, our finding that addition of the thiyl radical scavenger DMPO (10 mM) did not affect GSNO yield suggests strongly that thiyl radical is not an intermediate.

S-Nitrosation of GSH to form GSNO will not directly serve as a signal. However, it is well established that GSNO can participate in transnitrosation reactions where the S-nitroso functional group is reversibly transferred to another thiol [36,37]. Such transfer of nitroso groups is responsible for the very large increase in protein S-nitrosation that occurs when cells are exposed to S-nitrosocysteine [38]. We show here that when both SMPs and cell lysate are exposed to NO in the presence of cyt c, there is a robust formation of protein S-nitrosothiols. In the case of SMPs we show that protein S-nitrosation is paralleled with an inhibition of mitochondrial complex I, an enzyme complex known to be inhibited by thiol S-nitrosation. Although S-nitrosation of complex I has been implicated in a number of signaling pathways, it is very important to note that there are several aspects of our model SMP system that do not apply to normal physiology of the intact mitochondria. Superoxide generation by the mitochondrion, is likely to mitigate protein S-nitrosation. In addition, the localization of cytochrome c in the intermembrane space, where the concentration of GSH is relatively low may not be optimal for this nitrosative reaction to proceed. Thus, our experiments performed using the SMP system should be viewed as a demonstration that the cyt c/GSH/NO combination can result in protein S-nitrosation, but much more work is needed to demonstrate that this

actually occurs in the healthy mitochondria and there are several reasons for believing that it would not.

The experiments in this study are largely performed under anaerobic conditions. This was done largely to inhibit oxygen-dependent S-nitrosation. However, anaerobiosis is not required for this activity as NO still enhances cyt c reduction in the presence of 1% oxygen and robust S-nitrosation is still observed under these conditions. Acidification to pH 6.5 appears to result in a small but significant decrease in the yield of GSNO, indicating that the lower pH of the mitochondrial inter-membrane space will still allow for GSNO formation through this mechanism (Fig. 5). S-Nitrosation may also be turned on in hypoxia through an increase in NO via nitrite reduction by hemoglobin, [39,40] myoglobin [41,42] or cytochrome c oxidase, [43,44] potentially linking SNO/NO signaling to nitrite reduction. Nitrite reduction by the mitochondria results in the S-nitrosation of complex I [45]. Interestingly, cyt c acts as a nitrite reductase when the heme iron-methionine bond is broken whether through oxidative stress or interaction with anionic phospholipids such as those present in the inner membrane of the mitochondria as occurs during apoptosis [24,46]. In addition the release of cyt c in to the cytosol during apoptosis potentially releases a GSNO synthase activity and this may at least partially explain the observed inhibitory effects of NO on apoptosis. It is conceivable that cytochrome-c dependent protein S-nitrosation could be occurring in the apoptosome after the association of cyt c with apaf, and that such S-nitrosation could negatively influence apoptotic signaling. While methemoglobin and metmyoglobin do not appear to support efficient GSNO synthesis, it is possible that other heme proteins, or iron complexes are also able to generate S-nitrosothiols [12].

Cyt c-dependent GSNO formation clearly merits additional investigations to place this reaction in the context of physiology and pathophysiology. We demonstrate here a simple and efficient biologically relevant mechanism of S-nitrosothiol synthesis and the previous lack of such a mechanism has been a handicap to a full understanding of NO-signaling through S-nitrosothiol formation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

RSNO	S-nitrosothiols
cyt c	cytochrome c
GSNO	S-nitrosoglutathione
GSSG	glutathione disulfide
PROLI/NO	proliNONOate
DMPO	5,5-dimethyl-1-pyrroline N-oxide
TBAHS	tetra-butyl-ammoniumhydrogensulfite
SMP	submitochondrial particle
DTPA	diethylenetriamine penta-acetic acid



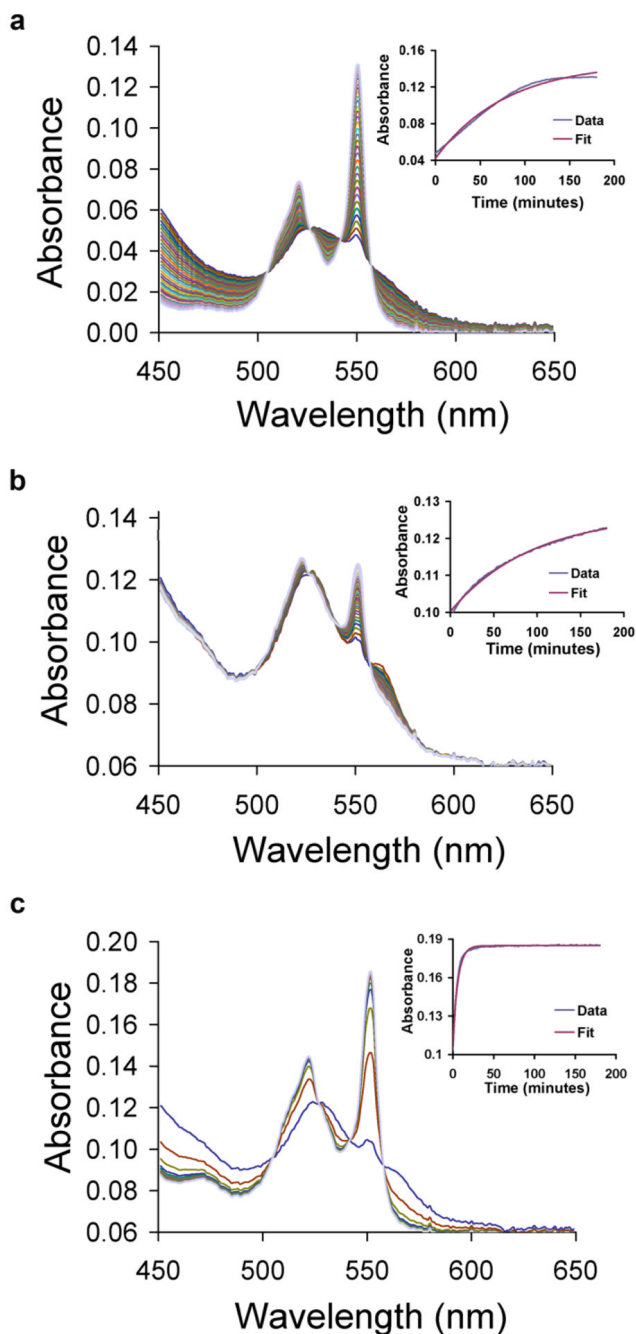
EDTA	ethylenediaminetetraacetic acid
NEM	5 mM N-ethylmaleimide
TFA	trifluoro-acetic acid

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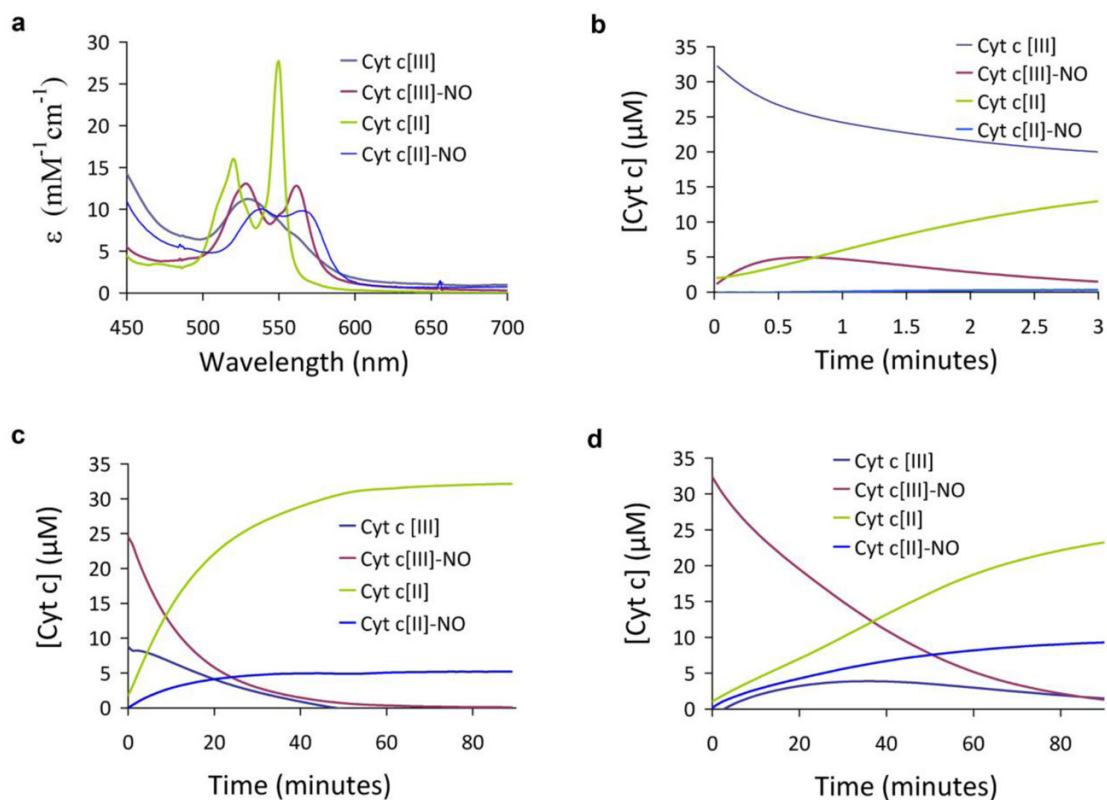
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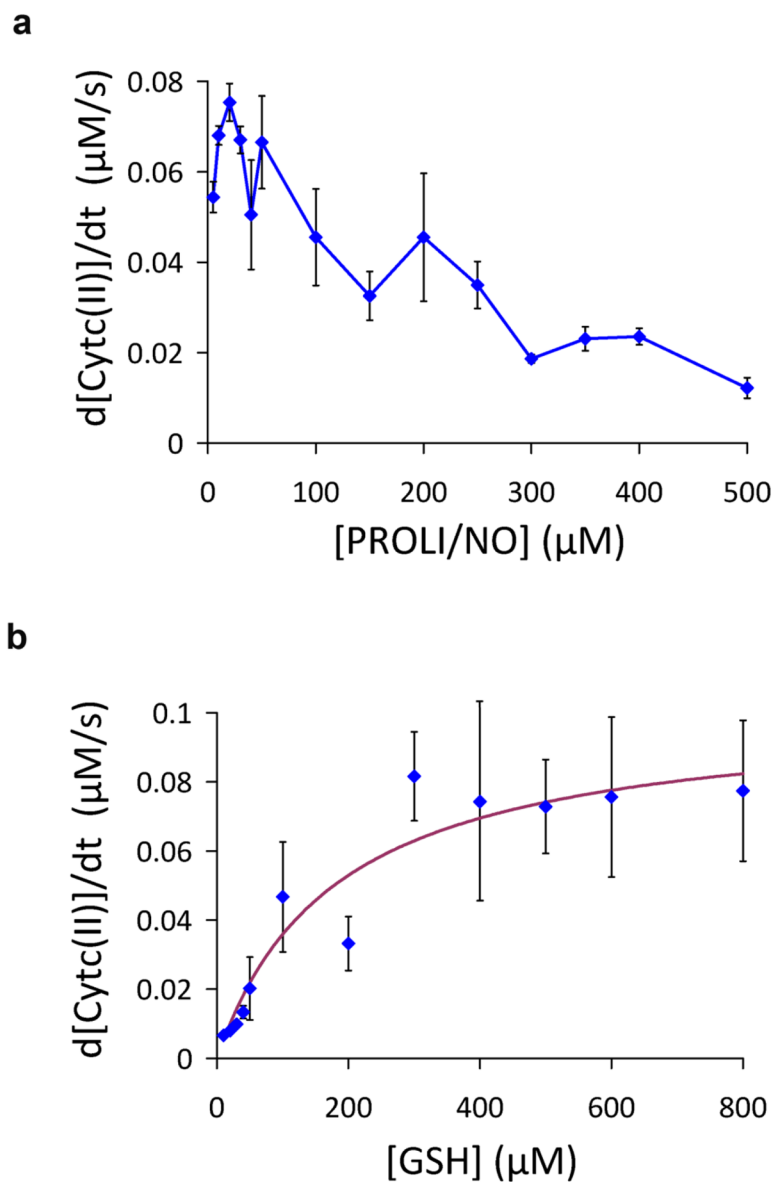


**Fig. 1.**

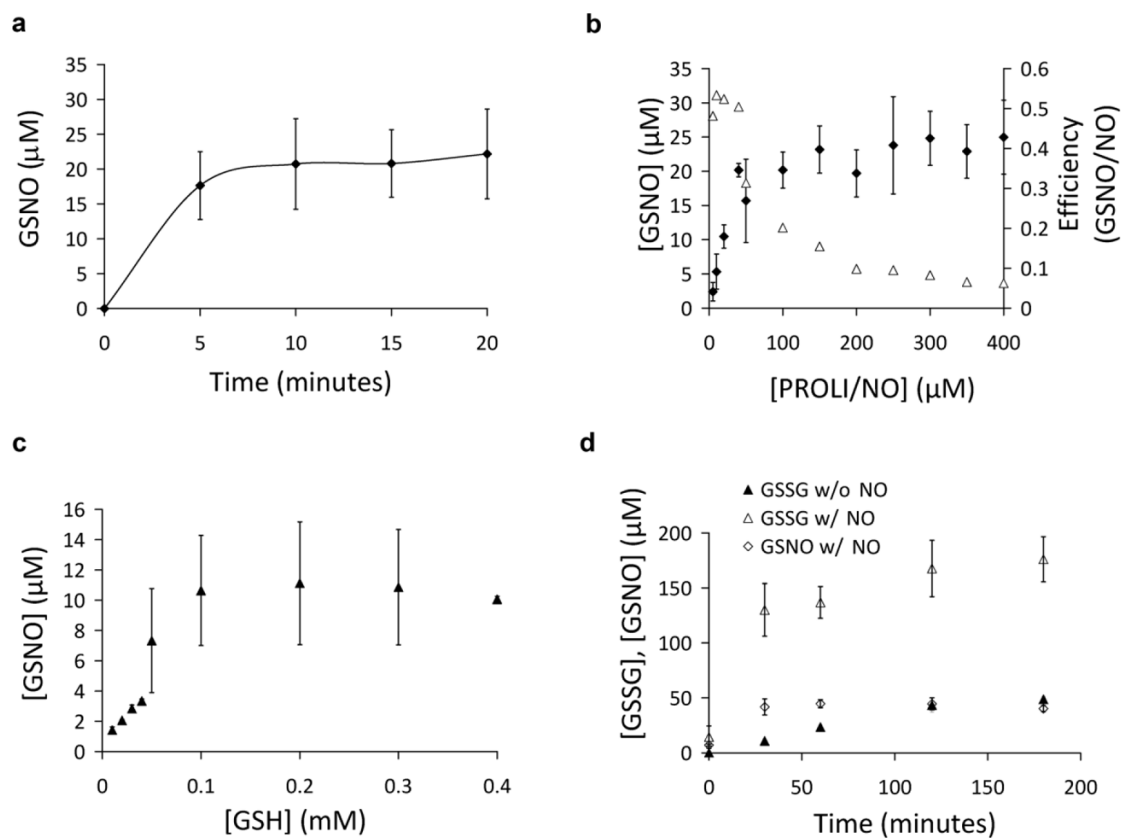
GSH/NO-dependent reduction of ferric cytochrome c Time-resolved absorption spectra following combination of cyt c (50  $\mu$ M) and (a) GSH (1 mM), (b) PROLI/NO (25  $\mu$ M), or (c) both of these under anaerobic conditions at room temperature and pH 7.4. Spectra were collected every two minutes following mixture of the reagents in a 0.1 cm pathlength cell. Insets show kinetic traces of absorption at 550 nm and the theoretical fit to a single exponential decay with observed rate constants of  $0.22 \times 10^{-3} \text{ s}^{-1}$ ,  $0.18 \times 10^{-3} \text{ s}^{-1}$ , and  $2.8 \times 10^{-3} \text{ s}^{-1}$  for conditions in (a), (b), and (c). The results shown here were for bovine heart cyt c. Similar results are shown in a supplementary figure (Figure S2) for horse heart cyt c (C-7752) that had been further purified using sedimentation and a G-25 column.



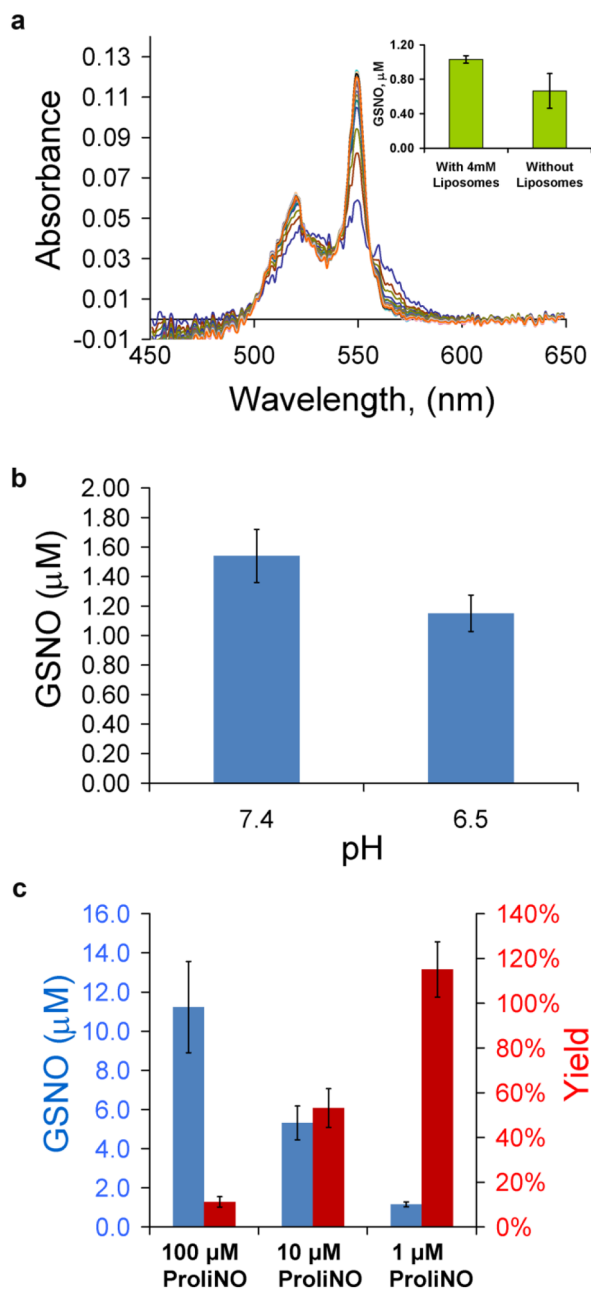
**Fig. 2.** Spectral analysis of cyt c during reduction by NO/GSH. (a) Basis spectra of 4 species used in multiple linear regression analysis: ferric cyt c, ferric nitrosyl cyt c, ferrous cyt c, and ferrous nitrosyl cyt c. b-d) Multiple linear regression analysis of spectral changes occurring during incubation of ferric cyt c (35  $\mu\text{M}$ ), GSH (1 mM) and PROLI/NO at a concentration of (b) 20  $\mu\text{M}$ , (c) 100  $\mu\text{M}$  and (d) 500  $\mu\text{M}$ . Reactions were performed anaerobically at room temperature, in 100 mM phosphate buffer, pH 7.4 containing 100  $\mu\text{M}$  DTPA.



**Fig. 3.** Concentration dependences of anaerobic cyt c reduction. (a) Initial rate of cyt c (35 μM) reduction (sum of ferrous cyt c and ferrous nitrosyl cyt c) by GSH (1 mM) as a function of PROLI/NO concentration, taken from similar data to that shown in Fig. 2. (b) Dependence of cyt c (35 μM) reduction (sum of ferrous cyt c and ferrous nitrosyl cyt c) by PROLI/NO (50 μM) as a function of with GSH concentration taken from similar data to that shown in fig 2. Data represent mean ± standard deviation (n=3)

**Fig. 4.**

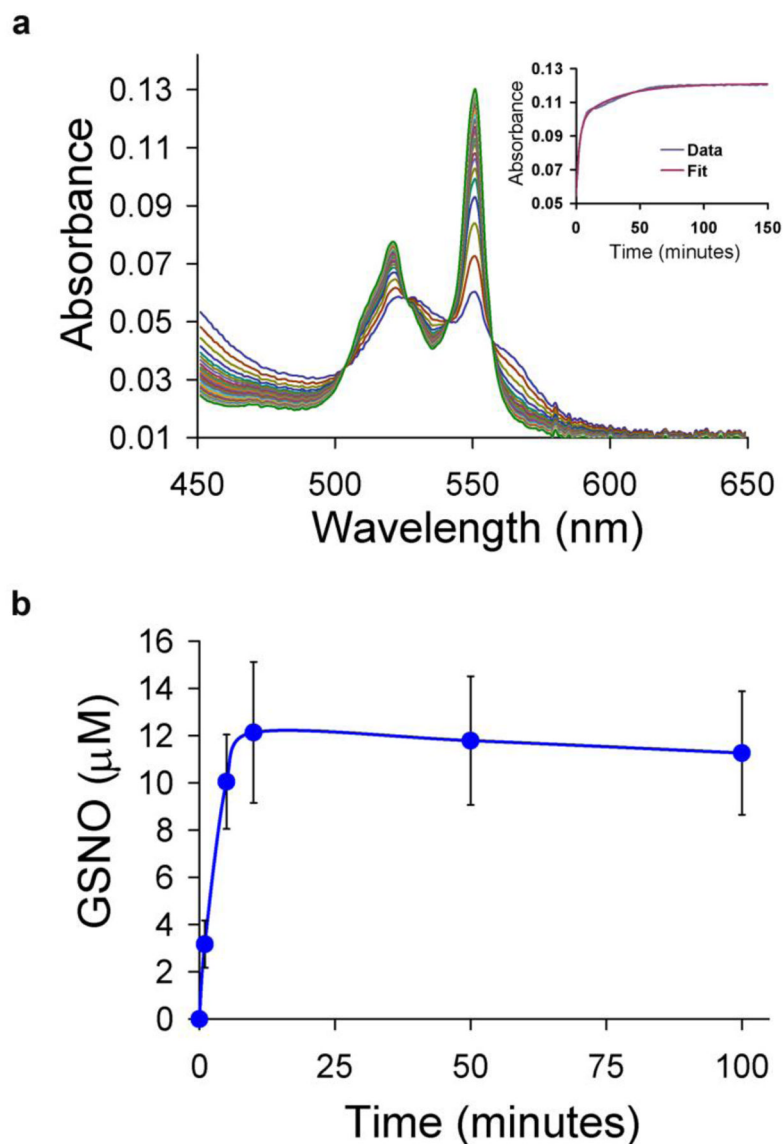
Cyt c-mediated GSNO synthesis (a) Cyt c (50  $\mu\text{M}$ ), PROLI/NO (25  $\mu\text{M}$ ) and GSH (1 mM) were incubated under anaerobic conditions at pH 7.4 and samples removed every 5 minutes. S-Nitrosothiol content was determined by chemiluminescence. (b) Cyt c (35  $\mu\text{M}$ ) was incubated with GSH (1 mM) and increasing amounts of PROLI/NO under anaerobic conditions in 100 mM phosphate buffer pH 7.4 containing 100  $\mu\text{M}$  DTPA at room temperature for 1 hour. GSNO levels ( $\blacklozenge$ ) were determined by HPLC (TFA method). Efficiency ( $\triangle$ ) was calculated as the ratio of GSNO formed divided by NO added, assuming 1 NO formed from each PROLI/NO molecule added. (c) Conditions as in panel “b”, but with 25  $\mu\text{M}$  PROLI/NO and variable GSH. (d) Cyt c (75  $\mu\text{M}$ ) was incubated with GSH (1 mM) and GSSG formation was determined by HPLC ( $\blacktriangle$ , TBAH method). An identical incubation was performed in the presence of PROLI/NO (500  $\mu\text{M}$ ) and both GSSG ( $\triangle$ ) and GSNO ( $\diamond$ ) were measured by HPLC (TBAH method). Data represent mean  $\pm$  standard deviation (n=3).



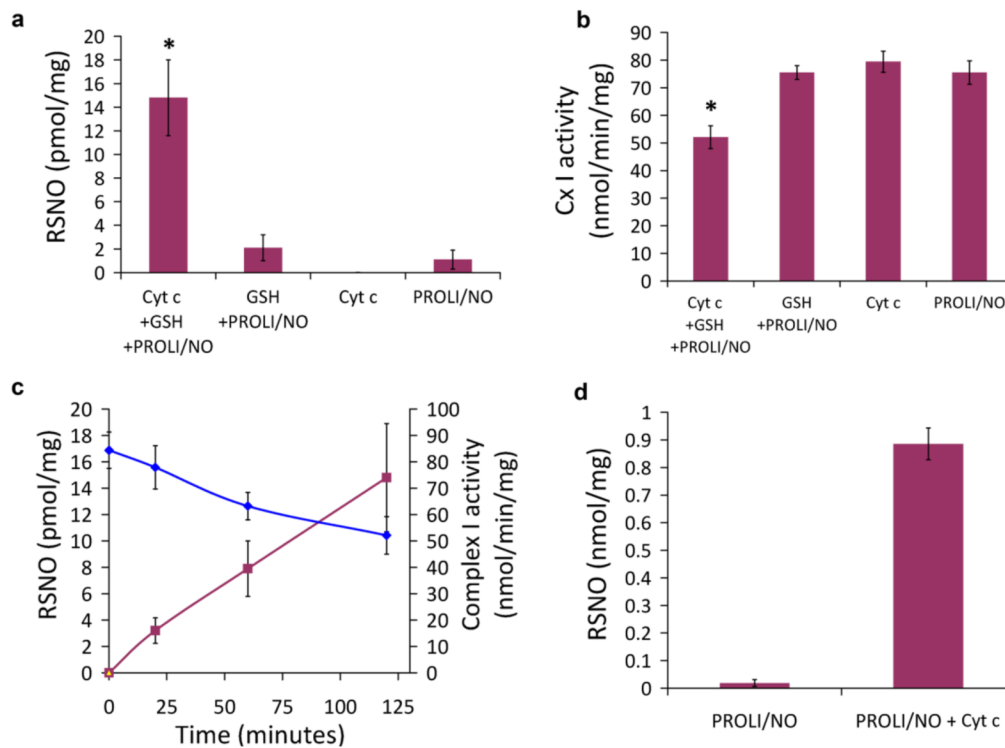
**Fig. 5.** Dependence of GSNO formation on the presence of anionic phospholipids and pH. (a) Liposomes (2 mM) were added (40:1 lipid:protein) and the reduction of cyt c (50  $\mu\text{M}$ ) was followed by time-resolved absorption in the presence of GSH (1 mM) and PROLI/NO (25  $\mu\text{M}$ ) in a 0.1 cm pathlength cell. The observed rate constant for cyt c reduction obtained by global analysis was  $2.0 \times 10^{-3} \text{ s}^{-1}$ . The inset shows the amount of GSNO made in the presence and absence of liposomes after a five minute incubation of GSH (1 mM) and PROLI/NO (1  $\mu\text{M}$ ), pH 6.5 ( $p = 0.019$ ) (b) GSNO yield after mixing 1  $\mu\text{M}$  PROLI/NO, 100  $\mu\text{M}$  cyt c, 4 mM liposomes, and 1 mM GSH at pH shown, ( $p = 0.018$ ). After five minutes, NEM was added and the sample was incubated for 40 minutes followed by filtration of low molecular weight compounds and GSNO analysis by chemiluminescence. (c) Dependence of GSNO formation



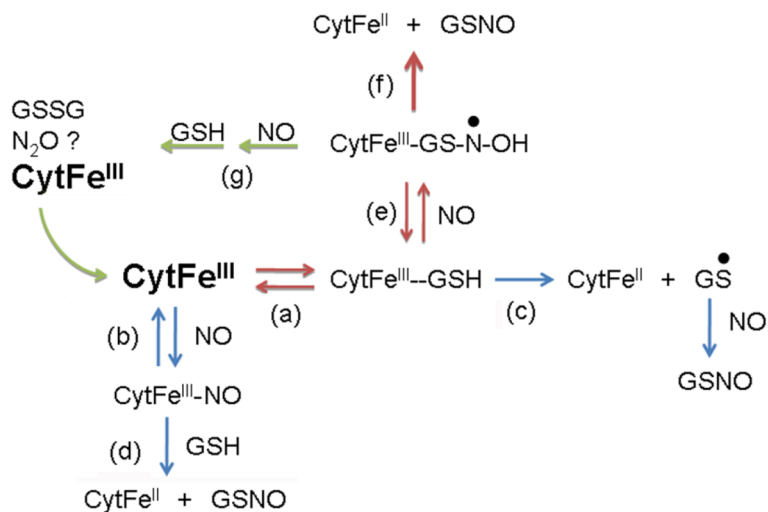
(blue) in experiments as described in panel “b” at pH 6.5 as a function of NO. GSNO yield (red) is calculated as a percentage of GSNO made compared to PROLI/NO added.



**Fig. 6.** Effect of Oxygen. (a) Cyt c (50  $\mu\text{M}$ ) was mixed with 1 mM GSH and 25  $\mu\text{M}$  PROLI/NO under a 1% oxygen atmosphere. Absorption spectra were collected in a 0.1 cm pathlength cell as a function of time, every 0.5 minutes for 10 minutes and then every two minutes until 170 minutes after mixing. The kinetics are shown in the inset and could not be fit well to a single exponential process but did fit to two exponentials with observed rate constants of  $6.3 \times 10^{-3} \text{ s}^{-1}$  for the fast phase and  $0.55 \times 10^{-3} \text{ s}^{-1}$  for the slow phase. (b) GSNO formation for the reaction described in (a) measured by chemiluminescence.



**Fig. 7.** S-nitrosation and catalytic activity in submitochondrial particles and S-nitrosation of cellular proteins. Submitochondrial particles (SMP; 2mg/ml) were incubated anaerobically with or without ferric cyt c (50µM), GSH (1mM), and PROLI/NO (25µM) for 0–120 minutes. (a) The S-nitrosothiol concentration in the SMPs at 120 minutes. (b) Complex I catalytic activity in the SMPs at 120 minutes, \*p< 0.001 (c) Time course of S-nitrosothiol formation in the SMPs (maroon line) and Complex I inhibition (blue line). (n ≥ 3 independent experiments) (d) Cell lysates (1.5 mg/ml) were incubated anaerobically with PROLI/NO (100 µM) in the absence or presence of ferric cyt c (100 µM) for 30 minutes. S-Nitrosothiol levels in the high molecular weight fraction were determined by tri-iodide-dependent chemiluminescence.



**Fig. 8.** Proposed kinetic scheme. The favored pathway responsible for GSNO formation is shown with red arrows. The non-productive pathway that becomes important with high [NO] is shown with green arrows. This results in a catalytic cycle producing GSSG and reduced NO forms which could oxidize ferrous cyt c. See text for further details.