New Insights into Protein S-Nitrosylation

MITOCHONDRIA AS A MODEL SYSTEM

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The biological effects of nitric oxide (NO) are in significant part mediated through S-nitrosylation of cysteine thiol. Work on model thiol substrates has raised the idea that molecular oxygen (O₂) is required for S-nitrosylation by NO; however, the relevance of this mechanism at the low physiological pO₂ of tissues is unclear. Here we have used a proteomic approach to study S-nitrosylation reactions in situ. We identify endogenously S-nitrosylated proteins in subcellular organelles, including dihydrolipoamide dehydrogenase and catalase, and show that these, as well as hydroxymethylglutaryl-CoA synthase and sarcosine dehydrogenase (SarDH), are S-nitrosylated by NO under strictly anaerobic conditions. S-Nitrosylation of SarDH by NO is best rationalized by a novel mechanism involving the covalently bound flavin of the enzyme. We also identify a set of mitochondrial proteins that can be S-nitrosylated through multiple reaction channels, including anaerobic/oxidative, NO/O₂, and GSNO-mediated transnitrosation. Finally, we demonstrate that steady state levels of S-nitrosylation are higher in mitochondrial extracts than the intact organelles, suggesting the importance of denitrosylation reactions. Collectively, our results provide new insight into the determinants of S-nitrosothiol levels in subcellular compartments.

A key feature of nitric oxide (NO) biology is the post-translational modification (S-nitrosylation) of cysteine thiol to form nitrosothiol (SNO) (1–3). Although protein S-nitrosylation is coupled to stimulation of all isoforms of NO synthase (4–6), the physiologically relevant pathways of SNO formation are not well understood. One candidate mechanism is the reaction of nitrosium ion (NO⁺) with thiolate (RS⁻). It is widely held that physiologically important NO⁻ equivalents are produced by the reaction of NO with molecular oxygen or superoxide (7–9). Whereas the reaction of NO with O₂ (to produce N₂O₃) is slow at physiological (submicromolar) NO concentrations, this kinetic barrier may be overcome by the partitioning of NO/O₂, at high concentrations, within the hydrophobic milieu of proteins or membranes (10–12). Even so, SNO levels are not positively correlated with O₂ or superoxide concentrations in many model biological systems (6, 13).

Protein S-nitrosylation may also occur by a variety of oxygen-independent mechanisms (14). For example, transnitrosylation (direct exchange of NO between nitrosothiol and thiolate) is suggested by functional genetics: cells that lack an S-nitrosoglutathione (GSNO) metabolizing enzyme (GSNO reductase) have elevated protein SNOs (3, 15). In addition, O₂-independent S-nitrosylation may be catalyzed enzymatically, as in the cases of hemoglobin and ceruloplasmin (16–19), which bind NO at their respective heme and copper centers and transfer the NO group to target cysteine or glutathione. Nitric oxide can also react directly with thyl radical, a reaction implicated in S-nitrosylation of hemoglobin under conditions where Cu/Zn-superoxide dismutase may serve a thiol-oxidizing role (17, 20). Finally, the direct reaction of NO with a thiolate may produce a radical anion intermediate, which is oxidized to nitrosothiol by an electron acceptor such as NAD⁺ (21).

Mechanistic studies of protein S-nitrosylation have primarily focused on a small number of endogenous substrates (e.g. serum albumin and hemoglobin). The chemical reactivities of these model SNO compounds are very different, and it is unlikely that they are representative of the entire nitrosoproteome (estimated to be >100 proteins). The identification of additional substrates of S-nitrosylation in situ, however, remains a challenge. Some popular chemical assays for SNOs, such as triiodide chemiluminescence and Greiss-based colorimetric and fluorimetric methods, introduce inevitable artifacts when applied to complex biological systems (22). In contrast, photolysis chemiluminescence (6, 23, 24) (which does not require processing of tissues in acids or the addition of multiple harsh reagents) has been shown to be sensitive and specific (24), as demonstrated most recently by the analyses of SNO and Fe-NO in yeast and mammalian cells deficient in enzymes that specifically metabolize NO and SNOs (3, 15, 25). But photolysis chemiluminescence is low throughput and better suited for analysis of isolated proteins. The recently described “biotin switch” assay (5), which replaces the NO moiety with a detectable biotin derivative, may be more easily adapted to the study of S-nitrosylation on a proteome-wide scale (e.g. whole cell or subcellular compartment), but its sensitivity may be limiting.

The mammalian mitochondrion has a rich association with NO biology. Mitochondrial NO synthase has been identified in a number of cell types (26–29). In the mitochondrion, NO inhibits respiration (30), degrades abundant iron-sulfur proteins (31), and has both pro- and anti-apoptotic effects (32, 33). Furthermore, both mitochondrial glutathione and proopiomelanocortin.
are endogenously S-nitrosoylated (33–35). These studies were performed in room air; however, the tissue pO2 is 3–30 torr (36–38) and the critical mitochondrial pO2 to support oxidative metabolism is <3 torr (39, 40). In this work, we use the biotin switch technique, photolysis chemiluminescence, and mass spectrometry to study S-nitrosoylation of mitochondrial proteins by NO and GSNO under in vitro and in situ conditions. We examine the O2 requirement for protein S-nitrosoylation and determine the extent to which protein environment and complexity of the system affects reaction mechanism.

EXPERIMENTAL PROCEDURES

Reagents—Diethylamine NONOate (DEANO) was purchased from Cayman Chemical. Stock solutions were freshly prepared in 10 mM NaOH. S-Nitrosoglutathione (GSNO) was synthesized from glutathione using acidified nitrite (41). Mouse monoclonal anti-catalase antibody was obtained from Sigma, and rabbit anti-lipoamide dehydrogenase antibody was from U. S. Biological.

Rat Liver Mitochondria—Rat liver mitochondria (RLM) were purified from adult male Sprague-Dawley rats by differential centrifugation and continuous Percoll gradient centrifugation (42, 43). RLM extracts were prepared by resuspending the mitochondria at ~10 mg/ml in lysis buffer (20 mM Heps, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and sonicating (3 × 5 min) on ice. CHAPS was added to 0.4% (w/v), and the homogenate was centrifuged at 20,000 × g for 10 min. This supernatant was used for S-nitrosoylation assays and protein purification. Outer and inner mitochondrial membrane fractions were obtained from freshly isolated mitochondria using a French pressure cell as described previously (44). Protein concentrations were determined using the BCA Protein Assay (Pierce).

Biotin Switch Assay—S-Nitrosoylated proteins were detected using the biotin switch assay as described by Jaffrey et al. (5) with some modifications. All steps were performed in amber centrifuge tubes (Eppendorf). Briefly, extracts were diluted to 1 mg/ml with a reaction buffer, e.g. HEN buffer (250 mM Heps, 1 mM EDTA, 0.1 mM neocuproine, pH 7.7), and incubated with NO donor for 1 h at room temperature. 75 μl of the lysate was loaded on a Micro Bio-Spin 6 column (Bio-Rad) previously equilibrated with HEN buffer and centrifuged at 1000 × g for 4 min. 7 μl of 25% SDS and 1.5 μl of MMTS (20% (v/v) in N,N-dimethylformamide) were added to 75 μl of eluate, and the free thiols were blocked at 50 °C for 20 min with frequent vortexing. The MMTS was removed by desalting with two successive Micro Bio-Spin 6 columns previously equilibrated with 2 ml of HEN buffer. Finally, 8 μl of 2 mM biotin-HDPD (Pierce) in Me2SO and 4 μl of 100 mM acetic acid in HEN buffer were added to 68 μl of the desalted eluate (to give a final concentration of 0.4 mM biotin-HDPD and 5 mM acetic acid). This 80-μl mixture was incubated for 1 h at room temperature, after which 20 μl of 50 mM CHAPS was added, and the mixture was centrifuged at 13,000 × g for 30 min. 0.25 mM HgCl2 in 100 mM glycine buffer (pH 10) was added, and the remaining eluent was separated by SDS-PAGE and visualized by silver staining.

For anaerobic experiments, RLM extracts were diluted to 5 mg/ml with dialysis buffer (25 mM Heps, 1 mM EDTA, 0.1 mM neocuproine, pH 7.4, 0.1 mM 4-(1-aminoethyl)benzenesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and dialyzed in a CO2 anaerobic chamber at room temperature against 1 liter of buffer for 4 h and 1 liter for an additional 16 h. Following dialysis, extracts were diluted with HEN buffer and treated anaerobically by dialysis, desalting, and the critical mitochondrial pO2 to support oxidative metabolism is <3 torr (39, 40). In this work, we use the biotin switch technique, photolysis chemiluminescence, and mass spectrometry to study S-nitrosoylation of mitochondrial proteins by NO and GSNO under in vitro and in situ conditions. We examine the O2 requirement for protein S-nitrosoylation and determine the extent to which protein environment and complexity of the system affects reaction mechanism.

S-Nitrosylation of Intact Mitochondria—Freshly prepared mitochondria were resuspended in RCR buffer (0.3 mM sucrose, 1 mM EGTA, 5 mM Mops, 5 mM KH2PO4, 1 mM EDTA, 0.1 mM neocuproine, pH 7.4) at ~50 mg/ml. Mitochondria were resuspended in dialysis buffer at 500 μl/ml aprotinin and dialyzed against 50 mM NaCl. The flow-through was collected and saved. The column was washed with buffer A and eluted with buffer A + 600 mM NaCl. The flow-through was concentrated with a Microcon-10 spin concentrator (Millipore) and was fractionated by fast protein liquid chromatography (Pharmacia) using a Superdex 200 column (Pharmacia) with buffer A at 0.5 ml/min. 1-ml fractions were collected, concentrated, and assayed using the biotin switch method. GSNO-sensitive fractions were further chromatographed on a Mono Q (5/5) column (Pharmacia) at 1 ml/min with a gradient from buffer A + 50 mM NaCl to buffer A + 500 mM NaCl. The flow-through from the initial Q-Sepharose column was also concentrated and analyzed after separation on the Superdex 200 column. GSNO-sensitive fractions were diluted with 50 mM KP, 1 mM cysteine HCl, and separated with a Hitrap Phenyl-Sepharose 6 FF column (Pharmacia) using a stepwise gradient from 1 to 0 mM (NH4)2SO4 in 50 mM KP. 7.4. Proteins were eluted manually in 2-ml fractions using a syringe. Fractions were again concentrated and assayed, and target proteins were separated by SDS-PAGE, visualized using Brilliant Blue-G Colloidal Stain (Sigma), and sequenced by MALDI-TOF MS. Sequencing was performed at the Howard Hughes Medical Institute/Columbia University Protein Core Facility by Dr. M. A. Gawinowicz.

RESULTS

Evaluating the Biotin Switch Assay to Study Protein S-Nitrosylation in Mitochondria—In anti-biotin immunoblots of control, detergent-solubilized extracts of RLM as well as other mitochondrial samples analyzed by the biotin switch assay (Fig. 1), two bands were observed that corresponded to endog-
enously biotinylated biotin carboxylases (47); pyruvate carboxylase (130 kDa), propionyl-CoA carboxylase (73 kDa for the biotinylated α-subunit), and β-methylcrotonyl-CoA carboxylase (75 kDa for the biotinylated α-subunit). The latter two proteins comigrated as a single band on most gels. These biotinylated proteins were used as internal standards on immunoblots.

Protein S-nitrosylation was first surveyed by the biotin switch assay after treatment of mitochondrial extracts for 30 min with 50 μM GSNO, a physiologically relevant nitrosylating agent (15). Whereas no labeling was seen with GSH alone, incubation with GSNO consistently produced four intense bands (between ~50 and 100 kDa) in addition to several minor lower mass species (Fig. 1). In the biotin switch, S-nitrosylated cysteines are identified by the ascorbate-dependent cleavage of S-NO followed by biotinylation of the free thiol (5). However, exclusion of ascorbate did not completely abrogate biotinylation, suggesting that protein SNOs may spontaneously degrade during the biotinylation step. An ascorbate-dependent band was also seen in control samples, suggesting endogenous S-nitrosylation (see below). The specificity of the assay was verified by treatment with 50 μM oxidized GSSG. It has been recently suggested that the biotin switch may detect protein S-glutathionylation (48). However, no GSSG-dependent labeling was observed (Fig. 1).

S-Nitrosylation of Mitochondrial Proteins by Nitric Oxide as a Function of Molecular Oxygen Concentration—Although O₂ is assumed to be necessary for NO-dependent protein S-nitrosylation, the requirement has not been carefully studied over a wide range of proteins (either in vitro or under more physiological conditions). We therefore probed the O₂ dependence of S-nitrosylation by NO, in both mitochondrial extracts and intact mitochondria. Mitochondrial extracts were assayed after extensive dialysis in an O₂-free chamber and subsequent treatment with 50 μM DEANO for 30 min (Fig. 2A). Under strict anaerobic conditions, all of the major SNO proteins were increased over background levels; however, labeling of the ~100-kDa protein (p100) was more prominent than the other species. S-Nitrosylation of p100 by NO was not increased by O₂. On the other hand, O₂ potentiated the NO-mediated S-nitrosylation of the other proteins (Fig. 2A). Thus, whereas some proteins show both O₂-dependent and -independent reactions with NO in vitro, p100 appears to be S-nitrosylated via a direct, O₂-independent mechanism.

Intact mitochondria were similarly probed to determine whether the same proteins were S-nitrosylated in situ as in extracts, and more importantly, if proteins were also S-nitrosylated in the intact organelle by NO under strict anaerobiosis (note that mitochondrial pO₂ is very low, perhaps <3 mm Hg, by contrast to ambient pO₂ of 150 mm Hg (40, 49)). Freshly prepared mitochondria were reacted with 50 μM DEANO for 30 min. Anaerobic incubation with DEANO resulted in S-nitrosylation of many of the same proteins (albeit to a lesser extent than in room air; Fig. 2A). By the same token, S-nitrosylation of p100 in intact mitochondria was seen at much lower levels than in mitochondrial extracts (Fig. 2, A and B). Furthermore, S-nitrosylation of the ~60-kDa species (p60) was barely detectable at 50 μM DEANO under either condition in situ (Fig. 2A), whereas substantial S-nitrosylation of this protein was detected (at similar levels to p50 and p55) when the mitochondria were exposed to 1 mM DEANO (not shown). Together, these results demonstrate that the protein environment (in vitro versus in situ) and physiological pO₂ have significant effects on steady-state levels of protein S-nitrosylation. Lower levels of steady-state SNOs are likely to be more physiological.

Identification of S-Nitrosylated Proteins in Rat Liver Mitochondria—To better understand the molecular basis underlying the unique reactivities of p100 and p60, we pursued their identification. The major (GSNO-reactive) proteins were identified by two different methods. First, following GSNO treatment and the biotin switch assay, biotinylated proteins were affinity purified using streptavidin-agarose, separated by SDS-PAGE (Fig. 3A), and tentatively identified by MALDI-TOF sequencing (5). Although immunoblotting of the same sample showed the presence of the major SNO proteins (Fig. 3A), only the 100-kDa protein was positively identified as sarcosine dehydrogenase, albeit at low (20%) coverage. We were unable to obtain adequate sequence information on the other proteins. In our hands, this method proved difficult for purifying adequate quantities of the SNO proteins for identification by mass spectrometry.

Alternatively, SNO proteins were purified by standard chromatography techniques on the basis of their reactivity with GSNO. A combination of anion exchange, size exclusion, and hydrophobic interaction chromatographies were used to identify six novel S-nitrosylated proteins. After each purification step, column fractions were treated with GSNO and screened by the biotin switch assay; fractions containing GSNO-sensitive species were subjected to further rounds of purification. Finally, fractions highly enriched in the GSNO-sensitive proteins were separated by SDS-PAGE and identified by MALDI-TOF MS analysis. Several steps in the purification of the 100-kDa protein are shown in Fig. 3B. None of the proteins identified displayed marked differences in their electrophoretic mobilities under reducing or non-reducing conditions, or when the proteins were biotinylated (not shown). Sarcosine dehydrogenase (SarDH),
methods were used for purification of nitrosylated proteins: A, 1 mg of mitochondrial extracts, untreated (−) or exposed (+) to 50 µM GSNO for 1 h, were analyzed by the biotin switch assay. Biotinylated proteins were purified with streptavidin-agarose, eluted under non-reducing conditions, separated by SDS-PAGE, and visualized by silver staining (SS) or immunoblotting with an anti-biotin antibody (IB). Protein loading for IB was 10% of that for SS; B, the 100-kDa protein (identified by arrow) was purified by column chromatography. Extracts were treated with GSNO and analyzed by the biotin switch assay before (lane 1) and after (lane 2) passage over an anion exchange resin (Q-Sepharose FF). The total protein eluted from the Q-Sepharose column was further purified (see “Experimental Procedures”). Following size exclusion chromatography (Superdex 200), several fractions contained a single GSNO-reactive species at ~100 kDa (lane 3). One of these fractions was separated by SDS-PAGE and visualized (lane 4), and the 100-kDa band was excised and sequenced.

which was the only observable SNO protein that bound to the initial Q-Sepharose column, was again identified (51% coverage). In addition, catalase (50% coverage to mouse protein), dihydrolipoamide dehydrogenase (DLDH; 40% coverage to mouse protein), hydroxymethylglutaryl-CoA synthase (38% coverage), glutamate oxaloacetate transaminase 2 (63% coverage), and malate dehydrogenase (43% coverage) were all purified from the flow-through of the initial Q-Sepharose column (Fig. 3B). The positions and sizes of these novel SNO proteins are shown in Fig. 4. Although DLDH was predicted to be ~3 kDa smaller than hydroxymethylglutaryl-CoA synthase, the latter protein had a lower apparent molecular weight.

To confirm the sequencing results, SarDH activity was identified in fractions that were also positive for the GSNO-sensitive 100-kDa protein (data not shown). GSNO-dependent S-nitrosylation of both catalase and DLDH, the two proteins for which antibodies were commercially available, was measured in RLM lysates by streptavidin-agarose purification and immunoblotting (Fig. 5A). As expected, the immunoreactivities of these proteins were both GSNO- and ascorbate-dependent. Whereas catalase has been identified as a mitochondrial matrix-associated protein in rat heart mitochondria (50), it is not known to localize to liver mitochondria, and has previously been used as a peroxisomal marker in Percoll gradient-purified mitochondrial preparations (42). To determine the localization of catalase, mitochondria were separated into outer membrane (including inter-membrane space and copurifying particles) and inner membrane (including matrix) fractions and analyzed by immunoblotting. Catalase was detected predominantly in the outer membrane fraction, consistent with a peroxisomal localization, whereas DLDH was predominantly in the inner membrane fraction, consistent with its matrix localization (Fig. 5B). Furthermore, immunostaining showed the presence of the peroxisomal membrane protein, PMP70 in the outer membrane fraction (not shown). The unique subcellular localization of catalase (copurifying peroxisomes) as compared with the other identified SNO proteins (mitochondrial matrix) may help to explain its distinct reactivity in situ.

Endogenous Mitochondrial S-Nitrosylation and Enzyme Inhibition—Giulivi and co-workers (34) have observed endogenous GSNO in rat liver mitochondria, and we and others have reported on endogenous S-nitrosylation of caspase-3 and -9 (33, 35). Indeed, photolysis chemiluminescence measurements on freshly isolated mitochondria confirmed the presence of both mercury-sensitive (nitrosothiols) and inert (e.g. metal-nitrosyl) NO-containing species (Fig. 6A) that are markers of constitutive NO production. Nitrosothiol levels were ~5 pmol of SNO per mg of protein. Furthermore, the biotin switch assay showed ascorbate-dependent immunoreactivity of both catalase and dihydrolipoamide dehydrogenase (Fig. 6B), suggesting that these proteins are endogenously S-nitrosylated. We also investigated whether S-nitrosylation might lead to inhibition of protein function. Both SarDH and DLDH could be modestly inhibited by NO (e.g. S-nitroso-cysteine), but only at high concentrations under our in vitro conditions (not shown). It remains to be determined if this inhibition by a short-lived nitrosothiol is of relevance to physiological or pathophysiological (nitrosative stress) situations.
Fig. 6. Endogenous S-nitrosylation. A, 2 mg of freshly isolated mitochondria (~20 mg/ml in 25 mM Hepes, 0.4% CHAPS, 0.1 mM EDTA, 0.2 mM DTPA, 10 mM neocuproine, pH 7.7) were analyzed using photolysis chemiluminescence (intensity A.U.) before (circle, 1) or after (inverted triangle, 2) treatment with 0.25 mM HgCl₂ for 5 min. The signal from buffer alone (square, 3) is also shown. B, 1 mg of mitochondrial extracts were analyzed with the biotin switch assay with or without addition of 5 mM ascorbate. Biotinylated proteins were purified with streptavidin-agarose, electrophoresed, and visualized by immunoblotting with anti-catalase (CAT) and anti-lipoamide dehydrogenase (DLDH) antibodies.

Comparison of NO- and GSNO-dependent S-Nitrosylation in Mitochondrial Extracts—Because NO and GSNO differ in their chemical and physical properties, they might be expected to target different subsets of mitochondrial proteins. However, under aerobic conditions, which favor S-nitrosylation by NO (see above), identical sets of proteins were S-nitrosylated by either 50 μM GSNO or 50 μM DEANO (Fig. 7A). This could suggest that intrinsic thiol reactivity is a more important determinant for protein S-nitrosylation than the specific nature of the nitrosylating agent. Alternatively, this lack of differential reactivity may be because of a common nitrosylating intermediate (e.g. NO released from both DEANO and GSNO). In particular, NO can be released from S-nitrosoglutathione via light- or metal-dependent mechanisms (51). English and co-workers (20, 52, 53) have recently shown that S-nitrosylation of hemoglobin and calbindin by GSNO, is inhibited by addition of the Cu²⁺ chelator DTPA, or both DTPA and the Cu¹⁺ chelator neocuproine. In these studies they concluded that copper-catalyzed NO release was required for S-nitrosylation by GSNO. However, our experiments were performed in the dark and in the presence of metal chelators.

We performed additional and complementary experiments to test for a role of metals in S-nitrosylation by GSNO. First, we examined the effect of metal chelators on GSNO-dependent protein S-nitrosylation in mitochondrial extracts (Fig. 7B). Little if any difference in protein S-nitrosylation was observed as a function of added DTPA or neocuproine, alone or in combination. However, this experiment does not exclude a role for redox-active copper proteins that are inert to demetallation by chelators. Next, we removed O₂ from the system. Protein S-nitrosylation by NO was attenuated in the absence of molecular oxygen, whereas S-nitrosylation by GSNO was unaffected (not shown). Together, these data suggest that under our conditions, where NO and GSNO modify identical targets, S-nitrosylation by GSNO does not occur via a free nitric oxide-dependent pathway.

DISCUSSION

Previous mechanistic studies of S-nitrosylation have been restricted to model systems, particularly small alkyl thiols. In vitro, the favored reaction pathway for S-nitrosylation involves NO/O₂ chemistry (to generate N₂O₃). However, it is unclear what role O₂ plays in vivo, or how well the chemistry of free cysteine emulates that of protein thiols, which vary widely in reactivity. The supreme example is the ryanodine receptor (RyR1), which contains as a many as 50 free thiols per subunit, many of which can be S-nitrosylated in room air (54), but only one of which is modified at physiological oxygen concentrations (13). Moreover, in the case of hemoglobin, molecular oxygen is required for S-nitrosylation, but principally because O₂ promotes a favorable conformation in the protein (55, 56), enabling intramolecular transfer of NO from heme to thiol (16, 57) (and not because it generates N₂O₃). Here we have studied the importance of O₂ in a complex mixture of proteins. We find that biological systems can fulfill the oxidative requirement for S-nitrosylation by NO, even in the complete absence of O₂.

Accordingly, we have shown that molecular oxygen and derived reactive oxygen species are not required for S-nitrosylation of mitochondrial proteins by NO, either in vitro or in situ. The abundance of redox-active cofactors in the mitochondrion suggests a number of plausible mechanisms for O₂-independent S-nitrosylation. For one, iron-sulfur proteins, constituents of the mitochondrial matrix and inner membrane, may react with NO to form dinitrosyl-iron complexes, effective NO⁺ donors (58). Dinitrosyl-iron complex derivatives of glutathione may also provide a facile mechanism for diffusion of NO⁺ equivalents within the mitochondrial matrix, and these complexes are in equilibrium with GSNO (59). However, additional mechanisms of S-nitrosylation must be operative in our experiments as the removal of low-mass thiol by dialysis, effectively precluding the formation of freely diffusible dinitrosyl-iron complexes or GSNO, did not prevent S-nitrosylation of SarDH by NO.

SarDH is not a metalloenzyme, but it does have a histidyl-bound FAD at its active site that may function in catalysis of S-nitrosylation. In this pathway, NO could first react with a thiolate to form a radical anion intermediate [RS-NO⁺] (60, 61). Structural studies of Hb and p21⁴[Ras have raised the possibility that the S-nitrosylated anion radical may be stabilized by protein conformation (56, 61). The oxidized RS-NO product could then form in the presence of an electron acceptor (21), and in sarcosine dehydrogenase, the bound FAD may fulfill this requirement. The enzyme has 20 cysteines, one of which is located (in the 1° sequence) within the flavin-binding domain (62). Although there is no known role for a thiol in the enzymatic activity of SarDH, the modest inhibition by CysNO suggests that NO⁺ may modify an allosteric site. In extracts, we
also observed the S-nitrosylation of catalase, DLDH, and hydroxymethylglutaryl-CoA synthase by NO under anaerobic conditions. Whereas higher levels of SNO proteins were detected in air, the amount should not be equated with physiological relevance. Indeed, the $p_{O_2}$ in situ is low, and elevated SNO protein may reflect a nitrosative stress. Thus, direct reac-
tion of NO with thiolate may well represent a principal mechanism of S-nitrosylation in situ. A common requirement of biological S-nitrosylation is the 1-electron oxidation of systems, and our studies make it clear that metals or other cofactors (besides $O_2$) may subserve this role.

Protein S-nitrosylation by DEANO was less evident in intact mitochondria than extracts. NO sequestration by membranes may have contributed to this effect, but the explanation we favor is that steady-state levels of SNO proteins reflect a balance between S-nitrosylation and denitrosylation, and that in situ, glutathione can both compete with protein thiols for NO$^+$ equivalents and remove NO groups from proteins via transnitrosylation (3), thereby preventing the accumulation of SNO. All of the mitochondrial proteins that we detected were denitrosylated upon addition of glutathione to S-nitrosylated extracts. Thus, for example, although SarDH is effectively S-nitrosylated under aerobic conditions in extracts, whereas higher levels of SNO proteins were detected in air, the amount should not be equated with physiological relevance. Indeed, the $p_{O_2}$ in situ is low, and elevated SNO protein may reflect a nitrosative stress. Thus, direct reaction of NO with thiolate may well represent a principal mechanism of S-nitrosylation in situ. A common requirement of biological S-nitrosylation is the 1-electron oxidation of systems, and our studies make it clear that metals or other cofactors (besides $O_2$) may subserve this role.

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