Current Topics in Biophysics 2002, 26(1), 115-127

NITROGEN OXIDES, THE GOOD, THE BAD, AND THE UGLY

NEIL R. BASTIAN, MEREDITH J.P. FOSTER, MARYJOY BALLANTYNE, YANXIANG LU

Division of Infectious Diseases, University of Utah School of Medicine,
30 North 1900 East, Salt Lake City, UT 84132-2405

Nitric oxide, one of the most stable radicals produced by biological systems, has been extensively studied. However, other reactive nitrogen species (RNS) are also formed in mammalian cells, either enzymatically by nitric oxide synthase or nonenzymatically as a reaction product of nitric oxide with other molecules. It is well known that RNS have a high affinity for iron and iron-containing proteins. A study of the effects of RNS on several iron containing proteins indicates that while nitric oxide (NO) inhibits the biological action of most iron proteins with concomitant production of iron-nitrosyl EPR signals, the effects are by and large reversible. Nitroxyl anion (NO^-) does not significantly inhibit most iron-containing proteins and in fact its reducing capability often is able to prevent the inhibitory effects of nitric oxide. Peroxynitrite ($ONOO^-$) irreversibility inhibits the activity of most iron enzymes tested without formation of EPR signals and often causes significant bleaching of the chromophores, suggesting that iron is removed from the proteins. S-nitrosoglutathione (GSNO) effects mimic those of nitric oxide with both reversible inhibition of biological activity and formation of iron-nitrosyl EPR signals. In the absence of added thiols, nitrosonium cation (NO^+) has little effect on iron proteins although in the presence of reduced thiols, the effects of nitrosonium are similar to those of nitric oxide, presumably due to the facile formation of S-nitrosothiols from nitrosonium and reduced thiols. At neutral pH, neither nitrite (NO_2^-) nor nitrate (NO_3^-) has a significant effect on biological activity of iron proteins and neither induces formation of EPR signals in the iron proteins tested. The bacterial molybdoenzyme, DMSO reductase forms an axial EPR signal when exposed to NO(g) but forms a rhombic EPR signal when incubated with NO^-. Incubation of DMSO reductase with nitric oxide synthase (NOS) produces an EPR signal that is a combination of axial and rhombic molybdenum (V) signals. This latter result shows that NOS enzymatically produces both NO(g) and another reductive compound, possibly NO^-.

INTRODUCTION

Nitric Oxide (NO) is formed from L-arginine as a component of the cell mediated immune response (Marletta, Yoon, Iyengar, Leaf & Wishnok, 1988). NO produced by macrophages has been shown to inhibit several key iron containing enzymes including ribonucleotide reductase (Granger, Taintor, Cook & Hibbs, 1980), aconitase (Drapier & Hibbs, 1988; Granger & Lehninger, 1982; Drapier & Hibbs 1986; Warton, Granger & Durack, 1988), complexes I and II of oxidative phosphorylation (Drapier & Hibbs, 1988; Granger & Lehninger, 1982; Drapier & Hibbs 1986; Warton *et al.*, 1988) and cytochrome oxidase (Torres, Cooper & Wilson, 1998; Borutaite & Brown, 1996). In addition, tumor cells co-cultivated with activated macrophages release a significant fraction of their intracellular iron in parallel with development of inhibition of DNA synthesis and mitochondrial respiration (Hibbs, Taintor & Vavrin, 1984).

In addition to NO(g), several other reactive nitrogen species (RNS) have potential biological effects, including nitrosonium ion (NO^+), Nitroxyl anion (NO^-), peroxynitrite ($ONOO^-$), and S-nitrosothiols (RSNO) (Hughes, 1999; Oshima, Yoshie, Auriol & Gilbert, 1998). Rusche, Spiering and Marletta (Rusche *et al.*, 1998) showed that tetrahydrobiopterin-free iNOS produces NO^- rather than NO(g) suggesting the possibility that NO^- may be enzymatically produced under some conditions. Indeed it has been suggested (Schmidt, Hofmann, Schindler, Shutenko, Cunningham & Feelisch, 1996; Komarov, Wink, Feelisch & Schmidt, 2000) that iNOS produces NO^- rather than NO(g) *in vivo*, although these results are contested by Xia and Zweier (1997). Sharpe and Cooper (1998) showed that nitric oxide(g) reacts with ferrocytochrome c to produce NO^- showing that reactions involving NO^- are biologically relevant even if iNOS produces only NO(g). Sharpe & Cooper (1998) also showed that reactions of either NO(g) or NO^- with reactive oxygen species produce $ONOO^-$. Vanin (1998) and Kostka, Xu & Skiles (1999) have shown that NO^+ is produced biologically during the degradation of dinitrosyl iron complexes. Thus, the biological effects of each of these NO congeners have relevance in

Table 1. Percent inhibition of iron-containing enzymes by RNS.

Enzyme	NO(g)	NO^-	NO+	GSNO	$ONOO^-$
Aconitase	80 (7)	25 (9)	65 (4)	73 (9)	88 (8)
NADH Dehydrogenase	75 (5)	2 (14)	9 (5)	73(6)	78 (2)
Myeloperoxidase	1 (10)	ND*	10 (13)	0 (9)	97 (3)
Catalase	77 (2)	18 (1)	0 (4)	24 (2)	95 (3)
Horseradish Peroxidase	26 (1)	28 (3)	5 (2)	18 (2)	70 (2)
Cytochrome c oxidase	51 (2)	ND*	36 (3)	35 (3)	ND*

ND* = not determined.

Enzymes were assayed by established methods (aconitase, Rose & O'Connell, 1967; NADH dehydrogenase, Hatefi, 1978; myeloperoxidase, Wright et al., 1990; catalase, Beers & Sizer, 1952; horseradish peroxidase, Ortiz de Montellano, David, Ator & Tew, 1988; cytochrome c oxidase, Wharton & Tzagoloff, 1967). Enzyme solutions (~0.2 mg/ml) were prepared in 0.25 M Tris buffer, pH 7.5 and were incubated anaerobically with 50 μM desired RNS for 15 minutes before assays were conducted. 50 μl aliquots of enzyme were added to each assay. Assays of RNS treated enzymes were done in the absence of RNS. Activities of RNS treated enzymes were taken as the initial enzymatic rate compared to an enzyme control incubated under identical conditions but in the absence of RNS. Inhibition is reported as the average of at least three separate determinations with the standard error in parentheses. Reversibility of inhibition was determined by anaerobically dialyzing 1 ml of RNS treated enzyme against 500 ml of 250 mM Tris·HCl pH 7.5 for three hours. After dialysis, enzymes were reassayed and activities were compared to a control treated under identical conditions. RNS were added as described in the text.

tissues containing any one of the three NOS isoenzymes.

Commoner, Woolum, Sentutia & Ternberg (1970) reported that a $g = 2.039$ electron paramagnetic resonance (EPR) signal appeared in the livers of rats which were fed various chemical carcinogens. They showed that this signal was due to formation of a paramagnetic Fe-NO-thiol complex in the tissues (Chiang, Woolum & Commoner, 1972). Vanin, Blyumenfel'd & Chetverikov (1967), Vanin (1991) and Butler, Glidewell, Hyde & Walton (1985) structurally characterized inorganic, EPR active Fe/RS/NO complexes and found them to have a general chemical formula of $Fe(RS)_2(NO)_2$. The complexes were found to be unstable with either oxidation or polymerization leading to loss of the EPR signal. This iron based EPR signal is observed in activated macrophages and their target cells after the induction of iNOS (Lancaster & Hibbs, 1990; Pellat, Henry & Drapier, 1990; Drapier, Pellat & Henry, 1991).

Heme-nitrosyl EPR signals have also been described in tumors from various sources. The characteristic spectrum of a heme-nitrosyl compound that contains six-coordinate iron is frequently observed in tumor tissues that have been exposed to endogenously produced nitric oxide (Bastian, Yim, Hibbs & Samlowski, 1994a). Brennan, Cole & Singley (1966) observed a second heme-nitrosyl signal with a characteristic three-line signal in two murine tumors, a reticulum cell sarcoma and a neuroblastoma. Subsequent papers by other groups described a similar signal in several other tumors (Emanuel, Saprin, Sharalkin, Kozlova & Krugtiakova, 1969). The three-line signal is found in nitrosylated heme-containing proteins containing five-coordinate iron (Kon, 1968; Kon & Kataoka, 1969; Scholler, Wang & Hoffman, 1979; Hille, Olson & Palmer, 1979; Morse & Chan, 1980). Formation of the triplet signal was once thought to be due to necrosis of the tissues, however, subsequent studies have shown that necrotic tissues do not develop this EPR signal unless they are exposed to nitrogen oxides such as NO(g) or nitrite (NO_2^-) (Bastian, Xu, Shao, Shelby, Granger & Hibbs, 1994b).

Because of the abundant evidence that multiple reactive nitrogen species (RNS) can be produced in mammalian cells we set out to determine what effect the different RNS have on iron-containing proteins and to determine whether these effects are reversible. It was also of interest to determine whether different RNS could be distinguished unambiguously from one another based on their effects on proteins. Effects were monitored by enzyme activity, electron paramagnetic resonance (EPR) spectroscopy, and uv-visible absorption spectroscopy. Proteins included both heme- and nonheme-iron-containing proteins. The proteins tested were either obtained commercially and used without further purification or were purified from the best available source using established methods. Enzymes included myeloperoxidase from human leukocytes (Sigma), horseradish peroxidase (Sigma, type VI-A), catalase from bovine liver (Sigma), cytochrome c oxidase from bovine heart

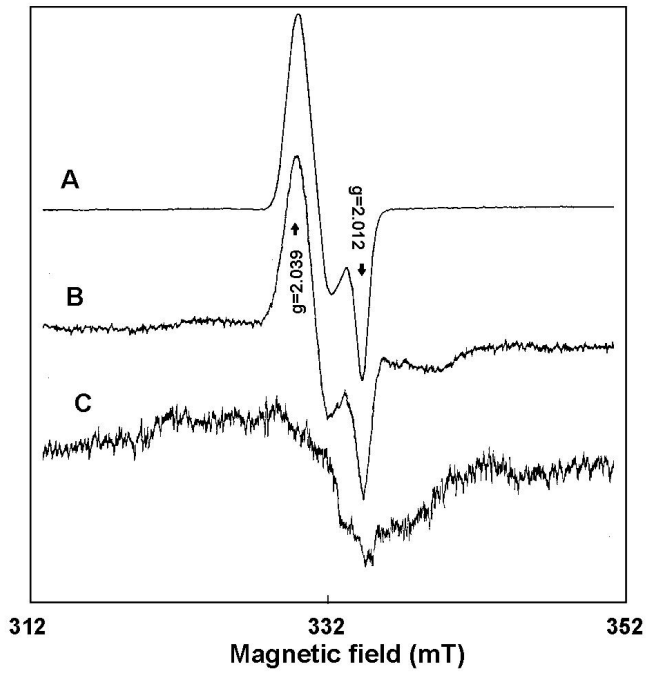


Fig. 1. EPR spectra of (A) GSNO (100 μM) treated ferritin. The ferritin was made up at an iron concentration of 200 μM. (B) 5 mg/ml aconitase treated with 100 μM NO(g). (C) Sample B after anaerobic dialysis against 250 mM Tris pH 7.5. EPR parameters were as follows: frequency, 9.42 GHz; temperature, 77 K; microwave power, 1.5 mW; modulation frequency, 100 kHz; and modulation amplitude, 1.0 mT.

(Ferguson-Miller, Brautigan & Margoliash, 1978), aconitase from bovine heart (Rose & O'Connell, 1967), and NADH dehydrogenase (Complex I) from bovine heart (Hatefi, 1978). Non-enzyme proteins included human hemoglobin (Sigma), horse heart cytochrome c (Sigma), horse spleen ferritin (Sigma), human transferrin (Sigma), and spinach ferredoxin (Tagawa & Arnon, 1962).

The RNS used in this study included NO(g) (administered as a saturated, buffered solution), NO^- in the form of Angeli's salt ($Na_2N_20_3$) (Hunt, Cox & Ray, 1962), NO^+ in the form of NOCl (Coleman, Lillis & Goheen, 1939) (administered as a gas), S-nitrosoglutathione (GSNO) (Park, 1988), and peroxynitrite anion ($ONOO^-$) (Wink, Darbyshire, Nims, Saavedra & Ford, 1993). The effects of nitrite (NO_2^-) and nitrate (NO_3^-), the end products of biological NO synthesis, were also tested. For enzyme inhibition studies and absorption spectroscopy, all RNS were tested at a final concentration of 50 μM unless otherwise noted. For spectroscopic studies where protein concentrations were greater, RNS concentrations were 100 μM. Preparation of samples for spectroscopy and enzyme assays were done anaerobically in order to avoid interactions between RNS and oxygen.

RNS EFFECTS ON IRON-SULFUR PROTEINS

Enzyme studies showed that both NO(g) and GSNO are effective inhibitors of the 4Fe/4S enzymes, aconitase and NADH dehydrogenase (Table 1). These effects were 70-100% reversible by anaerobic dialysis if the incubation times were relatively short (<6 hours). However, for longer exposure times (>12 hours) inhibition was not reversible, suggesting that iron was removed from the enzymes. No loss of the 420 nm absorbance peak was seen during early (<6 hours) NO(g) exposure but as incubation times increased, a bleaching of the Fe/S absorption peak occurred (data not shown). EPR spectroscopy of NO(g) or GSNO treated aconitase showed the formation of an $Fe(RS)_2(NO)_2$ EPR signal that disappeared upon dialysis (Table 2 and Figure 1B-C).

Neither NO^- nor NO^+ appreciably inhibited NADH dehydrogenase activity (Table 1), while aconitase activity was partially inhibited by both NO congeners. NO^+ inhibition of aconitase was only partially reversible (25-50%) even for short incubation times suggesting the possibility that NO^+ nitrosylates an enzyme thiol rather than iron. This is supported by the fact that EPR signal heights in NO^+ treated aconitase are significantly smaller than signals in GSNO treated enzyme (Table 2). Reversibility of NO^- inhibition of aconitase mirrored that of GSNO and a relatively small EPR signal was seen in NO^- treated enzyme (Table 2).

Peroxynitrite strongly and irreversibly inhibited both aconitase and NADH dehydrogenase activities even for incubation times as short as 10 minutes. Inhibition remained irreversible even in the presence of reduced thiols. Additionally, the characteristic 420 nm absorbance peak of aconitase was rapidly bleached bleached by $ONOO^-$ (data not shown) and no EPR signals were seen in $ONOO^-$ treated aconitase (Table 2). These data suggest that peroxynitrite rapidly oxidizes both these enzymes and causes loss of iron from the iron/sulfur center. Peroxynitrite must also cause nitrosation of protein amino acids since aconitase activity in $ONOO^-$ treated enzyme could not be reconstituted even in the presence of additional iron, sulfide, and DTT under anaerobic conditions.

Table 2. RNS induced EPR signals in Fe/S containing proteins.

	NO(g)	ONOO-	NO-	NO+	GSNO
Aconitase	++	-	+	+	+++
Ferredoxin red	+	-	-	++	+++
Ferredoxin ox	++	-	-	+	++
Ferritin w/RSH	+++	-	+++	+	+++
Transferrin w/RSH	++	-	-	++	++
Ferrous Sulfate w/RSH	++++	-	++	+	++++
Ferric Chloride w/RSH	+++	-	++	++	+++

Proteins (~5 mg/ml) were exposed to 100 μM RNS as described for Table 1. Symbols are as follows + < 25% of the iron is EPR active, ++ = 25-50% of the iron is EPR active, +++ = 50-75% of the iron is EPR active, ++++ = 75-100% of the iron is EPR active, - = no EPR signals detected. Iron concentrations were determined by the method of Carter (1971). For aconitase and ferredoxin, the iron concentration was divided by four in determining percent EPR activity since it was assumed that each Fe_4S_4 cluster will produce only one EPR active species. EPR signal formation by $FeSO_4$ and $FeCl_3$ are also listed. EPR spectra were recorded on a Bruker ER-200D spectrometer at 77K. Instrument settings were as follows: microwave frequency, 9.4 GHz; microwave power, 1.7 millitesla; modulation frequency, 100 kHz, and modulation amplitude, 1.0 millitesla. Signal amplitude was determined by double integration of the EPR signal with 20 μM $FeSO_4$ plus 100 μM GSH under 100% NO(g) used as a standard.

Neither nitrite nor nitrate, the final products of the reaction between NO(g) and O_2, inhibited either aconitase or NADH dehydrogenase activity (data not shown). Neither anion caused changes in the absorption spectrum of aconitase nor did either anion induce significant EPR signal formation in aconitase solutions.

EPR signal formation in the non-enzyme iron-proteins mirrored that of aconitase. The strongest signals were observed in GSNO treated proteins

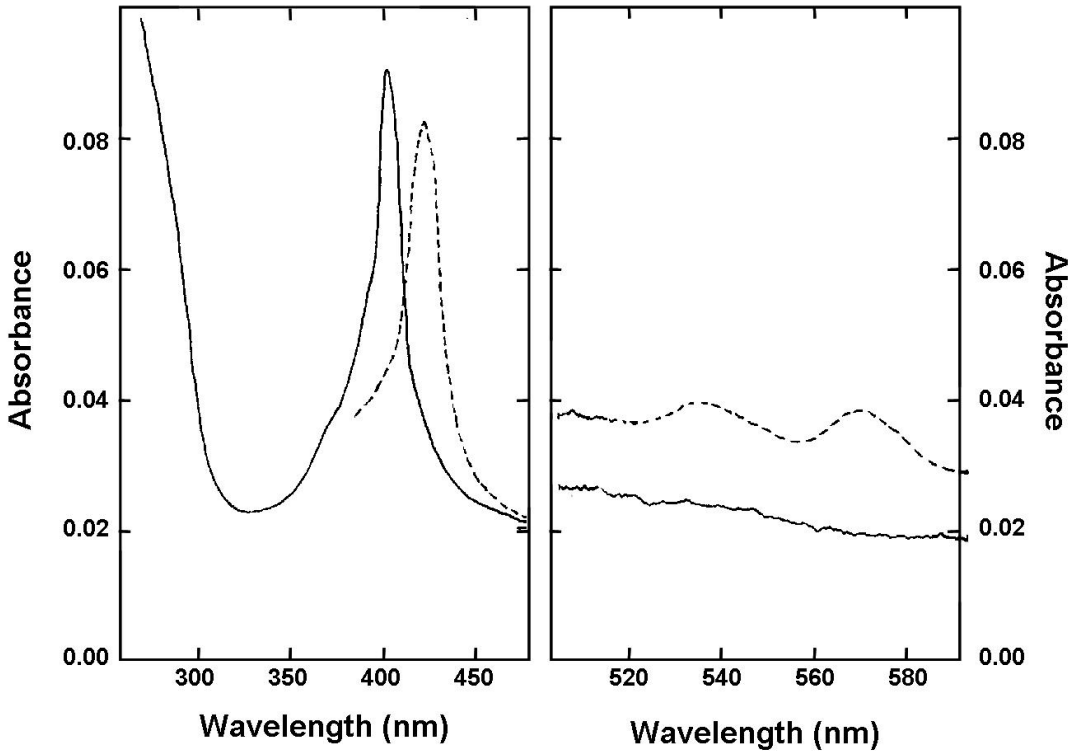


Fig. 2. uv-visible absorption spectra of untreated 0.2 mg/ml catalase (solid line) and 0.2 mg/ml catalase treated with 50 μM NO(g) (dashed line). GSNO treated catalase had an absorption spectrum identical to that of NO(g) treated enzyme. Spectra of catalase treated with any of the other RNS congeners was identical to that of the untreated enzyme.

Table 3. RNS induced EPR signals in heme-containing proteins.

	NO(g)	ONOO-	NO-	NO+	GSNO
Catalase ox	+	-	-	-	+
Catalase red	++	-	++	-	++
Horseradish Peroxidase ox	-	-	++	-	+
Horseradish Peroxidase red	++	-	++	++	++
Oxyhemoglobin	+	-	+++	+	+
Deoxyhemoglobin	++++	-	++++	++++	++++
Methemoglobin	+	-	++	+	+
Cytochrome c ox	-	-	+	+	+
Cytochrome c red	++	-	+	++	++

Proteins (~5 mg/ml) were exposed to 100 μM RNS as described for Table 1. Symbols and EPR parameters are as described in the legend for Table 2. Deoxyhemoglobin under 100% NO(g) was used as a standard.

while NO(g) also induced significant signal formation except with reduced ferredoxin (Table 2). For ferritin (Figure 1A) and transferrin it was necessary to add reduced glutathione (GSH) in order for significant EPR signal formation to develop (Table 2). NO^+ also induced signals in all proteins tested although, with the exception of reduced ferredoxin, the signals were small (Table 2). NO^- did not induce EPR signal formation except with ferritin (Table 2) where it is assumed that NO^- is able to reduce and solubilize Fe(III) in the protein core leaving soluble Fe(II) and NO(g) which readily react with GSH to form EPR active $Fe(RS)_2(NO)_2$. Peroxynitrite does not induce EPR signal formation in any of the iron-containing proteins tested (Table 2).

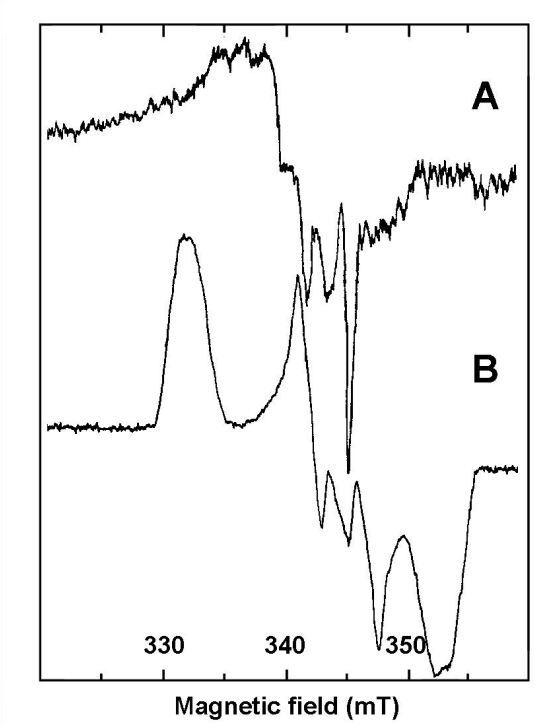


Fig. 3. EPR spectra of (A) 5 mg/ml catalase reduced with 50 μM dithionite then treated with 50 μM $Na_2N_2O_3$ and (B) 4.8 mg/ml horseradish peroxidase treated with 100 μM $Na_2N_2O_3$. EPR parameters were the same as those listed in the legend to Figure 1.

RNS EFFECTS ON HEME-CONTAINING PROTEINS

Catalase inhibition (Table 1) by nitric oxide gas was dose dependent and fully reversible by dialysis although extensive degassing under vacuum of the inhibited enzyme did not recover activity. Absorption spectra of catalase plus NO(g) indicated binding of NO(g) to the heme-iron (Figure 2) with a shift of the soret peak from 403 nm to 428 nm and formation of α and β peaks at 576 and 538 nm respectively. EPR spectroscopy also indicated NO binding to the heme-iron of ferric catalase (Table 3) although double integration of the signal indicated that only 15% of the iron in the sample was EPR active. On the other hand, almost 50% of dithionite reduced catalase was EPR active after incubation with NO(g) (Table 3). Treatment of catalase with 0.1 mM GSNO resulted in only moderate (24%), reversible inhibition of enzymatic activity (Table 1) although absorbance and EPR spectroscopy mirrored that of NO(g) treated enzyme for both ferric and ferrous enzyme (Figure 2 and Table 3). Nitroxyl anion inhibition was also reversible and dose dependent. 50 μM

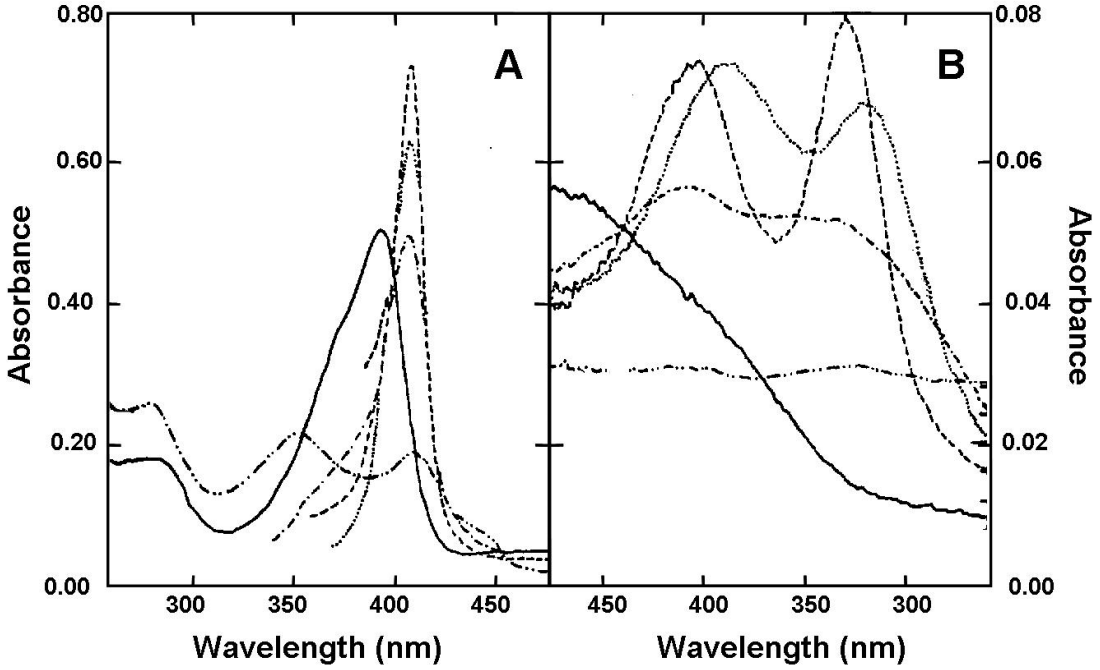


Fig. 4. Absorption spectra of 0.21 mg/ml horseradish peroxidase treated with 50 μM concentrations of RNS. Untreated (——), NO(g) treated (----), NO^- treated (·····), NO^+ treated (-·-·-), $ONOO^-$ treated (-··-··-).

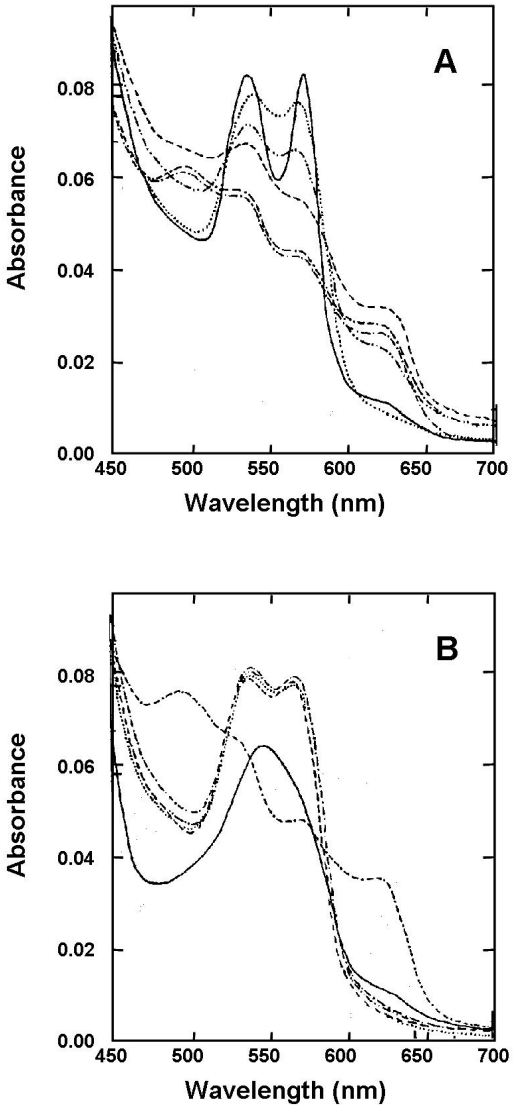
NO^- caused only 18% inhibition but at 8 mM NO^-, 73% of the activity was inhibited. The absorption spectrum of NO^- treated catalase was identical to that of untreated catalase and no heme-NO EPR signals (Table 3) were seen in NO^- treated ferric enzyme, indicating that NO^- is unable to reduce or bind to the heme iron. EPR spectroscopy showed that in dithionite reduced catalase solutions, approximately 50% of the heme-iron was nitrosylated NO^- (Figure 3A, Table 3).

Both nitrite and nitrate at 1 mM caused moderate, reversible inhibition of catalase activity although neither anion had any effect on the catalase absorption or EPR spectra. At 8 mM nitrite, 71% of catalase activity was inhibited suggesting that the inhibition seen with $Na_2N_2O_3$ was actually due to the presence of nitrite rather than to nitroxyl anion. NO^+ did not inhibit catalase activity (Table 1) or induce EPR signal formation in either oxidized or reduced catalase (Table 3) even at concentrations as high as 10 mM, nor did NO^+ addition cause changes in the absorption spectrum of catalase.

50 μM $ONOO^-$ caused a 95% inhibition of catalase activity (Table 1), although, as opposed to other heme-containing enzymes (see below), the inhibition of catalase by $ONOO^-$ was fully reversible. Absorption and EPR spectra of peroxynitrite inhibited catalase were identical to those of untreated enzyme, suggesting that $ONOO^-$ had no effect on the heme-iron.

It is notable that myeloperoxidase (MPO), which has a different heme-structure than the other hemeproteins tested (Wright, Bastian, Davis, Zuo, Yoshimoto, Orme-Johnson & Tauber, 1990), is not inhibited by any of the RNS tested except $ONOO^-$ (Table 1). In fact, a modest increase in activity was seen in both NO(g) and GSNO treated MPO. NO^- effects on MPO were not measured because Angeli's salt was shown to interfere with the enzyme assay. Inhibition by peroxynitrite was both immediate and irreversible with 50 μM $ONOO^-$ inhibiting 97% of myeloperoxidase activity. Since both iNOS and myeloperoxidase are components of the mammalian antibacterial arsenal, the lack of inhibition of myeloperoxidase by most NO congeners may prove useful to an infected animal. The fact that $ONOO^-$ does irreversibly inhibit myeloperoxidase suggests that this NO congener may target a site other than the heme-iron. Interestingly, both NO_2^- and NO_3^- at 0.5 mM concentrations reversibly inhibit myeloperoxidase, possibly by binding to an anion binding site and preventing binding of chloride ion. Neither absorbance nor EPR spectroscopy of myeloperoxidase were done.

The enzymatic activity of anaerobic solutions of Horseradish peroxidase (HRP) was only moderately inhibited by NO, NO^+, NO^-, or GSNO (Table 1). This inhibition was completely reversible by dialysis of the enzyme. 50 μM $ONOO^-$ on the other hand caused a 70% inhibition of HRP that was not reversible by dialysis. Inhibition by



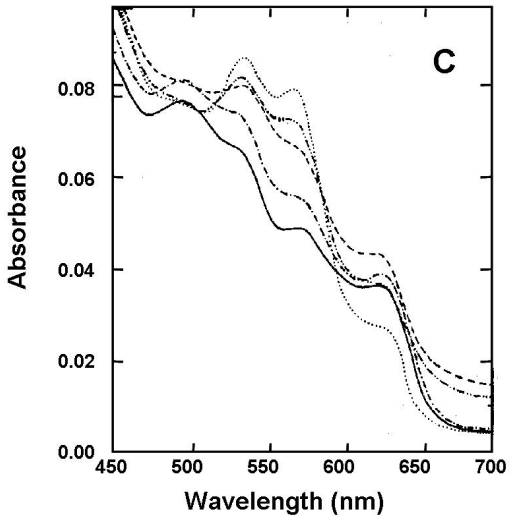


Fig. 5. Absorption spectra of 0.24 mg/ml (A) oxyhemoglobin, (B) deoxyhemoglobin, and (C) methemoglobin, treated with 50 μM concentrations of RNS. Untreated (——), NO(g) treated (----), NO^- treated (·····), NO^+ treated (-·-·-), GSNO treated (-··-··-), and $ONOO^-$ treated(-·-·-·-). In panel (C) the $ONOO^-$ spectrum is identical to the untreated methemoglobin and is not shown.

$ONOO^-$ was dose dependent (data not shown). Neither nitrite nor nitrate inhibited HRP.

Only NO^- and to a lesser extent, GSNO, among the RNS induced an EPR signal with ferric HRP (Table 3). The EPR signal seen for HRP (Figure 3B) is typical of a six coordinate heme-NO signal for this enzyme (Ascenzi, Brunori, Coletta & Desideri, 1989). Only three of the usual 9 hyperfine lines are visible in the spectrum due to the relatively high modulation amplitude used in obtaining the spectrum. When HRP was reduced by dithionite to form ferrous HRP prior to addition of RNS, all the RNS tested except $ONOO^-$ caused formation of a heme-NO signal that was identical to the signal formed with HRP and NO^- (Table 3). Neither nitrite nor nitrate induced EPR signal formation in either ferric or ferrous HRP.

Treatment of neutral (ferric) HRP with 50 μM NO(g) or GSNO caused an immediate increase in the amplitude of and a wavelength shift of the soret peak from 402 nm to 420 nm suggesting formation of a nitric oxide-ferric heme complex (Figure 4). The α and β peaks at 567 nm and 532 nm respectively were also apparent as previously described (Yonetani, Yamamoto, Erman, Leigh & Reed, 1972). 50 μM NO^+ caused a shift in the wavelength of the soret peak but not an increase in the amplitude of the signal (Figure 4) suggesting that only a part of the sample was converted to NO-ferric heme. When HRP was incubated with NO^-, the soret band shifted to 422 nm with α and β peaks visible at 572 nm and 542 nm respectively (Figure 4) suggesting reduction to form a ferrous HRP-NO complex. In agreement with enzyme activity assays and EPR, peroxynitrite caused a substantial bleaching of the absorbance of HRP (Figure 4) suggesting that $ONOO^-$ removes iron from the heme. Whether $ONOO^-$ causes additional damage to HRP by nitrosating amino acid residues cannot be inferred from the present data.

Cytochrome c oxidase activity was reversibly inhibited in a dose dependent manner by NO(g), NO^+, and GSNO (Table 1). NO^-, $ONOO^-$, NO_2^-, and NO_3^- were not assayed because of interference of these reagents with the cytochrome c oxidase assay. EPR and absorbance spectra of cytochrome c oxidase were not measured.

Neither hemoglobin (Hb) nor cytochrome c has enzymatic activity. However, exposure of these

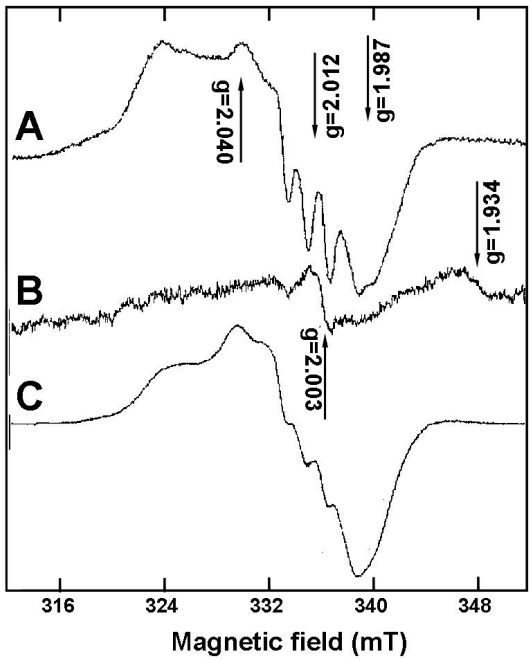


Fig. 6. EPR spectra of (A) NO(g) (100 μM) treated 4.9 mg/ml reduced cytochrome c. (B) Sample (A) after dialysis. (C) NO(g) (100 μM) treated 5.2 mg/ml deoxyhemoglobin. EPR parameters are as stated in the legend to Figure 1.

proteins to RNS was monitored by absorption and EPR spectroscopies. For these experiments, methemoglobin was reduced with $Na_2S_2O_4$ to form deoxyhemoglobin and the excess dithionite was removed by chromatography on a 10 cm Sephadex G25 (Pharmacia) column. The spectral identities of the solutions were monitored by absorbance spectroscopy. Oxyhemoglobin was formed by exposing deoxyhemoglobin to ambient air for 5 minutes, then the headspace gas over the solution was replaced with N_2 by a single 1 minute evacuation. Exposure of oxyhemoglobin to NO(g), NO^+, GSNO, or $ONOO^-$ produced predominantly methemoglobin as seen by absorbance spectroscopy (Figure 5A). EPR spectra of the samples agreed with these results, with less than 25% of NO(g)or GSNO exposed and only about 5% of NO^+ exposed HbO_2 being converted to EPR active HbNO (Table 3). None of the $ONOO^-$ treated HbO_2 was EPR active (Table 3). On the other hand, treatment of HbO_2 with NO^- produced predominantly HbNO as seen by both absorbance (Figure 5A) and EPR spectroscopy (Table 3).

Deoxyhemoblobin was rapidly converted to HbNO by exposure to NO(g), NO^-, NO^+, or GSNO as seen by both absorbance (Figure 5B) and EPR spectroscopies (Table 3, Figure 6C). Treatment of deoxyhemoglobin with $ONOO^-$ on the other hand, produced only methemoglobin as seen by the absence of an EPR signal (Table 3) and by absorbance spectroscopy (Figure 5B).

50 μM NO^- reduced approximately half of the methemoglobin in a 0.2 mg/ml sample and formed HbNO (Figure 5C and Table 3). Other RNS were significantly less effective at HbNO formation from methemoglobin (Table 3 and Figure 5C). Only about 10% of the methemoglobin in NO(g) or GSNO treated and 4% in NOCl treated methemoglobin formed EPR detectable HbNO. Absorbance spectroscopy of these samples also indicated that only a minor portion of the sample was detectable as HbNO. Peroxynitrite did not alter the absorbance spectra of methemoglobin, nor were HbNO EPR spectra detectable in $ONOO^-$ treated samples (Table 3).

Oxidized cytochrome c was also reduced by NO^- but only a modest heme-NO EPR signal developed in oxidized, NO^- treated cytochrome c (Table 3). Double integration of the EPR signal showed that only about 10% of the cytochrome c was nitrosylated. A modest EPR signal representing less than 5% of the cytochrome c in the sample also developed in NO^+ and GSNO treated oxidized cytochrome c although only minor changes in the absorbance spectra were seen (Figure 7A). Neither NO(g) nor $ONOO^-$ induced EPR signal formation or changes in the absorbance spectra of oxidized cytochrome c (Figure 7A and Table 3).

The absorption spectra of reduced cytochrome c were also largely unaffected by RNS treatment (Figure 7B). EPR spectra of NO(g) (Figure 6A), NO^+, and GSNO treated reduced cytochrome c did show that 30-50% of the cytochrome c was nitrosylated. EPR of NO^- treated reduced cytochrome c was only 10-15% nitrosylated while $ONOO^-$ failed to induce EPR signal formation in reduced cytochrome c (Table 3).

EFFECT OF RNS ON THE MOLYBDENUM-CONTAINING DMSO REDUCTASE

Dimethyl sulfoxide [DMSO] reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans* is a molybdenum-containing enzyme which reduces various sulfoxide and N-oxide compounds to the corresponding sulfide or amine (Satoh & Kurihara, 1987). Absorption and electron paramagnetic resonance (EPR) spectroscopies suggest that the molybdenum in DMSO reductase cycles between Mo(IV) and Mo(VI) during catalysis (Bastian, Kay, Barber & Rajagopalan, 1991). Titration of the reduced enzyme with substoichiometric amounts of substrate is accompanied by formation of EPR active Mo(V) (Bastian *et al.*, 1991), sug-

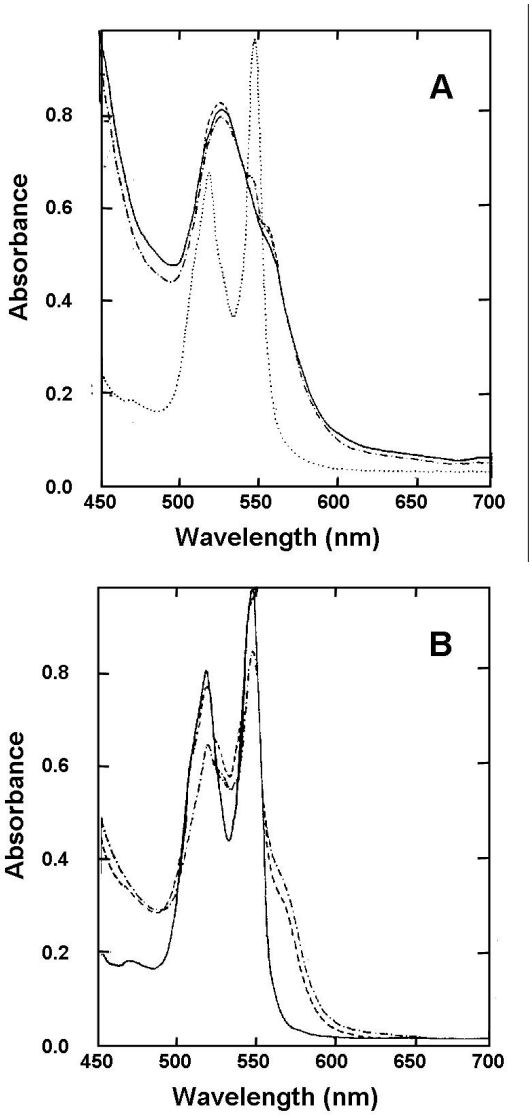


Fig. 7. Absorption spectra of 0.21 mg/ml (A) oxidized cytochrome c and (B) reduced cytochrome c, treated with 50 μM concentrations of RNS. Untreated (——), NO(g) treated (----), NO^- treated (·····), NO^+ treated (-·-·-). In both panels, GSNO treated protein is identical to that of NO(g) treated protein and the $ONOO^-$ spectrum is identical to the oxidized protein and are not shown. In panel (B), NO^- treated protein has a spectrum identical to the untreated sample and is not shown.

gesting sequential rather than concerted transfer of electrons from enzyme to substrate.

Nitric oxide (NO) resembles the trimethylamine-N-oxide substrate of DMSO reductase. It was therefore of interest to see whether RNS inhibit enzyme activity. Enzyme assays showed that DMSO reductase activity was not inhibited by any of the RNS tested, although it was noted that on incubation of DMSO reductase with NO(g), $Na_2N_2O_3$, GSNO, or a combination of nitrite and ascorbate, the usual brown color of the oxidized

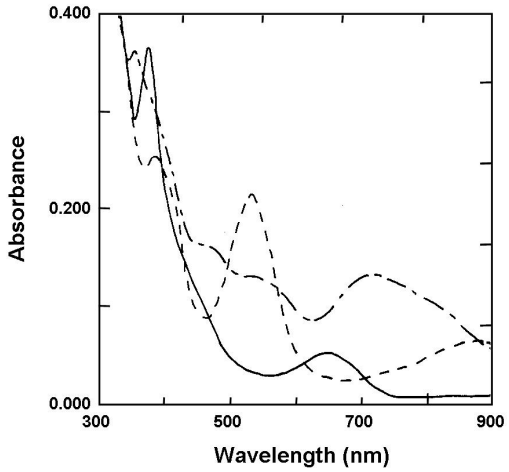


Fig. 8. Absorbance spectra of DMSO reductase. Oxidized enzyme at 6.9 mg/ml (---), dithionite reduced enzyme at 6.9 mg/ml (——), and NO(g) treated enzyme at 6.0 mg/ml (----).

enzyme turned to pink (Bastian, Foster & Pope, 1995). Similar results were observed with the reduced enzyme, with the usual yellow color of the reduced enzyme also turning to pink. The pink form of the enzyme was stable to oxidation by oxygen and was unable to reduce substrate. However, additional reduction of the pink enzyme with either reduced methyl viologen or dithionite restored enzyme activity. An absorbance spectrum of the pink enzyme showed peaks at 387 and 528 nm (Figure 8). EPR spectroscopy of the pink enzyme formed by ascorbate reduction (Figure 9A) showed a Mo(V) DMSO reductase spectrum that was indistinguishable from that produced by reduction of the Mo(VI) enzyme with reduced methyl viologen. Double integration of the EPR spectrum showed that 80-100% of the Mo in the sample was EPR active Mo(V).

To further characterize the reaction between DMSO reductase and NO(g), a 7.7 mg/ml DMSO reductase solution was diluted 1:2 with NO saturated buffer. Mo(V) formation was monitored optically by an increase in absorbance at 528 nm. EPR spectroscopy of this sample showed an axial signal with g-values of $g_\perp = 1.982$ and $g_\parallel = 1.961$ (Figure 9B), rather than the usual rhombic signal (Figure 9A). The absorbance spectra of samples showing rhombic and axial EPR signals were identical. Double integration of the axial EPR signal showed that it represented virtually all of the molybdenum in the sample. The presence of ^{95}Mo (I = 5/2, 15.72% natural abundance) and ^{97}Mo (I = 5/2, 9.46% natural abundance) hyperfine structure in the spectrum (seen in Figure 9B as small satellite peaks) confirmed that the signals were due to molybdenum. Similar hyperfine struc-

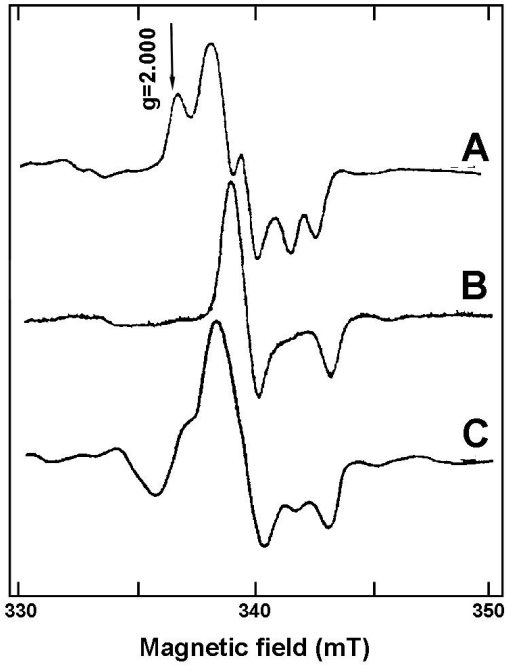


Fig. 9. EPR spectra of (A) Ascorbate reduced DMSO reductase. The spectrum of DMSO reductase reduced by NO^- is virtually identical. (B) DMSO reductase diluted 1:2 with NO(g) saturated Tris·HCl pH 7.5 and incubated at room temperature. (C) DMSO reductase incubated for 30 minutes with 2 μg/ml iNOS in the presence of an iNOS assay buffer consisting of 40 mM Tris buffer pH 7.8, 3 mM DTT, 1 mM L-arginine, 1 mM NADPH, and 4μM each of FAD, FMN, and tetrahydrobiopterin. At the end of the incubation period, the solution was transferred to an EPR tube, frozen in liquid nitrogen, and stored at –80 °C until spectra were recorded. Spectra were recorded at 77 K on a Bruker EMX spectrometer operating at a microwave frequency of 9.31 GHz, a microwave power of 6.3 mW, a modulation frequency of 100 kHz, and a modulation amplitude of 1.0 mT.

ture is seen in the rhombic signal produced by reduction of the oxidized enzyme. NO(g) treated enzyme was unable to reduce substrate without prior reduction by dithionite or reduced methyl viologen but NO(g) did not inhibit DMSO reductase activity.

Addition of $Na_2N_2O_3$ (NO^-) to DMSO reductase produced a rhombic Mo(V) species with an EPR signal identical to that shown in Figure 9A. Again, no enzyme inhibition was observed. Since NO_2^- is formed by the breakdown of $Na_2N_2O_3$, DMSO reductase was incubated with 1 mM NO_2^- In the absence of ascorbate, nitrite was unable to reduce the DMSO reductase, suggesting that nitroxyl anion can carry out a one electron reduction of DMSO reductase.

EFFECT OF NITRIC OXIDE SYNTHASE ON DMSO REDUCTASE

Because the EPR signal produced by incubation of DMSO reductase with NO(g) is different than that produced by NO^-, we wanted to see which signal would be produced if DMSO reductase was incubated with NO synthase. If only the axial signal was produced, NOS must produce only NO(g), whereas a rhombic signal would indicate that NO^- or another reductive species was produced. Accordingly, DMSO reductase (5.1 mg/ml) was incubated for thirty minutes at 24 °C with iNOS (2 μg/ml) in the presence of an NO synthase assay mixture. The assay mixture by itself did not induce formation of an EPR signal in the DMSO reductase, however, when iNOS was added, a Mo(V) signal developed. As can be seen in Figure 9C, the signal is a composite of roughly equal amounts of rhombic and axial signals. This suggests that under the conditions tested, NOS produces both NO(g) and another reductive species, possibly NO^-.

DISCUSSION

The exact identity of the active RNS produced by nitric oxide synthase is controversial, in fact, it is likely that the product of the NOS reaction is influenced by the conditions in the microenvironment. Rusche *et al.* (1998), for example have shown that NO^- is produced by NOS in the absence of tetrahydrobiopterin. Other confounding factors could include the redox status of the cell, the local concentration of NADPH and O_2, and even the presence of Ca^{2+} and calmodulin. NO(g) reacts with O_2 to form N_2O_3 which effectively nitrosylates thiols to S-nitrosyl thiols. NO(g) also reacts with O_2^- and NO^- can react with O_2 to also form $ONOO^-$ under some conditions (Sharpe & Cooper, 1998). NO^+ is formed by the degradation of iron-thiol-nitrosyl complexes (Vanin, 1998) and possibly also from the breakdown of S-nitrosothiols. Each of these RNS, therefore, may be responsible for some of the effects seen in cells upon induction of NOS.

As we have shown here, the effects of RNS on heme and Fe/S proteins are by and large reversible at least after a short-term exposure. This rule holds true for all RNS except peroxynitrite, which with few exceptions causes irreversible inhibition of iron-containing enzymes. The bleaching of the absorbance spectra of aconitase and HRP suggests that $ONOO^-$ can remove the iron in some proteins, although for most other enzymes the iron was

oxidized in $ONOO^-$ solutions but was apparently not removed. Thus, the effects of $ONOO^-$ on most iron-containing proteins are probably due to nitrosation of amino acid residues in the proteins rather than to direct interaction between iron and $ONOO^-$.

NO^+ at least in the case of aconitase can also cause irreversible inhibition of enzymatic activity presumably by nitrosylating a required thiol residue. Otherwise, NO^+ effects on iron-containing proteins are minor and reversible. NO^+ rapidly reacts with thiols to form S-nitrosothiols, so it is likely that in cells any NO^+ that is formed would form S-nitrosothiols rather than interacting with iron.

NO^- can act as a reductant for iron in proteins but otherwise its effects are also reversible. The likely products of NO^- release are NO(g) and reduced metals. In some cases, particularly in the cases of ferric horseradish peroxidase and methemoglobin, reduction of the metal by NO^- was accompanied by binding of NO to the ferrous iron to form an EPR active nitrosyl heme complex. Although NO^- is somewhat inhibitory to aconitase, its effects may be due to the NO(g) produced when NO^- loses an electron. In fact, when equimolar concentrations of NO^- and NO(g) were added to aconitase, the inhibition observed was less than half that seen when NO(g) alone was added suggesting that NO^- provided some protection against inhibition by NO(g) (data not shown).

NO(g) causes rapid and strong inhibition of most of the iron-containing enzymes tested, although inhibition is completely reversible in almost all instances. Inhibition appears to involve NO(g) binding to ferrous rather than ferric iron. The readily reversible nature of NO(g) inhibition agrees with results of Hibbs, Taintor, Vavrin & Rachlin (1988) who showed that induction of NO biosynthesis in tumor cell/macrophage co-cultures lead to cytostasis of the tumor cells but not to cytotoxicity.

Experiments with the molybdenum-containing DMSO reductase lend credence to the idea that iNOS produces NO^- but clearly show that under the conditions of the assay, NO(g) is also produced. Unfortunately, the assay does not distinguish between NO(g) produced directly by NOS and NO(g) produced by the one-electron oxidation of NO^- by DMSO reductase. The fact that the rhombic and axial Mo(V) EPR signals are produced in roughly equimolar concentrations suggests the latter, although further experiments will be necessary to confirm this result.

Acknowledgements

This work was supported by a grant from the Willard Eccles Charitable Foundation and by grant R01HL51963 from the National Institutes of Health.

Abbreviations

RNS, reactive nitrogen species; GSNO, S-nitrosoglutathione; EPR, electron paramagnetic resonance; Hb, hemoglobin; HRP, horseradish peroxidase; NOS, nitric oxide synthase; iNOS, immune/inflammatory nitric oxide synthase, MPO, myeloperoxidase; HbNO, nitrosylated hemoglobin; DMSO, Dimethylsulfoxide.

REFERENCES

Ascenzi P., Brunori M., Coletta M. & Desideri A. (1989). pH effects on the haem iron co-ordination state in the nitric oxide and deoxy derivatives of ferrous horseradish peroxidase and cytochrome c peroxidase. *Biochem J*., **258**, 473-478.

Bastian N. R., Kay C. J., Barber M. J., & Rajagopalan K. V. (1991). Spectroscopic studies of the molybdenum-containing dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* f. sp. *Denitrificans*. *J. Biol. Chem*., **266**, 45-51.

Bastian N. R., Yim C. -Y., Hibbs J. B., Jr., & Samlowski W. E. (1994a). Induction of iron-derived EPR signals in murine cancers by nitric oxide, evidence for multiple intracellular targets. *J. Biol. Chem*., **269**, 5127-5131.

Bastian N. R., Xu S., Shao X. L., Shelby J., Granger D. L. & Hibbs J. B., Jr. (1994b). N^{ω}- monomethyl-L-arginine inhibits nitric oxide production in murine cardiac allografts but does not affect graft rejection. *Biochim. Biophys. Acta*, **1226**, 225-231.

Bastian N. R., Foster M. J. P. & Pope, J. C. (1995). Nitric oxide stabilizes the Mo(V). oxidation state of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* without inhibiting enzyme activity. *BioFactors*, **5**, 5-10.

Beers R. F. Jr. & Sizer I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem*., **195**, 133-140.

Borutaite V. & Brown G. C. (1996). Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. *Biochem. J*., **315,** 295-299.

Brennan M. J., Cole T. & Singley J. A. (1966). A unique hyperfine ESR spectrum in mouse neoplasms analyzed by computer simulation. *Proc. Soc. Exptl. Biol. Med*., **123**, 715-718.

Butler A. R., Glidewell C., Hyde A. R. & Walton J. C. (1985). Formation of paramagnetic mononuclear iron nitrosyl complexes from diamagnetic di- and tetranuclear iron-sulfur nitrosyls: Characterization by EPR spectroscopy and study of thiolate and nitrosyl ligand exchange reactions. *Polyhedron*, **4**, 797-809.

Chiang R. W., Woolum J. C. & Commoner B. (1972). Further study on the properties of the rat liver protein involved in a paramagnetic complex in the livers of

carcinogen-treated rats. *Biochem. Biophys. Acta*, **257**, 452-460.

Coleman G. H., Lillis G. A. & Goheen G. E. (1939). Nitrosyl chloride. [In:] Booth, H. S. (Ed.), *Inorganic Syntheses*, (vol. 1, pp. 55-59), New York: McGraw-Hill.

Commoner B., Woolum J. C., Sentutia B. H. Jr. & Ternberg J. L. (1970). The effects of 2-acetylaminofluorene and nitrite on free radicals and carcinogenesis in rat liver. *Cancer Res.*, **30**, 2091-2097.

Drapier J.-C. & Hibbs J. B. Jr. (1986). Mutine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest.*, **78**, 790-797.

Drapier J.-C. & Hibbs J. B. Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J. Immunol.*, **140**, 2829-2838.

Drapier J.-C., Pellat C. & Henry Y. (1991). Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. *J. Biol. Chem.*, **266**, 10162-10167.

Emanuel N. M., Saprin A. N., Sharalkin V. A., Kozlova L. E. & Krugtiakova K. E. (1969). Detection and investigation of a new type of ESR signal characteristic of some tumor tissue. *Nature*, **222**, 165-167.

Ferguson-Miller S., Brautigan D. L. & Margoliash E. (1978). Definition of cytochrome c binding domains by chemical modification. III. Kinetics of reaction of carboxydinitrophenyl cytochromes c with cytochrome c oxidase. *J. Biol. Chem.*, **253**, 149-159.

Granger D. L., Taintor R. R., Cook J. L. & Hibbs J. B. Jr. (1980). Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. *J. Clin. Invest.*, **65**, 357-370.

Granger D. L. & Lehninger A. L. (1982). Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J. Cell Biol.*, **95**, 527-535.

Hatefi Y. (1978). Preparation and properties of NADH:ubiquinone oxidoreductase (complex I).. *Meth. Enzymol.*, **53**, 11-14.

Hibbs J. B. Jr., Taintor R. R. & Vavrin Z. (1984). Iron depletion: possible cause of tumor cell cytotoxicity induced by activated macrophages. *Biochem. Biophys. Res. Commun.*, **123**, 716-723.

Hibbs J. B. Jr., Taintor R. R., Vavrin Z. & Rachlin E. M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87-94.

Hille R., Olson J. S. & Palmer G. (1979). Spectral transitions of nitrosyl hemes during ligand binding to hemoglobin. *J. Biol. Chem.*, **254**, 12110-12120.

Hughes M. N. (1999). Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochim. Biophys. Acta*, **1411**, 263-272.

Hunt H. R. Jr., Cox J. R. Jr. & Ray J. D. (1962). The heat of formation of crystalline sodium α-oxyhyponitrite. The structure of aqueous α-oxyhyponitrite ion. *Inorganic Chem.*, **1**, 938-941.

Komarov A. M., Wink D. A., Feelisch M. & Schmidt H. H. (2000). Electron-paramagnetic resonance spectroscopy using N-methyl-D-glucamine dithiocarbamate iron cannot discriminate between nitric oxide and nitroxyl: implications for the detection of reaction products for nitric oxide synthase. *Free Radic. Biol. Med.*, **28**, 739-742.

Kon H. (1968). Paramagnetic resonance study of nitric oxide hemoglobin. *J. Biol. Chem.*, **243**, 4350-4357.

Kon H. & Kataoka N. (1969). Electron paramagnetic resonance of nitric oxide-protoheme complexes with some nitrogenous base model systems of nitric oxide hemoproteins. *Biochemistry*, **8**, 4757-4762.

Kostka P., Xu B. & Skiles E. H. (1999). Degradation of S-nitrosocysteine in vascular tissue homogenates: role of divalent ions. *J. Cardiovasc. Pharmacol.*, **33**, 665-670.

Lancaster J. R. Jr. & Hibbs J. B. Jr. (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. USA*, **87**, 1223-1227.

Marletta M. A., Yoon P. S., Iyengar R., Leaf C. D. & Wishnok J. S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706- 8711.

Morse R. H. & Chan S. I. (1980). Electron paramagnetic resonance studies of nitrosyl ferrous heme complexes. Determination of an equilibrium between two confirmations. *J. Biol. Chem.*, **255**, 7876-7882.

Oshima H., Yoshie Y., Auriol S. & Gilibert I. (1998). Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite, and nitroxyl anion. *Free Radic. Biol. Med.*, **25**, 1057-1065.

Park J.-W. (1988). Reaction of S-nitrosoglutathione with sulfhydryl groups in protein. *Biochem. Biophys. Res. Commun.*, **152**, 916-920.

Pellat C., Henry Y. & Drapier J.-C. (1990). IFN-γ-activated macrophages: Detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. *Biochem. Biophys. Res. Commun.*, **166,** 119-125.

Rose I. A. & O'Connell E. L. (1967). Mechanism of aconitase action. The hydrogen transfer reaction. *J. Biol. Chem.*, **242**, 1870-1879.

Rusche K. M. Spiering M. M. & Marletta M. A. (1998). Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry*, **37**, 15503-15512.

Satoh T. & Kurihara F. N. (1987). Purification and properties of dimethylsulfoxide reductase containing a molybdenum cofactor from a photodenitrifier, *Rhodopseudomonas sphaeroides* f. s. *denitrificans*. *J. Biochem.*, **102**, 191-197.

Schmidt H. H. H. W., Hofmann H., Schindler U., Shutenko Z. S., Cunningham D. D. & Feelisch M. (1996). No. NO from NO synthase. *Proc. Natl. Acad. Sci. USA*, **93**, 14492-14497.

Scholler D. M., Wang M. -Y. R. & Hoffman B. M. (1979). Resonance raman and EPR of nitrosyl human

hemoglobin and chains, carp hemoglobin, and model compounds. *J. Biol. Chem.*, **254**, 4072-4078.

Sharpe M. A. & Cooper C. E. (1998). Reactions of nitric oxide with mitochondrial cytochrome c: a novel mechanism for the formation of nitroxyl anion and peroxynitrite. *Biochem. J.*, **332**, 9-19.

Tagawa K. & Arnon D. I. (1962). Ferredoxins as electron carriers in photosynthesis and in biological production and consumption of hydrogen gas. *Nature*, **195**, 537-543.

Torres J., Cooper C. E. & Wilson M. T. (1988). A common mechanism for the interaction of nitric oxide with the oxidized binuclear centre and oxygen intermediates of cytochrome c oxidase. *J. Biol. Chem.*, **273**, 8756-8766.

Vanin A. F., Blyumenfel'd L. A. & Chetverikov A. G. (1967). Investigation by the EPR method of complexes of non-haem iron in cells and tissues. *Biofizika*, **12**, 829-838.

Vanin A. F. (1991). Endothelium-derived relaxing factor is a nitrosyl iron complex with thiol ligands. *FEBS Lett.*, **289**, 1-3.

Vanin A. F. (1998). Dinitrosyl iron complexes and S-nitrosothiols are two possible forms for stabilization and transport of nitric oxide in biological systems. *Biochemistry-Mosc.*, **63**, 782-793.

Warton M., Granger D. L. & Durack D. T. (1988). Mitochondrial iron loss from leukemia cells injured by macrophages. A possible mechanism for electron transport chain defects. *J. Immunol.*, **141**, 1311-1317.

Wink D. A., Darbyshire J. F., Nims R. W., Saavedra J. E. & Ford P. C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxygenation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.*, **6**, 23-27.

Wright J., Bastian N., Davis T. A., Zuo C., Yoshimoto S., Orme-Johnson W. H. & Tauber A. I. (1990). Structural characterization of the isoenzymatic forms of human myeloperoxidase: Evaluation of the iron-containing prosthetic group. *Blood*, **75**, 238-241.

Xia Y. & Zweier J. L. (1997). Direct measurement of nitric oxide generation from nitric oxide synthase. *Proc. Natl. Acad. Sci. USA*, **94**, 12705-12710.

Yonetani T., Yamamoto H., Erman J. E., Leigh, J. S. Jr. & Reed G. H. (1972). Electromagnetic properties of hemoproteins. V. Optical and electron paramagnetic resonance characteristics of nitric oxide derivatives of metalloporphryn-apohemoprotein complexes. *J. Biol. Chem.*, **247**, 2447-2455.