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NITROXIDE-STIMULATED H_2O_2 DECOMPOSITION BY PEROXIDASES AND PSEUDOPEROXIDASES

ROLF J. MEHLHORN and CHRISTOPHER E. SWANSON

Energy and Environment Division, Lawrence Berkeley Laboratory

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Nitroxide free radicals interact with Hb/metHb, Mb/metMb and with peroxidases/phenols to induce a catalase-like conversion of H_2O_2 to O_2 (catalatic activity), without being substantially consumed in the process. The mechanism of this reaction is postulated to involve a one-electron oxidation of the nitroxide to the immonium oxene, which then reacts further to release oxygen and the nitroxide. An involvement of the immonium oxene in the reaction mechanism is consistent with ferryl heme reduction by nitroxides and a detection of the reduced nitroxide when the reaction mixture is supplemented with the two-electron reductant sodium borohydride. The nitroxide-induced catalatic activity is completely inhibited when the reaction mixture is supplemented with glutathione. Nitroxides suppress free radical formation by hydroperoxide-activated heme proteins, as inferred from their inhibition of the spin-trapping of glutathionyl radicals. H_2O_2 decomposition and a suppression of reactive free radical formation by heme proteins appears to be an antioxidant activity of nitroxides, which is distinct from their previously reported superoxide dismutating activity and which may be a factor in their protective action in models of cardiac reperfusion injury.

KEY WORDS: Peroxidase inhibitor, ferryl heme reduction, protein free radical scavenger, spin trap destruction, antioxidant—heme specific, nitric oxide oxidation.

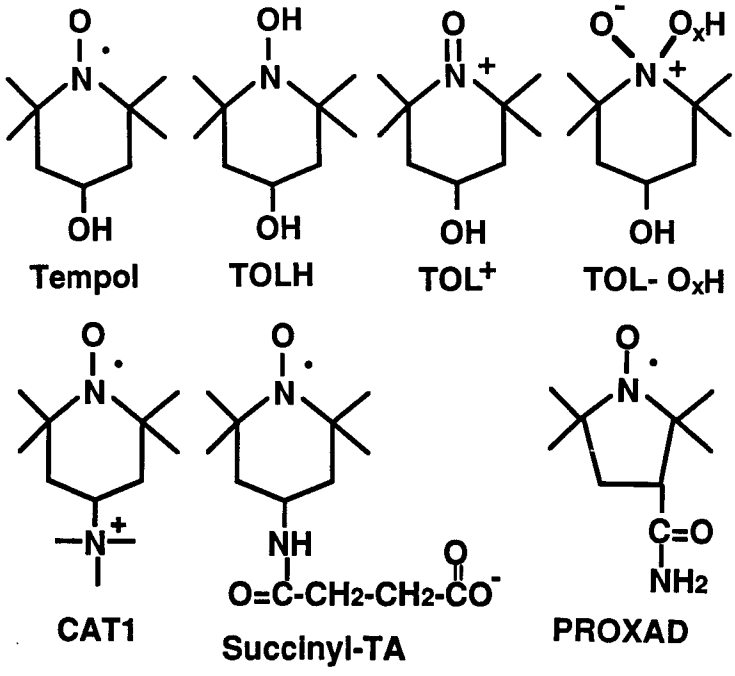
INTRODUCTION

Peroxides can be activated to biologically destructive oxidants by heme proteins or transition metal ions.[1] The reaction of H_2O_2 with reduced transition metal ions, often referred to as the Fenton or Haber-Weiss reaction, has stimulated considerable interest because it produces the destructive hydroxyl radical, implicated in the toxicity of ionizing radiation. However, "free' transition metal ions in cells are not likely to persist and their catalysis of hydroxyl radical production requires reducing agents. Only a few reaction cycles with one-electron reducing agents like ascorbic acid are likely to occur before released transition metal ions are sequestered in storage proteins like ferritin. On the other hand, Mb, Hb, peroxidases as well as the "pseudoperoxidases" metHb and metMb can activate peroxides catalytically independently of reducing agents. Hb and Mb oxidation products catalyze lipid peroxidation,[2–6] protein degradation and crosslinking[2–4,7–10] and other destructive reactions.[11] These reactions are complex and their details have yet to be fully elucidated.[12] However, it is clear that reactive intermediates, including protein free radicals, arise when metHb and metMb are exposed to hydroperoxides.[13] The reactions of heme proteins with excess organic hydroperoxides have been relatively well characterized and appear to involve principally the formation of peroxyl radicals and little, if any, direct formation of alkoxyl species.[14] Because of their abundance in animals, Hb and Mb may play significant roles in oxidant damage, including chronic free radical exposure resulting from the steady production of low levels of

hydrogen peroxide during normal physiological processes,[15] or from the autoxidation of reduced quinonoids, possibly including reducing compounds present in cigareete smoke.[16] Here we report that nitroxide free radicals reduce peroxide-activated heme proteins, stimulate their catalatic activity and suppress the formation of secondary free radicals. Previously a protective effect of nitroxides observed in H_2O_2-treated cells had been ascribed to an SOD activity of nitroxides or maintenance of transition metal ions in their oxidized states.[17] Results reported here suggest that nitroxides may exert their protective effect, at least partially, by a direct effect on ferryl heme proteins.

MATERIALS AND METHODS

All experiments were performed at room temperature (22 to 25°C). Results shown are representative of several experiments under similar conditions. Oxygen was measured polarographically (Rank Brothers Ltd oxygen polarograph Model 10) in reaction mixtures made up with PBS buffer, pH 7.4. Nitroxides were synthesized: SuccinylTA (N-Succinyl-4-amino-2,2,6,6-tetramethyl-piperidin-N-oxyl),[18] CAT1 (4-Trimethylammonium-2,2,6,6-tetramethyl piperidin-N-oxyl bromide),[19] TOLH (1,4-dihydroxy-2,2,6,6-tetramethyl piperidine),[20] or purchased (Aldrich Chemical Co.): Tempol (4-Hydroxy-2,2,6,6-tetramethyl piperidin-N-oxyl), PROXAD (3-Carbamoyl-2,2,6,6-tetramethyl pyrrolidin-N-oxyl). Nitroxides, the hydroxylamine TOLH, the immonium oxene TOL^+ and the complex of the latter with the hydroxide ion TOL-OH or with the protonated superoxide radical TOL-OOH, are shown in the probe structures.



ESR measurements were performed with a Varian E109E spectrometer (X-band). Hemoglobin absorption spectra were obtained with a Shimadzu UV-160U spectrophotometer. Changes in the peak absorbance in the Soret region according to published procedures[21] were used to estimate the rate of formation and decay of the ferryl species of Hb.

RESULTS

Oxygen evolution by H_2O_2-treated Hb

O_2 is known to be evolved when a variety of peroxidases (e.g., lactoperoxidase) or pseudoperoxidases (e.g., metMb) are treated with an excess of H_2O_3.[13,22] An example of O_2 evolution with excess H_2O_2 in a mixture of Hb and metHb and a subsequent consumption of the evolved O_2 is shown in Figure 1b. If the concentration of added H_2O_2 is less than the heme concentration, no such oxygen evolution is observed, even if the H_2O_2 is added repeatedly so that its cumulative concentration substantially exceeds that of the hemes (Figure 1a). At any stage of treatments with small aliquots of H_2O_2, addition of excess H_2O_2 elicits O_2 evolution (similar to that shown in Figure 1b). Oxygen evolved with excess H_2O_2 is consumed again; the time required for this O_2 consumption increases with the number of H_2O_2 treatments (Figure 1b).

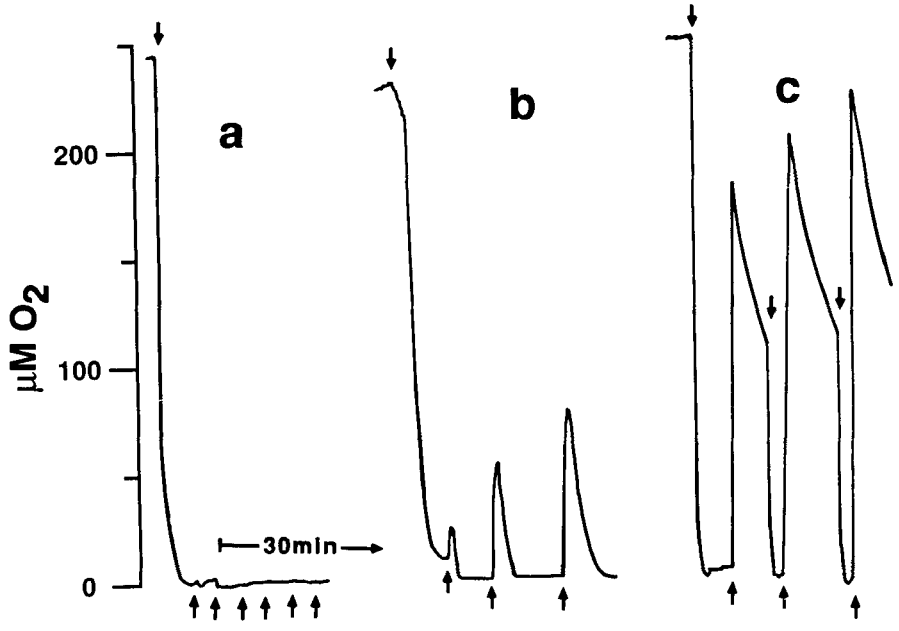


FIGURE 1 Decomposition of H_2O_2 to O_2 by Hb/metHb. Bovine Hb (200 μM; Sigma product H2500, which is a mixture of Hb and metHb) in PBS buffer, pH 7.4, was rendered anaerobic by N_2 flushing (initiated at times indicated by downward arrows) and treated with H_2O_2 at times indicated by upward arrows. Aliquots of 0.5 mM H_2O_2 (a); aliquots of 1.6 mM H_2O_2 (b); 1 mM Tempol added before N_2 flushing, then aliquots of 0.5 mM H_2O_2 (c).

O_2 consumption subsequent to treatment with H_2O_2 implies that chemical reactions occur between the heme proteins and molecular oxygen, which must necessarily contribute to the protein modification that has been characterized for oxidation by H_2O_2 alone.[23,24]

Stimulation of O_2 evolution by nitroxides

Although O_2 consumption by pseudoperoxidases subsequent to treatment with H_2O_2 was first reported nearly four decades ago,[13] the mechanism of this O_2 consumption has not been resolved. One possible reaction pathway is incorporation of oxygen into proteins via peroxyl radical intermediates. To test this possibility we used nitroxide stable free radicals as oxygen mimics. Like oxygen, nitroxides combine with carbon- and sulfur-centered free radicals. The nitroxides are thereby destroyed, which can be monitored by ESR methods.[25] ESR measurements of Hb and Hb treated with H_2O_2 showed limited nitroxide destruction (see section on nitroxide reduction/destruction).

When the effect of nitroxides on oxygen consumption was analyzed, oxygen evolution was observed even for low H_2O_2:heme concentration ratios (Figure 1c). Consumption of the newly evolved O_2 was much slower than had been observed with excess peroxide (cf. Figure 1b), suggesting that formation of O_2-reactive Hb intermediates was inhibited in the presence of the nitroxides. A plot of oxygen evolution vs. H_2O_2 concentration extrapolated nearly to zero (Figure 2), suggesting that nitroxides facilitate oxygen-evolution under conditions that would otherwise induce formation of mostly ferryl Hb. With 1 mM Tempol, Hb produced about half of the catalatic yield of O_2, whereas Mb (equine), at an equivalent heme concentration produced about 80% of the catalatic O_2 yield. Since Mb does not contain any cysteine residues part of the difference in O_2 consumption by these two proteins may be due to oxygen consumption by cysteinyl radicals in Hb.[26–28]

Since nitroxides have been reported to dismutate superoxide radicals,[29,30] it was of interest to assess whether the nitroxide-induced catalatic activity of Hb could also be elicited by SOD. However, no catalase-like activity was observed by treating the Hb or Mb solutions with Cu–Zn superoxide dismutase (up to 4000 U/ml). Thus the H_2O_2-decomposing effect of nitroxides does not appear to involve their SOD mimicking activity.

The Hb experiments were performed with a commercial preparation, which contains a substantial admixture of metHb. Purified metHb evolved oxygen (Figure 2), indicating that the catalatic activity stimulated by nitroxides is elicited by metHb alone (we cannot ascertain whether this activity would be elicited by Hb alone). Part of the newly evolved oxygen in the Hb/metHb and Mb/metMb preparations would be expected to bind to the ferro species and not be detected by the polarographic assay and this could account for the lower oxygen evolution in Hb/metHb relative to metHb. Since both Hb and oxyHb are rapidly converted to metHb by H_2O_2,[31] and since the evolved O_2 would be prohibitively difficult to quantify with varying concentrations of Hb, no attempt was made here to resolve the effects of nitroxides on purified Hb.

Suppression of O_2 evolution and potentiation of nitroxide reduction/destruction by glutathione and ascorbate

GSH (1 mM) substantially suppressed the nitroxide-induced catalatic activity of Hb

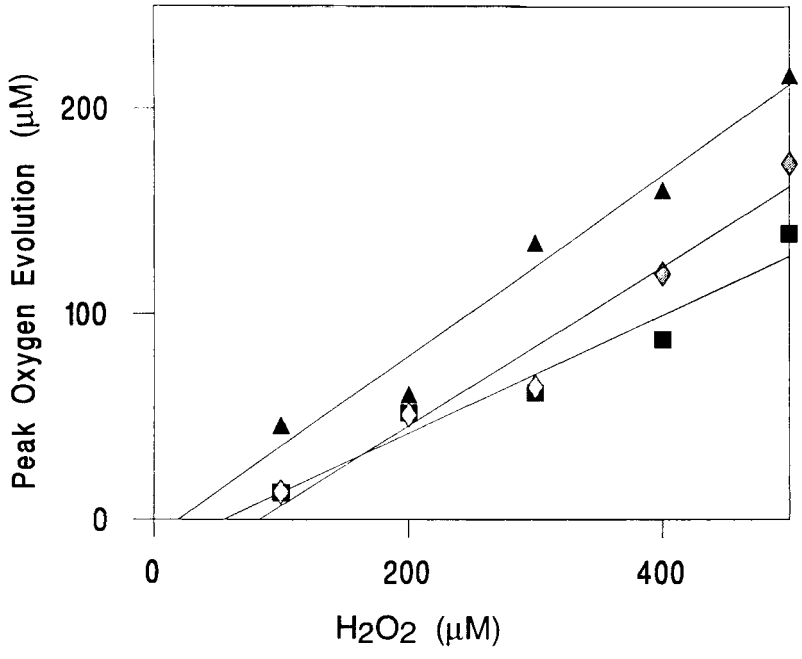


FIGURE 2 Tempol-stimulated oxygen evolution. Hb (■, 200 uM), metHb (◇, 200 uM; Sigma product M 9250) and Mb (▲, 800 uM; Sigma product M0630). All reaction mixtures contained 1 mM Tempol.

and Mb. With a more than two-fold excess of Tempol relative to GSH, the nitroxide was destroyed until all of the GSH was consumed; at this point approximately two nitroxides had been destroyed per GSH. Destruction was inferred from a failure to reoxidize the nitroxide by treatment with excess ferricyanide and from the observation that the same extent of nitroxide destruction was observed with Tempol as with PROXAD. Dihydropyrrolines like PROXAD are not reduced by thiols in the presence of a superoxide generating source, indicating that the previously postulated superoxide/nitroxide complex[29] is not implicated in the GSH-dependent nitroxide loss. If H_2O_2 was added incrementally to a reaction mixture containing an excess of Tempol, oxygen evolution became apparent only when the H_2O_2 concentration exceeded approximately twice the concentration of GSH.

Suppression of glutathionyl spin trapping by nitroxides

Spin trapping of glutathionyl radicals showed that Tempol (1 mM) did not prevent the formation of strong one-electron oxidants when Mb was treated with H_2O_2 (Figure 3). However, the decay of the ESR signal of the DMPO-GS adduct was greatly accelerated in the presence of Tempol, consistent with the rapid depletion of H_2O_2 by the Tempol-induced catalatic activity of Mb (Figure 3). A relatively minor component of the decay kinetics of the DMPO-GS adduct (Figure 3, diamonds) may be due to a reaction of the adduct with Tempol, e.g., a hydrogen atom transfer from DMPO-GS to Tempol to yield a nitrone and TOLH; the maximum magnitude

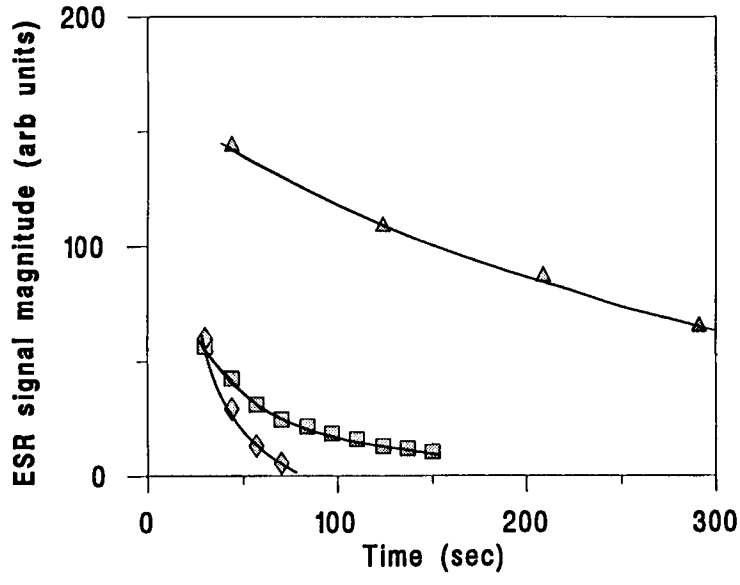


FIGURE 3 Stimulation of the decay of the ESR signal of glutathionyl-DMPO adducts by Tempol. To facilitate the measurement of the low field line of spin trap signals relative to the much larger ESR signals of 1 mM Tempol, ^{15}N Tempol was used. The reaction mixture included 0.9 mM Mb, 1 mM GSH, 0.1 M DMPO, and 0.4 mM H_2O_2. Control (Δ); Tempol present prior to H_2O_2 (◇), Tempol added after H_2O_2 (□).

of this effect would presumably be represented by the decay kinetics observed when Tempol was added after the H_2O_2 treatment (Figure 3, squares).

In contrast to the potent suppression of the nitroxide-dependent oxygen evolution by GSH, ascorbate exerted only a modest inhibitory effect on the nitroxide-stimulated oxygen evolution by myoglobin (1 mM Tempol and 0.4 mM ascorbate elicited about half the oxygen evolution seen with Tempol alone, data not shown). ESR analysis indicated that Mb and H_2O_2 induced a slight stimulation of the rate of nitroxide reduction by ascorbate (about two-fold relative to the reduction rate that would have occurred in the absence of the Mb and H_2O_2).

Suppression of spin trap adduct ESR signals by H_2O_2 and Hb

Since free radical adducts of spin traps are nitroxides, it seemed plausible that spin trap adducts would also react with H_2O_2-treated heme proteins. To examine this possibility, methyl radicals were spin trapped in a Fenton-type system in the presence of absence of Hb. Ascorabte-driven redox cycling of Fe-EDTA was used to generate methyl radicals according to the reaction:

$$Fe^{2+} + (Me)_3COOH \dashrightarrow Fe^{3+} + (Me)_3CO^{\cdot} + OH^-$$

$$(Me)_3CO\cdot \dashrightarrow (Me)_2\ C=O + CH_3^{\cdot}$$

Treatment of this reaction mixture with metHb completely eradicated the ESR signals

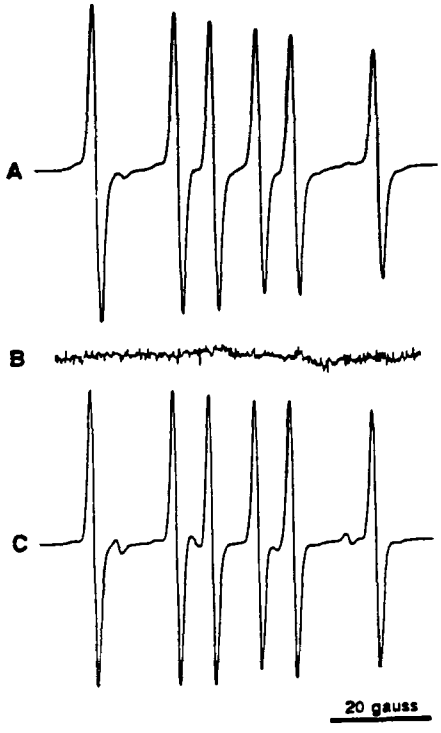


FIGURE 4 Suppression of DMPO-methyl ESR signals by H_2O_2-treated Hb. DMPO-Me adducts generated by 2 mM Fe-EDTA, 2 mM ascorbate, 4 mM tBH in PBS buffer, pH 7.4 with 0.1 M DMPO (A). Reaction mixture A treated with 25 mg/ml metHb (B). Reaction mixture A treated with 25 mg/ml metHb and 5 mM KCN (C).

of the DMPO adducts of methyl radicals (Figure 4). When the activation of metHb by H_2O_2 was blocked by cyanide the ESR signal magnitudes of spin trapped methyl radicals were similar to those observed in the absence of Hb. Since cyanide blocks the activation of metHb by H_2O_2, these results are consistent with a destruction of DMPO-methyl radical adducts by ferryl Hb. Part of the suppression of the DMPO-methyl ESR signals by metHb may be due to an accelerated depletion of hydroperoxide; however the rate of tBH depletion does not appear to be sufficient to explain all of the suppression (see Figure 8).

Nitroxide reduction/destruction by H_2O_2-treated Hb or Mb

The oxygen evolving activity of Hb depends on the nitroxide concentration (Figure 5 and Table I). Tempol concentrations exceeding 2 mM yielded the maximum oxygen evolution (Figure 5). With multiple H_2O_2 treatments, oxygen evolution was a transient phenomenon at the lowest Tempol concentrations (Table I). Electron spin resonance (ESR) measurements showed that the nitroxide had been substantially destroyed when oxygen evolution ceased. Quantification of nitroxide concentrations requires that the linebroadening effects of oxygen be taken into account—whenever

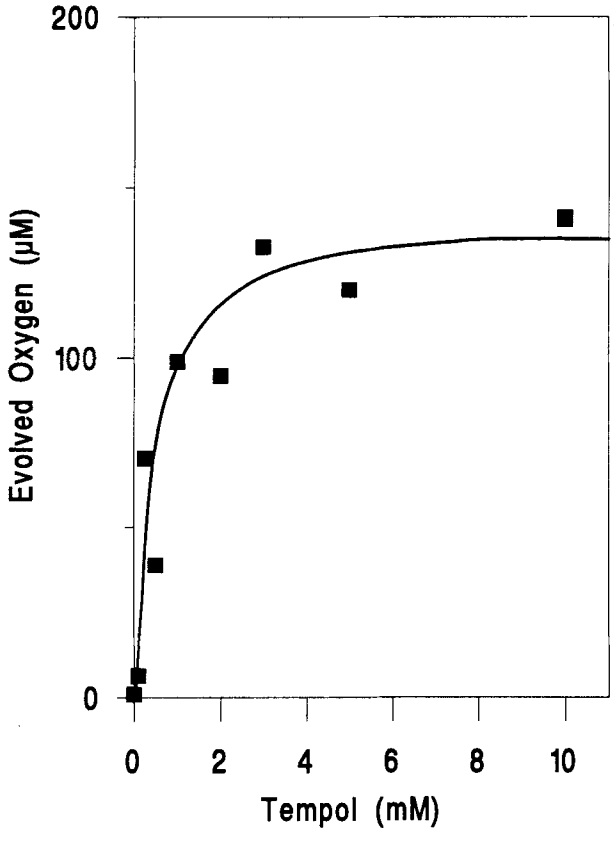


FIGURE 5 Oxygen evolution vs. Tempol concentration. Hb (200 μM) was treated with 300 μM H_2O_2.

TABLE I
O_2 (μM) evolution in H_2O_2-treated Hb as a function of the initial Tempol concentration

	Total H_2O_2 (mM)		
Tempol (mM)	0.5	1.0	1.5
0	0	0	0
0.1	35	0	0
0.3	82	82	15
1.0	172	198	222

Up to three aliquots of 0.5 mM H_2O_2 were added to an anaerobic solution of 200 μM Hb. The O_2 concentration refers to the peak value. Flushing with N_2 was used to render the reaction mixture anaerobic (see Figure 1).

TABLE II
Tempol destruction by H_2O_2-activated Mb
A. Sequential H_2O_2 treatments of 4 mM Mb

	H_2O_2 additions			
	None	4 mM	2 × 4 mM	3 × 4 mM
Tempol*	0.4 (100%)	0.242 (60%)	0.213 (53%)	0.141 (35%)
Tempol*	2 (100%)	1.71 (85%)	1.64 (82%)	1.66 (83%)

* Remainder (mM); also expressed as percentage of untreated sample.

B. Single H_2O_2 treatments of 1 mM Mb

	H_2O_2 addition	
	None	1 mM
Tempol‡	50 (100%)	29 (58%)
Tempol‡	100 (100%)	66 (66%)
Tempol‡	200 (100%)	160 (80%)

‡ Remainder (μM); also expressed as percentage of untreated sample.

possible, samples were fully equilibrated with air prior to the ESR measurements to avoid making these corrections. About 0.3 mM Tempol was destroyed in the sample that initially contained 1 mM of the nitroxide and most of this destruction occurred with the first H_2O_2 aliquot. A study of the extent of Tempol destruction by Mb/H_2O_2 at different concentrations of the nitroxide showed that destruction was greatest at low nitroxide:Mb ratios (Table II). Tempol destruction by Hb was more extensive than that by Mb (data not shown), probably because of a reaction of the nitroxide with the protein thiyl radical, which has been inferred to arise from the oxidation of the cysteine residue at the 93 position of the β chain.[26–28] Since equine Mb lacks cysteine, the modest loss of Tempol with H_2O_2-treated Mb suggests little, if any, formation of carbon-centered radicals at protein sites accessible to the nitroxide.

Effect of nitroxide structure on O_2 evolution

The induction of a catalase-like activity of Hb or Mb was greater by six-membered ring (piperidine) nitroxides than by five-membered ring (pyrroline) nitroxides, the latter being represented by PROXAD (Table II). Charged nitroxides elicited less oxygen evolution from the initial aliquot of H_2O_2 than was observed with the neutral Tempol. The cationic CAT1 consistently induced less O_2 evolution that did two-fold lower concentrations of Tempol or the negatively charged SuccinylTA.

Nitroxide reduction in the presence of sodium borohydride

The potent reducing agent borohydride readily reduces the ketone nitroxide Tempone to the secondary alcohol Tempol without affecting the paramagnetism of the nitroxide.[32] Therefore, borohydride does not act as a one-electron reductant towards Tempone or Tempol. However, when borohydride was added to a reaction mixture

TABLE III
Effect of nitroxide structure on peak oxygen evolution (μM) by Hb

Nitroxide	H_2O_2 additions (mM)		
	0.5	2 × 0.5	3 × 0.5
Tempol (1 mM)	172	199	222
SuccinylTA (1 mM)	120	188	231
CAT1 (2 mM)	124	178	175
PROXAD (1 mM)	48	42	42

See Table I for experimental procedure.

containing Hb or Mb and H_2O_2, a substantial loss of the Tempol ESR signal was observed (Table IV). The magnitude of the initial ESR signal was completely restored by treatment of the reaction mixture with 2 mM $K_3Fe(CN)_6$, indicating that the signal loss was entirely due to a reduction of Tempol to TOLH. The Tempol signal slowly reappeared in the control sample, but was relatively constant in the presence of DTPA. Iron is known to be released from H_2O_2-treated Mb and Hb,[21] and many iron chelates, e.g., Fe-EDTA, oxidize TOLH. However, Fe-DTPA does not oxidize TOLH at an appreciable rate (data not shown). Therefore it appears that the slow reoxidation of TOLH in the control sample is due to released iron.

O_2 evolution by peroxidases

The nitroxide-induced catalase activity was also observed with two peroxidases. Although oxygen evolution is known to occur when peroxidases are treated with excess H_2O_2,[20] little or no such oxygen evolution is observed in the presence of sufficient substrate concentrations (Figure 6). Tempol altered this normal reaction pattern of horseradish peroxidase (hrp), inducing a catalase-like activity in the enzyme even when the concentration of the substrate phenol exceeded that of the nitroxide (Figure 6a). Lactoperoxidase treated with H_2O_2 exhibited some O_2 decomposition even with 10 mM phenol. Tempol stimulated this catalatic activity (Figure 6b).

Optical spectroscopy

Optical spectroscopy indicated that Tempol as well as TOLH reduced the ferryl species (Figure 7a). From an analysis of ferryl reduction at two different Tempol concentrations we estimate a rate constant of $34 \pm 5\ M^{-1}\ s^{-1}$ for the reduction of ferryl Mb by Tempol. Others have estimated rate constants of $15\ M^{-1}\ s^{-1}$ and $153\ M^{-1}\ s^{-1}$ for the reactions of ascorbate and urate with ferryl Hb,[33] respectively. In our hands ascorbate appeared to reduce pre-formed ferryl Hb more effectively than did Tempol. Ascorbate did not appear to reduce the ferryl species when Hb was treated with an excess of H_2O_2 (Figure 7a). This suggests ascorbate autoxidation, possibly catalyzed by iron released from Hb by an excess of H_2O_2 relative to the heme concentration. Such transition metal ion-catalyzed autoxidation is not expected to occur with Tempol. Since ascorbate readily reduces Tempol (the standard reduction potential of semihydroascorbic acid is 282 mV),[34] the efficacy of TOLH in reducing the ferryl species may be due not to TOLH, but rather, to an accumulation of Tempol, which is readily formed from TOLH by H_2O_2-treated Hb or Mb (data not shown).

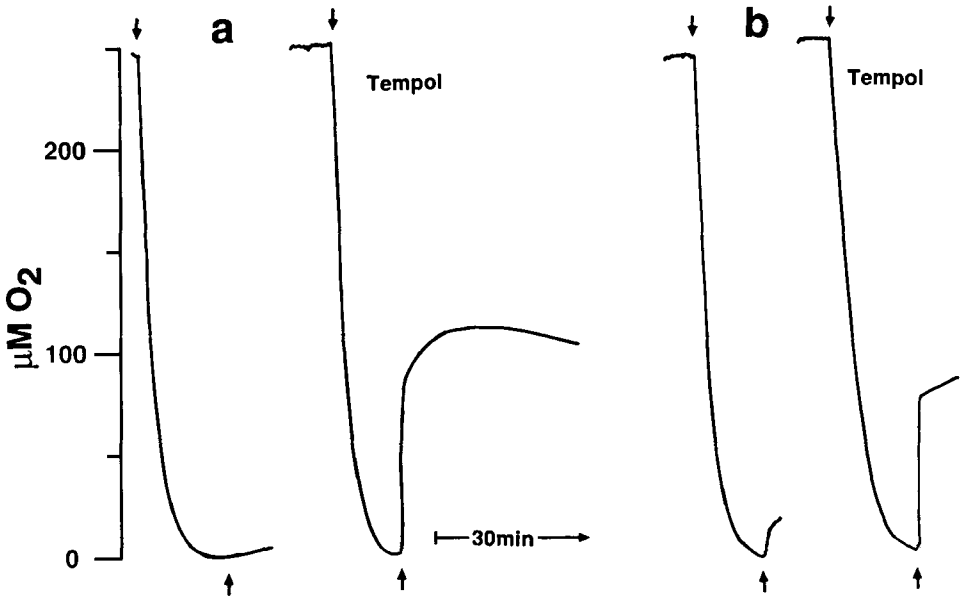


FIGURE 6 Tempol-stimulated oxygen evolution by peroxidases treated with 0.5 mM H_2O_2. (a) Horseradish peroxidase (10 U/ml), 2 mM phenol and the effect of Tempol (1 mM); (b) Lactoperoxidase (10 U/ml), 10 mM phenol and the effect of Tempol (2 mM).

TABLE IV
Tempol reduction by Mb, H_2O_2 and $NaBH_4$ and its subsequent DTPA-inhibitable reoxidation

Time (min)	Control	DTPA (2 mM)
2	300	190
3	365	200
4	425	205
5	495	215

Remaining Tempol concentrations (μM) are shown. The initial reaction mixture containing 1 mM Mb and 1 mM Tempol in PBS buffer was treated with 1 mM H_2O_2, followed by three aliquots of 2 mM $NaBH_4$, added at 15 sec intervals. The Tempol concentration was determined by ESR. The indicated times refer to the time elapsed after addition of the last borohydride aliquot.

The reduction of ferryl Hb by Tempol produced metHb with no obvious spectral features of other Hb species (Figure 7b). Tempol (1 mM) substantially inhibited the oxidation of metHb to ferryl Hb (Figure 7b vs. 7c).

Comparison of Mb oxidation by H_2O_2 and tBH

The high rate of destruction of nitroxides in the presence of GSH was used to compare the rate of Mb activation by H_2O_2 and tBH (Figure 8). With the plausible assumption

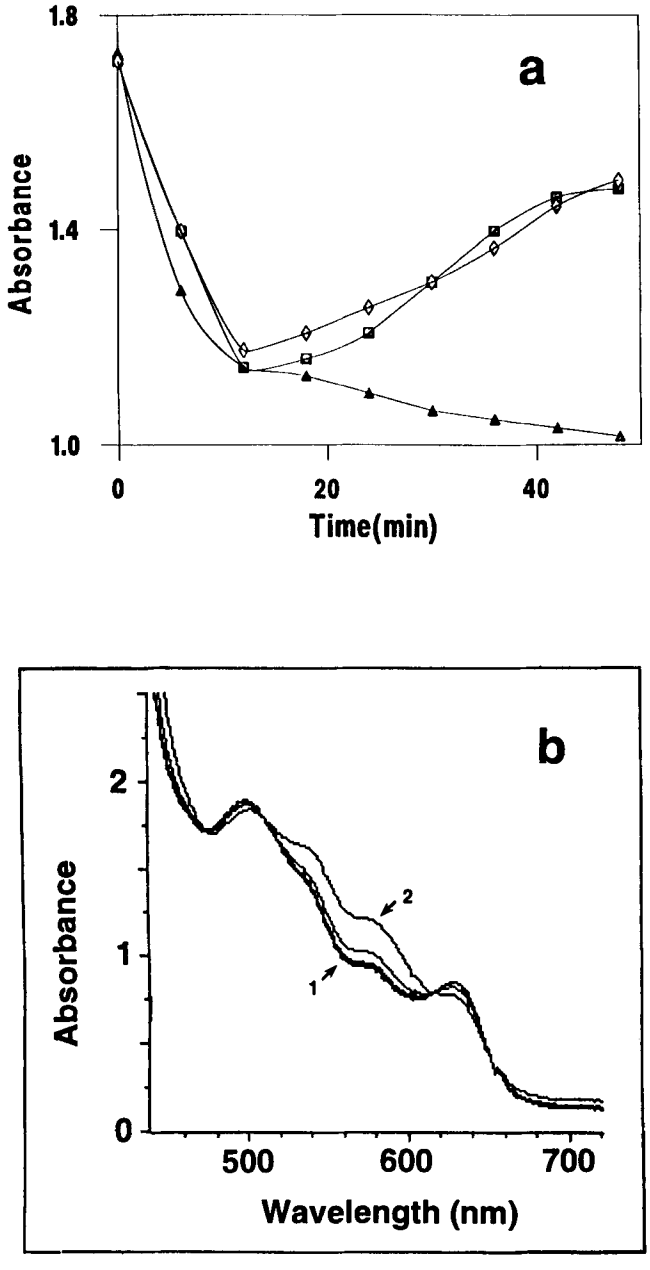


FIGURE 7 (a) Formation and decay of the ferryl Hb species in the presence of excess H_2O_2 and reducing agents. Absorbance refers to the peak at about 400 nm. Hb (7 μM) was treated with 0.5 mM H_2O_2, followed immediately by 0.5 mM of the reductant. Tempol (■); TOLH (◇); ascorbate (△); (b) formation of ferryl Hb—metHb (50 μM) was treated with 0.2 mM H_2O_2 in the presence of 1 mM Tempol—1 min between scans (spectrum 1 was H_2O_2-free control).

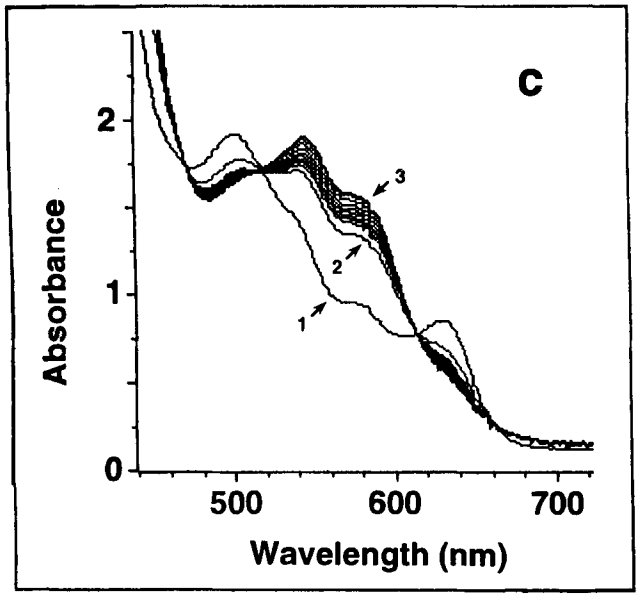


FIGURE 7 *continued.* (c) same as b, but without Tempol.

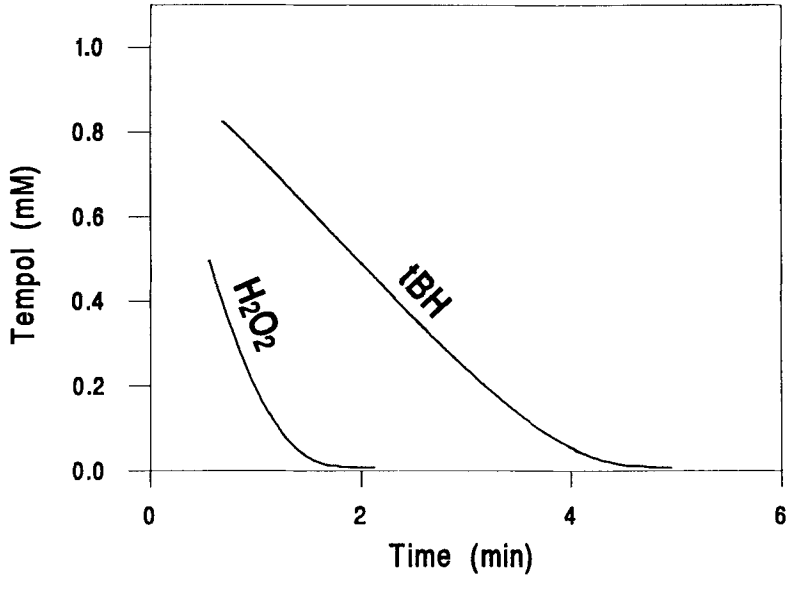


FIGURE 8 Tempol destruction by Mb/GSH and 2 mM H_2O_2 or tBH. 200 μM Mb in PBS buffer, pH 7.4, 2 mM GSH were treated with the hydroperoxide at zero time.

that the rate limiting step in the nitroxide destruction is the activation of the heme by the peroxide and that nitroxide destruction by carbon-centered radicals derived from tBH can be neglected, it can be estimated that H_2O_2 reacts about three times more rapidly with Mb than does the organic hydroperoxide. This reaction of nitroxides with ferryl Hb and Mb derived radicals appears to be useful for evaluating the activation of a variety of hydroperoxides by heme proteins (work in progress).

DISCUSSION

Nitroxides as heme-specific antioxidants

Our results indicate that nitroxides can act as novel heme-specific antioxidants that could potentially serve to decrease free radical formation by peroxidases as well as by Hb/metHb and Mb/metMb. Previously other antioxidant activities of nitroxides have been observed or inferred, including termination of lipid peroxidation chain reactions by hydrophobic nitroxides, superoxide dismutating activity and hydrogen atom donation by hydroxylamine reduction products of nitroxides.[25] It has been estimated that a significant fraction of intracellular H_2O_2 would bypass catalase and react with the much more concentrated Hb in the erythrocyte,[33] an analysis that seems appropriate only for glutathione-depleted cells, where the more active glutathione peroxidase pathway of hydroperoxide reduction would be curtailed. It was suggested that comproportionation of the resulting ferryl Hb with Hb would protect cells from peroxide activation.[33] As we have shown here, nitroxides are potent ferryl heme reductants. While they appear to be somewhat less effective than uric acid, their water solubility is greater, suggesting that they could be used to increase the scavenging of ferryl heme-derived free radicals. Nitroxide intermediates may also be involved in the effects of other reductants of ferryl heme species, e.g., desferal and DMPO,[35] which form nitroxides upon one-electron oxidation and during spin trapping reactions, respectively. It has been noted that many nitroxides, including Tempol, are highly membrane permeable so that they could conceivably be used to augment intracellular protective mechanisms.[36] Among potential applications of nitroxides as antioxidants is their use as research tools to help resolve oxidative damage mechanisms. Previously it had been documented that nitroxides act like superoxide dismutase and inhibit oxidative damage in a model system of cardiac reperfusion injury.[37] Piperidines are more effective than are pyrroline nitroxides as superoxide dismutase mimics, just as they have now been shown to be more potent inducers of the catalase activity of Hb and Mb. It had been suggested that the protective effect of nitroxides in an ischemia-reperfusion model is due to their superoxide dismutating activity.[37] Our experiments suggest that there may be an alternative explanation of their protective effect. Assuming that much of the damage associated with cardiac reperfusion injury is due to peroxides interacting with Mb,[38] it appears plausible that nitroxides may curtail the formation of reactive radicals by Mb/H_2O_2 during tissue reperfusion.

Postulated reaction mechanism

A mechanism that accounts for the stimulation of catalase activity with little

consumption of nitroxides is:

$$G\cdot Fe^{IV}\text{–}OH + Tempol \dashrightarrow GFe^{IV}\text{–}OH + TOL^+ \quad (1)$$

$$GFe^{IV}\text{–}OH + H_2O_2 \dashrightarrow GFe^{II}\text{–}OO + H_2O \quad (2)$$

$$GFe^{II}\text{–}OO + TOL^+ \dashrightarrow GFe^{III} + O_2 + Tempol \quad (3)$$

Other reactions that may be involved are:

$$TOL^+ + H_2O^- \dashrightarrow Tempol\text{–}OOH \leftrightarrow Tempol + H^+ + O_2^- \quad (4)$$

$$TOL^+ + Tempol\text{–}OOH \dashrightarrow 2\ Tempol + H^+ + O_2 \quad (5)$$

$$G\cdot Fe^{IV}\text{–}OH + 2\ Tempol\text{–}OOH \dashrightarrow GFe^{III} + 2\ Tempol + 2O_2 \quad (6)$$

where G· refers to a globin radical and Fe^{IV}–OH refers to the ferryl heme within the activated protein in the case of metHb or metMb. Reaction (4) produces the previously postulated reversible superoxide adduct with piperidine nitroxides.[39] O_2 evolution by pseudoperoxidases extending to low H_2O_2 concentrations (Figure 2) can be explained by assuming that the above reaction sequence has a much higher effective rate constant than ferric heme activation by H_2O_2. In water TOL^+ is likely to form the reversible hydroxide complex (TOL-OH, see probe schematics).[32] Since Tempol is not extensively destroyed, the reaction mechanism should allow that TOL^+ be reduced after it is formed. Possible routes of TOL^+ reduction are given by reactions (3), (4) and (5). Other postulated reactions are:

$$G\cdot Fe^{IV}\text{–}OH + GSH \dashrightarrow GFe^{IV}\text{–}OH + GS\cdot \quad (7)$$

$$TOL^+ + GS^- \dashrightarrow TOL\text{-}GS \quad (8)$$

$$Tempol + GS\cdot \dashrightarrow TOL\text{-}GS \quad (9)$$

$$TOL^+ + BH_4^- \dashrightarrow TOLH + BH_3 \quad (10)$$

$$TOL^+ + AscH^- \dashrightarrow TOLH + DHA \quad (11)$$

$$PhO\cdot + Tempol + H^+ \dashrightarrow PhOH + TOL^+ \quad (12)$$

PhOH is phenol, $AscH^-$ ascorbic acid and DHA dehydroascorbic acid. The immonium oxide cation TOL^+ decomposes in aqueous solution;[32] this decomposition could account for the modest loss of ESR signals in H_2O_2-treated Mb. Reaction (8) is analogous to the destruction of nitroxides by thiyl radicals (reaction 9).[40] The reaction of TOL^+ with GS^- (GSH pKa ~ 9), would probably be favored to that with HOO^- at an equivalent concentration of H_2O_2 (pKa ~ 12) at physiological pH, possibly accounting for the suppression of oxygen evolution by GSH. If significant Tempol-OOH formation were to occur in the presence of GSH, this would produce TOLH,[39] which seems to be ruled out by the observation that Tempol is destroyed rather than reduced. Reaction 10 explains the formation of TOLH in the presence of borohydride. The analogous reaction with ascorbate (reaction 11) appears to be sluggish, judging from the relative ineffectiveness of ascorbate in suppressing the nitroxide-induced oxygen evolution. We suggest that reaction (12) is implicated in oxygen production by hrp and lactoperoxidase. The latter enzymes, when activated by H_2O_2, do not appear to oxidize TOL directly, as judged by a lack of TOL destruction in a reaction mixture containing only hrp, H_2O_2 and GSH. However when this reaction mixture is supplemented with the

substrate phenol, a rapid destruction of Tempol is observed (data not shown), consistent with the one-electron oxidation of GSH by phenoxyl radicals, followed by reaction of the thiyl radical with Tempol (reaction 9).

Spin trapping implications

Spin trap adducts are pyrroline-type nitroxides (like PROXAD) and are likely to undergo oxidation to immonium oxene species as well. However, unlike the fully hindered nitroxides, they would be expected to undergo deprotonation to yield a stable non-paramagnetic nitrone. Therefore, the oxidation pathway we have described would destroy adducts of the commonly used nitrone spin traps and would limit the magnitudes of ESR signals of spin trap adducts detectable in hydroperoxide-treated solutions of simple heme compounds, peroxidases and pseudoperoxidases. Such spin trap oxidation could explain, at least in part, the complete suppression of DMPO-methyl radical adducts by peroxide-activated Hb (Figure 4).

Possible implications for nitric oxide catabolism

Nitric oxide forms a complex with superoxide just as nitroxides do.[39,41] It has been suggested that the homolytic scission of this complex into nitrogen dioxide and hydroxyl radicals could be a physiologically significant damage mechanism.[41] We suggest that nitric oxide could act as a one-electron reducing agent of ferryl heme proteins in a reaction analogous to (1). Being a small unchanged molecule, NO could have access to sites that are sequestered from larger or charged reductants. The nitrosonium ion (NO^+) formed in this reaction would readily hydrolyze and yield the relatively stable nitrite anion upon deprotonation.[42] Thus, not only may nitric oxide may act synergistically with superoxide to promote oxidative damage, it may also reduce compound I of peroxidases, pseudoperoxidases or catalase to compound II, being oxidized to nitrous acid in the process. Oxidation of nitric oxide by compound I of catalase may have played a role in some of the catalase effects reported by Murphy and Sies.[43] Subsequent reaction of compound II with H_2O_2 could yield the inactive enzyme form, compound III.[21] This reaction pathway with catalase could enhance pro-oxidant activities of nitric oxide.

Nitroxides as prototypes for novel drugs

Nitroxides may be useful models for the development of new drugs for suppressing or enhancing activities of heme proteins, including the suppression of the peroxidase activities. In considering their clinical potential, it is noteworthy that acute toxicity studies of nitroxides have been conducted to evaluate their possible application as contrast enhancing agents for use in magnetic resonance imaging. These studies have shown that several nitroxides are tolerated remarkably well by animals.[44] However, under some circumstances nitroxides may potentiate oxidative damage. With extracellular sources of superoxide radicals, they may stimulate mutagenic effects in susceptible cells,[45] and they are well-established radiation sensitizers.[46] These pro-oxidant effects may be offset by the benefits of their antioxidant activity, particularly under acute oxidative stress.

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