The ability of stable nitroxide radicals to detoxify hypervalent heme proteins such as ferrylmyoglobin (MbFeIV) produced in the reaction of metmyoglobin (MbFeIII) and H2O2 was evaluated by monitoring O2 evolution, H2O2 depletion, and redox changes of the heme prosthetic group. The rate of H2O2 depletion and O2 evolution catalyzed by MbFeIII was enhanced by stable nitroxides such as 4-OH-2,2,6,6-tetramethyl-piperidinooxy (TPL) in a catalytic fashion. The reduction of MbFeIV to MbFeIII was the rate-limiting step. Excess TPL aminated, and the initial concentration of TPL was reduced. The reaction between H2O2 and metmyoglobin has been shown to produce a two-electron oxidation product of MbFeIII and O2, which disproportionate to MbFeIII and O2 which disproportionate to MbFeIII and O2 (1). Such reactions were noted for hypervalent heme proteins such as ferrylmyoglobin (6, 7), FeIV=O, which is denoted as FeIV throughout the text. Although the lifetime of the globin radical has been estimated to be in the range of 50–280 ms, the lifetime of the oxoferryl species ranged between minutes and hours (8, 9). Both the globin radical and the oxoferryl species are chemically reactive and have access to components in the bulk solution and initiate free radical-mediated reactions that could result in biologic damage (10) by chemical oxidation reactions (11). Considerable efforts have been made to characterize the globin radical. Although earlier studies suggested the globin radical to be a tyrosine phenoxyl radical (12), recent high resolution EPR-spin trapping studies provide strong evidence to identify the amino acid radical on the globin to be centered on tryptophan (13). The globin radical has been proposed to mediate oxidation, peroxidation, and epoxidation of substrates and induce biologic damage (10, 14). The oxoferryl moiety on the other hand has also been implicated in oxidation of cellular components and xenobiotics (9).

The decay of the globin radical proceeds through mechanisms that are not conclusively identified. In the absence of other reductants, the ferryl moiety and possibly also the globin radical can oxidize another H2O2 molecule to O2 and consequently yield molecular O2 (Reactions 2 and 3). Reactions of hematin/hydroperoxide to generate alkoxyl radicals have been also reported (15). There is, however, no direct evidence for H2O2-induced decay of the globin radical (Equation 2), and an alternative pathway might involve an intramolecular reduction of globin radical to yield the perferrylMb (MbFeV). Whatever is the mechanism of globin radical decay, MbFeIV can be reduced to MbFeIII by reacting with H2O2 (Equation 3):

\[
\text{MbFeIV} + \text{H}_2\text{O}_2 \rightarrow \text{MbFeIII} + \text{O}_2^+ + 2\text{H}^+
\]

One oxidation equivalent has been shown to reside on the globin as a transient radical located on an aromatic amino acid residue (3, 4). The second and longer lived oxidizing equivalent was shown to be the oxoferryl complex (6, 7), FeIV=O, which is denoted as FeIV throughout the text. Although the lifetime of the globin radical has been estimated to be in the range of 50–280 ms, the lifetime of the oxoferryl species ranged between minutes and hours (8, 9). Both the globin radical and the oxoferryl species are chemically reactive and have access to components in the bulk solution and initiate free radical-mediated reactions that could result in biologic damage (10) by chemical oxidation reactions (11). Considerable efforts have been made to characterize the globin radical. Although earlier studies suggested the globin radical to be a tyrosine phenoxyl radical (12), recent high resolution EPR-spin trapping studies provide strong evidence to identify the amino acid radical on the globin to be centered on tryptophan (13). The globin radical has been proposed to mediate oxidation, peroxidation, and epoxidation of substrates and induce biologic damage (10, 14). The oxoferryl moiety on the other hand has also been implicated in oxidation of cellular components and xenobiotics (9).

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Nitroxide-facilitated Catalase Activity of Myoglobin

**RESULTS**

**General**—The effect of stable nitroxides on the kinetics of MbFe$^{III}$ reaction with H$_2$O$_2$ was studied by measuring O$_2$ evolution, depletion of H$_2$O$_2$, and redox transitions between various oxidation states of the heme prosthetic group in MbFe$^{III}$. All reactions were carried out at 25 °C or at room temperature in 50 mM phosphate-buffered solutions, pH 7.4, containing 50 μM DTPA to minimize undesired reactions catalyzed by adventitious transition metal ions.

**Dismutation of H$_2$O$_2$ and Evolution of O$_2$**—Fig. 1A shows O$_2$ evolution as a function of time at 25 °C, in 50 mM phosphate buffer, pH 7.4, containing MbFe$^{III}$ and H$_2$O$_2$ in the absence and the presence of 100 μM TPL. A steady rate of O$_2$ evolution was established with additional 50-fold excess of H$_2$O$_2$ to solutions containing 25 μM MbFe$^{III}$. The rate linearly depended on MbFe$^{III}$, exhibiting a turnover number of 0.15 ± 0.04 mmol O$_2$·min$^{-1}$·μmol MbFe$^{III}$$^{-1}$.

**MATERIALS AND METHODS**

**Chemicals**—Mb (ferric form, horse heart, type III) was purchased from Sigma. The stable nitroxides 4-OH-2,2,6,6-tetramethylpiperidine-N-oxyl (TPO), 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (TPA), and TPO were purchased from Aldrich. 4-Dihydroxy-2,2,6,6-tetramethylpiperidine-1-hydroxyamine (TPL-H) was prepared by catalytic reduction of TPL in anoxic solution bubbled with H$_2$ over PtO$_2$, CuZn-Superoxide dismutase and NADH were purchased from Boehringer Mannheim. H$_2$O$_2$ was obtained from Fisher, and the solution was standardized using an ε$_{240}$ of 43.6 M$^{-1}$·cm$^{-1}$.
turnover number about 4–6-fold. This rate persisted until H$_2$O$_2$ was consumed and was not sensitive to the presence of superoxide dismutase (data not shown).

The decay of H$_2$O$_2$ concentration as a function of time was monitored by sampling aliquots of the reaction mixture and estimating [H$_2$O$_2$] by the iodometric assay (36). As seen in Fig. 1A, the rate of [H$_2$O$_2$] decay in the presence of MbFe$^{III}$ increased upon the addition of TPL. Comparing the rates of O$_2$ evolution and H$_2$O$_2$ decay yields a ratio of 0.5 ± 0.05, which indicates a 1:2 stoichiometry as anticipated for a genuine catalase-like dismutation reaction of H$_2$O$_2$ (37). Cyanide (1 mM) completely inhibited O$_2$ evolution, and H$_2$O$_2$ depletion mediated by MbFe$^{III}$ both in the absence and the presence of nitroxide.

The rate of H$_2$O$_2$ dismutation depended on the concentrations of Mb, H$_2$O$_2$, and nitroxide. The rate increased linearly with the increase in [MbFe$^{III}$]. However, an increase in [H$_2$O$_2$] beyond 10:1 [H$_2$O$_2$]/[MbFe$^{III}$] or in [nitroxide] beyond 2:1 [nitroxide]/[MbFe$^{III}$] resulted in a slight increase of the rate of dismutation. The enhancing effect of the nitroxide was practically the same over the pH range 5.5–8 (data not shown).

Superoxide dismutase had no significant effect on O$_2$ evolution or on H$_2$O$_2$ depletion induced by TPL + Mb under these conditions. The three-electron reduced form of TPL, namely the cyclic amine, did not enhance O$_2$ evolution. The cyclic hydroxylamine TPL-H progressively enhanced oxygen evolution mediated by MbFe$^{III}$, concomitant with the hydroxylamine’s rapid oxidation to TPL (data not shown).

The predominant heme iron species—The distribution of the heme iron among its various oxidation states was determined by recording the uv-visible spectra as a function of time and

**Fig. 1.** TPL effect on O$_2$ evolution, H$_2$O$_2$ depletion, and MbFe$^{IV}$ reduction during H$_2$O$_2$ dismutation. The dismutation of H$_2$O$_2$ at room temperature, mediated by 25 μM MbFe$^{III}$ in 50 mM phosphate buffer, pH 7.4, containing 50 μM DTPA and 1 mM H$_2$O$_2$ in the absence and in the presence of 100 μM TPL (the arrows show when the reagents were added) was followed by monitoring A, oxygen evolution measured by O$_2$ selective electrode. B, H$_2$O$_2$ depletion was measured spectrophotometrically at 350 nm using the iodometric assay with (open circles) and without (closed circles) TPL. C, conversion of MbFe$^{IV}$ into MbFe$^{III}$ measured spectrophotometrically at 556 nm.

**Fig. 2.** The effect of [TPL] and [MbFe$^{III}$] on the rate of oxygen evolution during H$_2$O$_2$ dismutation. The evolution of oxygen during H$_2$O$_2$ dismutation at room temperature in 50 mM phosphate buffer, pH 7.4, containing 50 μM DTPA and 1 mM H$_2$O$_2$ was followed by O$_2$ selective electrode and studied using 25 μM MbFe$^{III}$ with various concentrations of TPL (A) or various concentrations of MbFe$^{III}$ in the absence (circles) and in the presence (squares) of TPL at a constant concentrations ratio [TPL]/[MbFe$^{III}$] = 4 (B).
deriving the respective concentrations of MbFe^{III} and MbFe^{IV} according to Whitburn’s algorithms (38). Upon the addition of a large excess of H_2O_2, as indicated by the arrow, the absorption spectrum of MbFe^{III} was replaced by that of MbFe^{IV}; however, TPL enhanced the rate of H_2O_2 depletion (Fig. 1B) associated with a corresponding restoration of MbFe^{III} (Fig. 1C).

The Persistence of TPL—The complete reaction mixture containing MbFe^{III}, H_2O_2 and TPL was analyzed for the TPL signal using EPR spectroscopy. In the absence of reducing agent, such as NADH, the nitroxide persisted without any appreciable spin loss throughout the complete MbFe^{III}-induced dismutation of a large excess of H_2O_2 (data not shown). No change of the shape of the EPR spectrum, indicative of any spin-spin interaction between TPL and the heme iron, was observed throughout the course of the reaction. The persistence of TPL concentration indicated that it acts catalytically as a self-replenishing reagent in the dismutation of H_2O_2 with a concomitant O_2 evolution.

The Catalytic Species—TPL accelerates the catalase-like dismutation of H_2O_2 in a reaction in which TPL is not consumed in the overall process. Furthermore, there is no stoichiometric relationship between TPL and the quantity of the substrate altered, all of which indicates TPL functions as a true catalyst. The observed stoichiometry of 1:2 between O_2 evolution accompanying H_2O_2 decay mediated by MbFe^{III} with nitroxide provides further evidence for the genuine catalytic nature of the process. Similar catalytic decomposition facilitated by manganese/bicarbonate has been observed in earlier studies (37). Evidently such catalysis implies a time invariance of the concentration of MbFe^{IV} and of the nitroxide (see Fig. 1).

The rate of H_2O_2 dismutation induced by MbFe^{III} increased with the increase of either [MbFe^{III}] and/or [TPL]. Fig. 2A shows that in the presence of excess H_2O_2 and with constant [MbFe^{III}] the rate of O_2 evolution increased with the increase of [TPL]. However, beyond [TPL]/[MbFe^{III}] ratio of 2–3, the rate of dismutation increased very little with an increase of [TPL]. A similar result was obtained with various concentrations of MbFe^{III}. Fig. 2B shows that varying [MbFe^{III}] while maintaining [TPL]/[MbFe^{III}] ratio at 4 yielded a straight line. The dependence of the rate of H_2O_2 decomposition on [TPL] demon-
stratred a similar behavior, where almost a maximal rate of \( \text{H}_2\text{O}_2 \) decay is achieved beyond [TPL]/[MbFe\(^{III}\)] = 2–3.

**NADH Effect on \( \text{O}_2 \) Evolution**—The two electron-deficient sites formed in MbFe\(^{III}\) upon reaction with \( \text{H}_2\text{O}_2 \) are strong oxidants and can potentially oxidize many substrates, including TPL yielding oxoammonium cation TPL\(^+\) (25, 27). In order to test for the intermediacy of TPL\(^+\) in facilitating the catalase-like activity of the MbFe\(^{III}\), the effect of NADH has been studied. Upon the addition of NADH to the oxygraph chamber containing MbFe\(^{III}\) and \( \text{H}_2\text{O}_2 \), the evolution of oxygen was replaced by \( \text{O}_2 \) consumption in the absence of TPL. The rate of \( \text{O}_2 \) consumption was significantly greater than the original rate of \( \text{O}_2 \) evolution and lasted until all NADH was oxidized; whereupon, \( \text{O}_2 \) evolution was re-established.

The effect of NADH on \( \text{O}_2 \) evolution by MbFe\(^{III}\) and \( \text{H}_2\text{O}_2 \) in the presence of TPL depended on the relative concentrations of TPL and NADH. At [NADH] < [TPL], oxygen evolution fully stopped, and \( \text{O}_2 \) did not change until NADH was consumed and \( \text{O}_2 \) evolution was resumed (Fig. 3A). However, at higher NADH concentrations as when [NADH] > [TPL], \( \text{O}_2 \) was consumed at a rate comparable with that observed in the absence of nitroxide. This effect was also transient, and with the progressive depletion of NADH, the consumption of oxygen ended, and later evolution of \( \text{O}_2 \) was gradually resumed to the original rate. Successive transient inhibitions of \( \text{O}_2 \) evolution could be demonstrated by fractionated addition of NADH aliquots, and the inhibition duration increased with the increase of [NADH] added (data not shown). Control experiments showed that the two electron oxidation product of NADH, namely NAD\(^+\), had no effect on the rate of \( \text{O}_2 \) evolution (data not shown).

**NADH Effect on \( \text{H}_2\text{O}_2 \) Decay**—Upon addition of NADH (200 \( \mu \text{M} \)) to a reaction mixture containing \( \text{H}_2\text{O}_2 \), MbFe\(^{III}\), and TPL, inhibition in the rate of \( \text{H}_2\text{O}_2 \) depletion was noted (Fig. 3B). No significant depletion of \( \text{H}_2\text{O}_2 \) was noted for the first 10 min when NADH (200 \( \mu \text{M} \)) was included in the reaction mixture initially (open circles); subsequently \( \text{H}_2\text{O}_2 \) depletion was re-established. The addition of NADH (200 \( \mu \text{M} \)) 20 min after the initiation of the reaction (open squares) resulted in the inhibition of \( \text{H}_2\text{O}_2 \) depletion followed by a 20–30% inhibition compared with that observed in the absence of NADH (closed squares).

**NADH Effect on TPL Level**—The effect of NADH on the level of TPL was studied by following the intensity of the EPR signal of the nitroxide throughout the course of the reaction in a sample containing MbFe\(^{III}\) and \( \text{H}_2\text{O}_2 \). Upon the addition of NADH, the TPL level progressively decreased for several minutes, and when NADH was consumed the EPR signal of [TPL] increased, achieving its original level (Fig. 3C). The restoration of EPR signal of TPL paralleled the restoration of the original level of \( \text{O}_2 \) evolution. The transient decrease of EPR signal resulted from the reduction of TPL to its respective hydroxylamine, TPL-H, as this EPR-silent form was oxidizable to TPL like activity of the MbFe\(^{III}\), the effect of NADH has been studied. Upon the addition of NADH to the oxygraph chamber containing MbFe\(^{III}\) and \( \text{H}_2\text{O}_2 \), the evolution of oxygen was replaced by \( \text{O}_2 \) consumption in the absence of TPL. The rate of \( \text{O}_2 \) consumption was significantly greater than the original rate of \( \text{O}_2 \) evolution and lasted until all NADH was oxidized; whereupon, \( \text{O}_2 \) evolution was re-established.

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**NADH Oxidation**—The oxidation of NADH by the MbFe\(^{III}\) (25 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (1 \( \text{mM} \)) in the presence and the absence of TPL was followed spectrophotometrically at 340 nm, and the results are shown in Fig. 3D. The presence of TPL enhanced the rate of oxidation of NADH.

**The Kinetics of MbFe\(^{IV}\) Reduction by TPL**—The effect of TPL on the rate of \( \text{O}_2 \) evolution seemed to depend on its direct reaction with the hypervalent iron. To study the kinetics of MbFe\(^{IV}\) reaction with TPL, its reduction to MbFe\(^{III}\) was spectrophotometrically followed. MbFe\(^{IV}\) was prepared by treating

10 \( \mu \text{M} \) MbFe\(^{III}\) with an excess of \( \text{H}_2\text{O}_2 \) in 50 \( \text{mM} \) phosphate buffer, pH 7, followed by 500 units/ml catalase to remove unreacted \( \text{H}_2\text{O}_2 \). In the absence of nitroxide, MbFe\(^{IV}\) decayed slowly to MbFe\(^{III}\); however, TPL facilitated this reduction. To study the reaction kinetics, the change in OD\(_{408\text{nm}}\) was monitored and \( \Delta \text{OD} = \text{OD}_0 - \text{OD}_t \) was determined, where \( \text{OD}_0 \) represented the constant absorbance achieved upon completion of the reaction.

The decay of \( \Delta \text{OD} \) exhibited a single exponential kinetics irrespective of the nitroxide concentration (see Fig. 4A) even at [TPL] < [MbFe\(^{IV}\)], and the first order reaction rate constant \( k_{\text{obs}} \) was evaluated. The reaction was conducted at various concentrations of TPL, and the dependence of \( k_{\text{obs}} \) on [TPL], which shows a biphasic character, is presented in Fig. 4. The value of \( k_{\text{obs}} \) linearly increased with the increase of [TPL]; however, at [TPL] < 100 \( \mu \text{M} \), the dependence exhibited a downward curvature. A similar dependence of \( k_{\text{obs}} \) on [nitroxide] was obtained when TPL was replaced by TPA or TPO as seen in Fig. 4.

**The Reaction of \( \text{H}_2\text{O}_2 \) with TPL**—The initial step of superoxide dismutation catalyzed by TPL involves the generation of TPL\(^+\) and \( \text{H}_2\text{O}_2 \) (27). In order to study the reverse reaction, TPL\(^+\) was generated electrochemically and added into an oxygen chamber containing deaerated, continually stirred, buffered solution of \( \text{H}_2\text{O}_2 \). An instantaneous evolution of \( \text{O}_2 \) was observed upon the addition of TPL\(^+\) (data not shown), which indicates that with large excess of \( \text{H}_2\text{O}_2 \) the reverse reaction does indeed take place.

**Cellular Cytoprotection**—The protective effect of the combination of MbFe\(^{III}\) and TPL against \( \text{H}_2\text{O}_2 \) injury to mammalian

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**Fig. 4. Nitroxide concentration effect on the kinetics of MbFe\(^{IV}\) reduction.** The reduction of MbFe\(^{IV}\) to MbFe\(^{III}\) in 50 \( \text{mM} \) phosphate buffer, pH 7, containing 10 \( \mu \text{M} \) MbFe\(^{III}\) and 5 \( \text{mM} \) \( \text{H}_2\text{O}_2 \) following the addition of 500 units/ml catalase and various concentrations of TPL (circles), TPO (triangles), and TPA (squares) was monitored spectrophotometrically at 408 nm. A, a typical decay curve of MbFe\(^{IV}\) in the presence of 400 \( \mu \text{M} \) TPL obeying first-order kinetics. B, the dependence of the pseudo-first-order rate constant \( k_{\text{obs}} \), calculated from the decay curve, on [nitroxide]. The straight lines represent linear regression analysis of the experimental points at the linear portion of each biphasic curve, by which the values of \( k_a \) (slope) and \( k_b \) (intercept) were determined (see text).
cells was tested. Chinese hamster V79 cells were exposed to 750 μM H₂O₂ for 1 h in the absence or the presence of 50 μM MbFeIII or 50 μM TPL alone or their combination, and H₂O₂ cytotoxicity was assessed (28, 29). Incubation with H₂O₂ alone caused a 98% loss in cell survival, and modest protection was provided by MbFeIII, which can be associated with its pseudo-peroxidase activity; whereas, TPL alone was without effect. However, the combination of MbFeIII and TPL protected the cells from H₂O₂ (Fig. 5). The large synergistic activity of TPL on the protective effect of MbFeIII is attributed to the facilitation of H₂O₂ dismutation.

**DISCUSSION**

The pseudo-peroxidase activity of heme protein (3) is known to involve the intermediacy of MbFeIV (6), a strong oxidant capable of inflicting significant damage by oxidizing a number of biologic targets (10, 17, 19). Therefore, its detoxification is being attempted using a variety of antioxidants (9). Generally, reagents effective in reducing MbFeIV to MbFeIII operate in a stoichiometric manner. Conversely, nitroxide radicals, which shuttle among three oxidation states can detoxify hypervalent stoichiometric manner. Conversely, nitroxide radicals, which shuttle among three oxidation states can detoxify hypervalent oxygen and depletes its level.

**Kinetic Considerations**—The predominance of MbFeIV over MbFeIII shows that k₁ ≫ k₃, thus indicating that the reduction of MbFeIV by H₂O₂ (Reaction 3) is the rate-limiting step in the catalytic cycle of H₂O₂ dismutation. The rates of O₂ evolution and H₂O₂ depletion increased about 4–6-fold in the presence of TPL (see Fig. 1, A and B) most likely due to a reduction of MbFeIV by nitroxide, yielding the oxoammonium cation TPL⁺, which can oxidize another H₂O₂ through Reaction 5,

\[
\text{MbFeIV + TPL} \rightarrow \text{MbFeIII + TPL}^+ \\
\text{REACTION 4}
\]

or generate O₂ by oxidizing O₇⁻ through Reaction 6.

\[
O_7^- + \text{TPL}^+ \rightarrow O_2 + \text{TPL} \\
\text{REACTION 6}
\]

Considering the values of 1.2 × 10⁶, 3.2 × 10⁷, and 1.5 × 10¹⁰ M⁻¹ s⁻¹ reported for k₅, k₆, and k₇ (39, 40), respectively, the rate of oxygen evolution with an excess of H₂O₂ would be the same whether TPL⁺ disappears via Reaction 5 or 6. The higher rate of H₂O₂ dismutation in the presence of TPL shows that k₄ ≫ k₅. The time invariance of the concentrations of MbFeIV and TPL provides direct evidence that both reagents act as true catalysts. The intensity of the EPR signal of TPL did not change during the course of the reaction because the steady-state concentration of the oxoammonium cation TPL⁺ was below detection. Nevertheless, TPL⁺ was the species mediating the depletion of H₂O₂ because in the presence of NADH the EPR signal of TPL was lost due to two-electron reduction of TPL⁺ to the respective cyclic hydroxylamine TPL-H via Reaction 7.

\[
\text{NADH + TPL}^+ \rightarrow \text{NAD}^- + \text{TPL-H} \\
\text{REACTION 7}
\]

In the presence of NADH alone, the evolution of oxygen was replaced by O₂ consumption because MbFeIV was reduced via Reaction 8,

\[
\text{MbFeIV + NADH} \rightarrow \text{MbFeIII + H}^+ + \text{NAD}^+ \\
\text{REACTION 8}
\]

yielding NAD⁺, which is capable of reducing oxygen to O₂. Even at [NADH] < [H₂O₂], Reaction 8 prevailed rather than Reaction 3, indicating that k₅ ≫ k₆. This conclusion apparently accounts for the observation that upon the addition of NADH the rate of O₂ consumption was faster than that of O₂ evolution and the finding that even low [NADH] fully blocked O₂ evolution in the presence of TPL. In this case, provided that k₇[NADH] > k₅[H₂O₂], the oxoammonium cation TPL⁺ is reduced by NADH rather than by H₂O₂. The situation is different for a vast excess of NADH where k₇[NADH] > k₅[TPL] > k₆[H₂O₂] and MbFeIV reacts with NADH rather than with TPL or H₂O₂. Under such conditions the nitroxide does not play any role; whereas NAD⁺ which is formed by MbFeIII reduces molecular oxygen and depletes its level.

Scheme 2 displays the processes underlying the heme-induced dismutation of H₂O₂ in the presence and the absence of nitroxide. Competing with H₂O₂ for MbFeIV (see Scheme 2), the

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**Scheme 2**

![Scheme 2](https://example.com/scheme2.png)

**REACTION 4**

\[
\text{MbFeIV + TPL} \rightarrow \text{MbFeIII + TPL}^+ \\
\]

**REACTION 5**

\[
\text{H}_2\text{O}_2 + \text{TPL}^+ \rightarrow \text{O}_2^+ + \text{TPL} + 2\text{H}^- \\
\]

**REACTION 6**

\[
\text{O}_7^- + \text{TPL}^+ \rightarrow \text{O}_2 + \text{TPL} \\
\]

**REACTION 7**

\[
\text{NADH + TPL}^+ \rightarrow \text{NAD}^- + \text{TPL-H} \\
\]

**REACTION 8**

\[
\text{MbFeIV + NADH} \rightarrow \text{MbFeIII + H}^+ + \text{NAD}^+ \\
\]
Nitroxide accelerates the replenishment of MbFe$^{III}$ and consequently the entire cycle of reactions.

Mechanism of Ferryl Reduction—The combined presence of MbFe$^{IV}$, H$_2$O$_2$, and nitroxide did not result in any detectable modification of the EPR spectrum of the nitroxide or the absorption spectrum of the heme iron. Nevertheless, the biphase behavior of the reduction of MbFe$^{IV}$ in the presence of increasing concentration of nitroxides (Fig. 4B) suggests that ferrylmyoglobin exists in two forms, which decay via Reactions 9 and 10. Such behavior was also observed in the reaction between MbFe$^{IV}$ and Trolox (41).

\[
\text{Turnover number} = (\text{rate of } H_2O_2 \text{ decay})/[\text{MbFe}^{IV}] = k_{obs} = k_9 + k_4 \cdot [\text{TPL}] \quad (\text{Eq. 4})
\]

The results displayed in Fig. 2B indicating a turnover number of 0.65 min$^{-1}$ for $O_2$ with 0.4 mM TPL can be compared with $k_{obs} = 1.26$ min$^{-1}$ seen in Fig. 4 for similar experimental conditions.

In conclusion, the present results further elucidate the role played by nitroxides in detoxifying hypervalent metals and enhancing the catalase-like activity of heme proteins. It is possible that in addition to the previously recognized modes of nitroxide action, these mechanisms underlie the protective biological activity of nitroxide antioxidants. Furthermore, nitroxides having a wide variety of analogues with different ring structures and substituents, which confer them with differences in charge and redox behavior might be helpful tools in elucidating the redox reactions of hypervalent heme species. In addition, covalent linking of nitroxides to proteins at different sites is feasible (42) and would provide an interesting chemical system to extend the study of the redox reactions between nitroxides and myoglobin.

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J. Biol. Chem. 1996, 271:26018-26025.
doi: 10.1074/jbc.271.42.26018

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