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HYDROXYL AND ALKOXYL RADICAL PRODUCTION BY OXIDATION PRODUCTS OF METMYOGLOBIN

ROLF J. MEHLHORN and JEANNETTE GOMEZ

Energy and Environment Division, Lawrence Berkeley Laboratory, Berkeley, CA 94720, USA

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The one-electron oxidation of a reduced nitroxide (2,2,6,6-tetramethyl-1,4-dihydroxypiperidine, TOLH), detected by ESR, was used to resolve and quantify oxidants arising from the reaction of heme proteins with hydroperoxides, including chelatable iron released subsequent to oxidative cleavage of the porphyrin ring. Released iron was distinguished from protein radicals and ferryl heme by analyzing TOLH oxidation in the presence of different chelating agents. Metmyoglobin (metMb) treatment with one mole of H_2O_2 per mole of heme produced protein-bound oxidants that oxidized about two molecules of TOLH per heme. Some of the oxidizing species responsible for TOLH oxidation were highly persistent ($t_{1/2}$ for the decay was 3 hrs at 25°C). Iron release, metMb bleaching and the catalysis of Fenton-type chemistry were compared in metMb solutions treated with *tert*-butyl hydroperoxide (tBH). Iron release required about five-fold higher hydroperoxide concentrations than did metMb bleaching. Alkoxyl and methyl radical production was catalyzed by iron released from metMb but not by protein-bound iron in oxidized metMb solutions treated with tBH and ascorbic acid. The results suggest that ascorbate-mediated hydroxyl and alkoxyl radical production by hydroperoxide-treated metMb is due to released iron and that the protein-bound non-heme iron that arises during bleaching is at most a weak Fenton reagent.

KEY WORDS: Fenton reaction, ferryl heme, persistence, protein free radicals, heme iron release, alkoxyl radicals, porphyrin, oxidative cleavage.

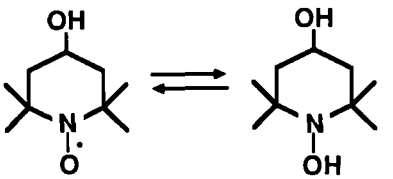
INTRODUCTION

When heme proteins such as hemoglobin (Hb) and myoglobin (Mb) are exposed to hydroperoxides protein free radicals are formed and free iron can be detected in the reaction mixture.[1-6] Hb and Mb oxidation products promote lipid peroxidation,[2-6] protein modification[7-13] and other deleterious reactions.[2] The molecular details of these reactions are complex and have yet to be fully resolved.[14] In particular, the relative roles of protein-bound oxidants and released iron in promoting or suppressing these reactions remain to be elucidated. The reactions of heme proteins with a large excess of 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.[15] Because of their abundance in animals, Hb and Mb may play significant roles in oxidant damage, including chronic free radical exposure resulting from the production of low levels of hydrogen peroxide during normal physiological processes,[16] or from the redox cycling of quinonoids, possibly including

compounds present in cigarette smoke.[17] This study was undertaken to improve our understanding of the role of heme-bound oxidants and free iron in catalyzing free radical reactions. An ESR assay that resolves protein-bound oxidants from chelatable iron and that does not require a separation of proteins from the assay medium was developed for this purpose. By comparing iron release, bleaching and the formation of free radicals by a Fenton-type mechanism in metMb solutions after various hydroperoxide treatments we have resolved some of the effects of free iron and protein-bound iron species in catalyzing free radical reactions.

MATERIALS AND METHODS

MetMb. Myoglobin (Mb) from horse skeletal muscle, tert-butyl hydroperoxide (tBH), diethylenetriaminepentaacetic acid; free acid (DTPA), ethylenediaminetetraacetic acid; disodium salt (EDTA), ferrous sulfate were obtained from Sigma Chemical Co. Analysis of the Mb by visible spectroscopy, in conjunction with ferricyanide oxidation and dithionite reduction, showed that the Mb was largely (ca 93%) in the metMb form and that the content of oxyMb was at most 7%. Hydrogen peroxide was from Mallinckrodt. Ascorbic acid and 5,5-dimethylpyrroline-N-oxide (DMPO) were purchased from Aldrich Chemical Co. Chelex 100 was from Biorad. The reduced nitroxide, 2,2,6,6-tetramethyl-1,4-dihydroxypiperidine (TOLH) was synthesized.[18] Nitroxides structures are shown below.



TEMPOL TOLH

Reagents. All experiments were performed at room temperature (22 to 25°C). MetMb solutions were diluted from either 1 mM or 4 mM stock solutions. Reaction mixtures were in 150 mM NaCl, 10 mM sodium phosphate (PBS) buffer, pH 7.4, which had been passed twice through a Chelex 100 column. Some of the results were confirmed using Chelex 100-treated 50 mM acetate buffer, pH 7.0.

ESR spectroscopy. The measurements were performed with a Varian E109E (X-band) spectrometer. Instrument settings were: modulation amplitude 0.125 mT, microwave power 10 mW, scan range 10 mT. Iron quantification experiments were carried out in acid-washed capillaries (50 μl) to remove transition metals.

Spectrophotometry. MetMb absorption spectra were obtained with a Shimadzu UV-160U spectrophotometer interfaced with an IBM Model 70 computer and using the PC160 Plus Personal Spectroscopy Software. Decreases in the peak absorbance of the Soret region (ca 410 nm) were used to analyze metMb bleaching.

Hydroperoxide treatments used for iron release, bleaching and spin trapping experiments. Samples (containing 30 μM metMb and 1 mM DTPA) were prepared in sodium phosphate buffer, pH 7.4, and treated with various concentrations of tBH (30 μM–2 mM). The hydroperoxide was added in discrete portions to the metMb solution, incubated for 10 minutes and an aliquot was removed before adding the

next portion of tBH. The samples were incubated with tBH for at least 24 hours before assaying them for released iron or bleaching. For comparison of hydroperoxide treatments under anaerobic and aerobic incubations, the anaerobic samples were flushed with nitrogen and treated with H_2O_2 in a Clark-type oxygen polarograph; the oxygen tension was monitored throughout the experiment to ensure that the samples remained anaerobic.

RESULTS

Fe-EDTA Calibration Curves

Oxidants were analyzed by ESR using the oxidation of the probe TOLH to the stable free radical Tempol. TOLH does not react appreciably with hydrogen peroxide or molecular oxygen in transition metal ion-free buffers at neutral or acidic pH but is reactive with many free radical oxidants. In an earlier study, we used the facile oxidation of TOLH by phenoxyl radicals to analyze free radical processes in peroxidase systems.[19] Whereas the direct oxidation of TOLH by horseradish peroxidase is sluggish and is greatly stimulated by phenol, the direct oxidation by peroxide-treated metMb and metHb is a facile process in the absence of phenols. The one-electron oxidation of hydroxylamines like TOLH is also facilitated by transition metal ions as previously reported in a study of the reaction of superoxide radicals with nitroxide-derived hydroxylamines.[20]

Ferrous iron readily autoxidizes in aerobic solution and ferric iron is not soluble at neutral pH in the absence of chelating agents. Therefore, in studies of iron release from heme proteins, care should be taken to control for precipitation of iron. To help analyze the fate of released iron, we performed a recovery experiment. MetMb (1 mM) in acetate buffer (50 mM, pH 7) or in PBS buffer was analyzed for the persistence of added iron. The control was treated with 1 mM EDTA, followed by 1 mM ferrous sulfate (ferrous sulfate stock solutions were used to facilitate accurate quantification and to simulate iron persistence under conditions where either the ferric or ferrous species could be protein-bound). The treated sample was incubated with 1 mM ferrous sulfate for various times followed by addition of 1 mM EDTA. Even after 24 hour incubation of the sample containing iron without EDTA, more than 80% of the iron was detectable as the EDTA complex by ESR, suggesting that metMb aids in solubilizing released iron. In most of the experients reported here, much lower concentrations of the heme proteins were used. Moreover, iron precipitation in protein solutions may be accelerated in the presence of hydroperoxides. Therefore the chelating agents DTPA and EDTA were present during the incubations of heme proteins with hydroperoxides to ensure that all of the released iron would remain in solution.

Fe-EDTA oxidized the reduced nitroxide TOLH, whereas Fe-DTPA was virtually inert towards TOLH. The oxidation of TOLH by Fe-EDTA in aerobic solution, measured as the increase in the magnitude of the ESR signal of Tempol, was a catalytic process in that a linear rate of oxidation was observed, even when the extent of TOLH oxidation greatly exceeded the concentration of Fe-EDTA. The rates of TOLH oxidation by various concentrations of Fe-EDTA were used to obtain a calibration curve for quantifying free iron (Figure 1). Linear regression analysis, excluding the data at the highest and lowest concentrations of Fe-EDTA, yielded the

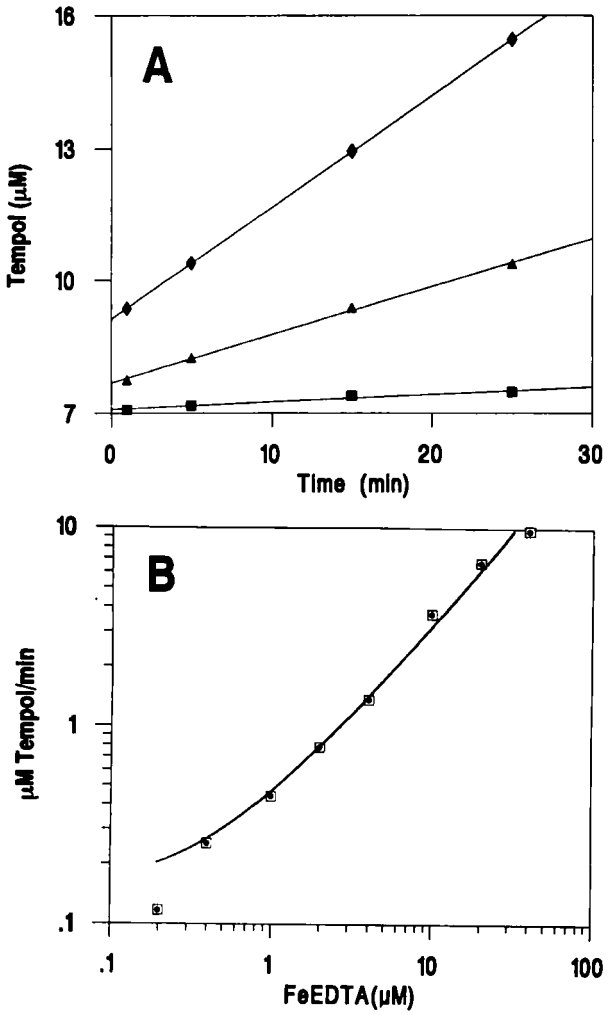


Figure 1 ESR assay for Fe-EDTA. (A) Low concentrations of Fe-EDTA in 10 mM of sodium phosphate buffer, pH 7.4 were treated with 300 μM of TOLH and the rate of Tempol formation was measured by ESR. These and similar data for intermediate and high concentration ranges were fitted by least squares, and the slopes were used to derive the calibration curve for quantifying Fe-EDTA (B).

formula

$$[\text{Fe-EDTA}] = 3.27\, d[\text{TOLH}]/dt - 0.48$$

where [Fe-EDTA] refers to the iron chelate concentration in μM and d[TOLH]/dt is the rate of TOLH oxidation expressed in μM/min. This expression was used to determine the concentration of free iron when a heme protein solution was treated with EDTA, after subtracting the effects of protein-bound oxidants, e.g., heme ferryl species. The activity of protein-bound oxidants was inferred from the TOLH

oxidation rate in the presence of DTPA, which forms an iron chelate that does not appreciably oxidize TOLH.

Decay of Ferryl Mb

When metMb is treated with hydroperoxides heme-bound oxidants, e.g., ferryl species as well as protein free radicals, e.g., tyrosinyl residues, oxidize TOLH much more rapidly than does Fe-EDTA (at iron concentrations equivalent to the heme concentrations). Therefore, during the initial stages of hydroperoxide treatment of metMb it was difficult to resolve the effects of released iron from those of protein-bound oxidants. To identify conditions that would be suitable for quantifying iron release and to learn more about the persistence of protein-bound oxidants in the absence of exogenous electron donors, TOLH oxidation by H_2O_2-treated metMb was analyzed in the presence of DTPA. Protein-bound oxidants were analyzed in the presence of DTPA to exclude any contribution of released iron to the TOLH oxidation. The oxidation of TOLH by metMb and H_2O_2 was sufficiently slow to measure without rapid mixing procedures (Figure 2A). The oxidation curve was characterized by a plateau, which provided a measure of total oxidants associated with the metMb. The maximum observed TOLH oxidation corresponded to nearly twice the concentration of H_2O_2. Treatment of the initial reaction mixture with catalase completely inhibited the formation of TOLH oxidants, consistent with a removal of H_2O_2 and an inhibition of significant formation of Mb-bound oxidants. When catalase was added after pre-incubation with H_2O_2, the extent of TOLH oxidation increased with incubation time during the first hour and thereafter was indistinguishable from the catalase-free samples (representative traces are shown in Figure 2A).

The decay of metMb-bound oxidants, estimated from the magnitudes of the oxidation plateaus, was a slow process, with significant TOLH oxidizing capacity remaining several hours after metMb was treated with H_2O_2 (Figure 2A). When these decay data were plotted semiexponentially, the kinetics appeared to be well represented by an exponential decay (Figure 2B). The $t_{1/2}$ for this exponential decay was 3 hrs. Treatment of the incubation mixtures with catalase effectively removed H_2O_2—the quantification of TOLH oxidants in these samples represented metMb-bound species that had formed up to the time of the catalase addition. The difference between catalase-treated and catalase-free samples gave the decomposition rate of H_2O_2 in this reaction mixture—the $t_{1/2}$ for the logarithmic fit (Figure 2B) was 4 min.

Free Iron Measurements in H_2O_2-treated MetMb

Iron was released when metMb was incubated with hydrogen peroxide or tBH (Figure 3). Iron release in the presence of oxygen was slightly greater than that observed in anaerobic solution, particularly at the lowest concentrations of H_2O_2 (Figure 3A). The degree of iron release varied among experiments, as reflected in the error bars for the intermediate range of hydroperoxide treatments (Figure 3B). At high peroxide levels iron release reached a plateau corresponding to 100% of the heme iron. At low peroxide concentrations, iron release was nearly a linear function of the cumulative hydroperoxide concentration. In this linear range approximately 50 equivalents of peroxide yielded one equivalent of iron.

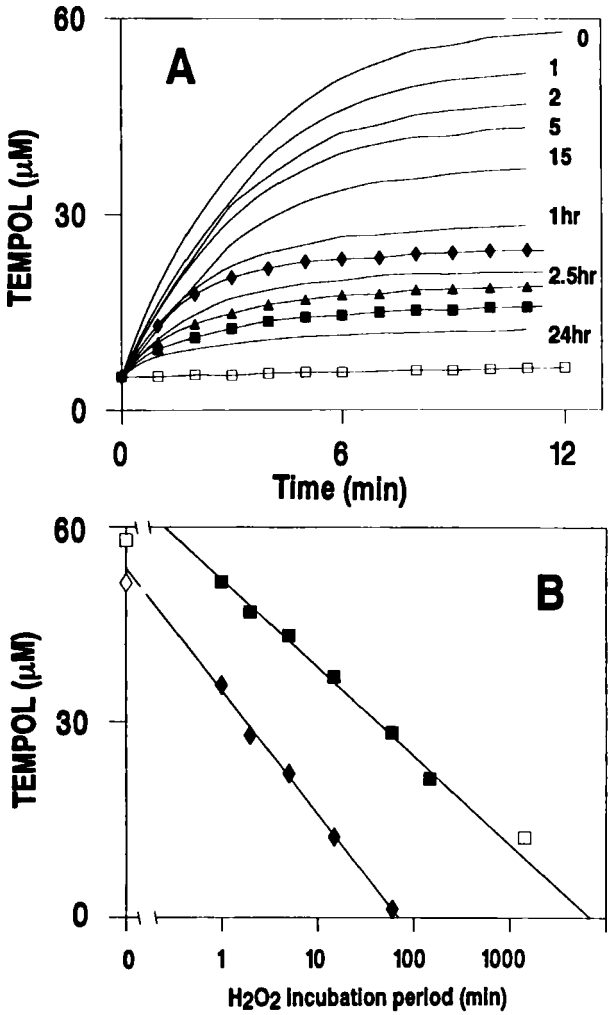
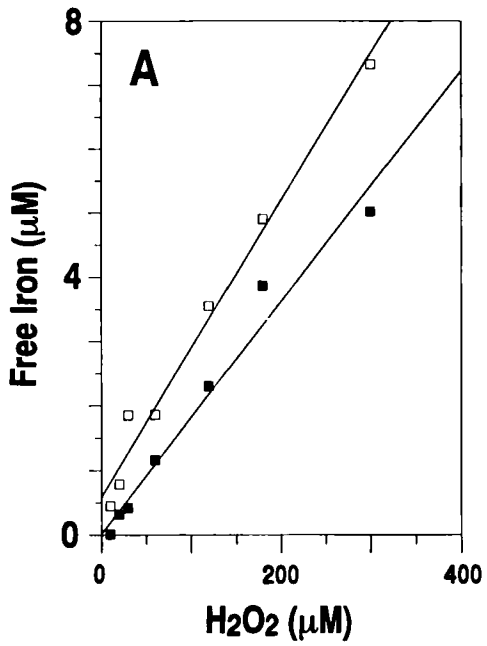


Figure 2 Ferryl decay. (A) Time course of TOLH oxidation of 30 μM metMb treated with 30 μM H_2O_2 for different incubation times (numbers refer to min unless shown as hrs) followed by addition of 300 μM TOLH and measurement of Tempol formation. Control of Mb without H_2O_2 □; results with H_2O_2 alone were indistinguishable from this control. Effect of catalase (1.3 kU/ml, 10 sec prior to TOLH addition) 1 min ■, 2 min ▲, and 15 min after H_2O_2 treatment ◆. (B) Semiexponential plot of the TOLH oxidation plateaus in the absence of catalase ■,□ and of the difference between oxidation plateaus observed with or without catalase treatment after the H_2O_2 incubation ◆,◇. Open symbols were excluded from the logarithmic fit shown by the straight lines.

Comparison of MetMb Bleaching and Iron Release with tBH Treatment

Optical absorption measurements of tBH-treated metMb showed the extent of heme bleaching with progressive peroxide treatments. With a cumulative tBH concentration of 120 μM tBH, the Soret band absorbance had decreased by about 50% and after treatment with 500 μM tBH the Soret band had virtually disappeared (Figure 4A). Iron release was analyzed in the same samples used for the bleaching analysis. Iron release



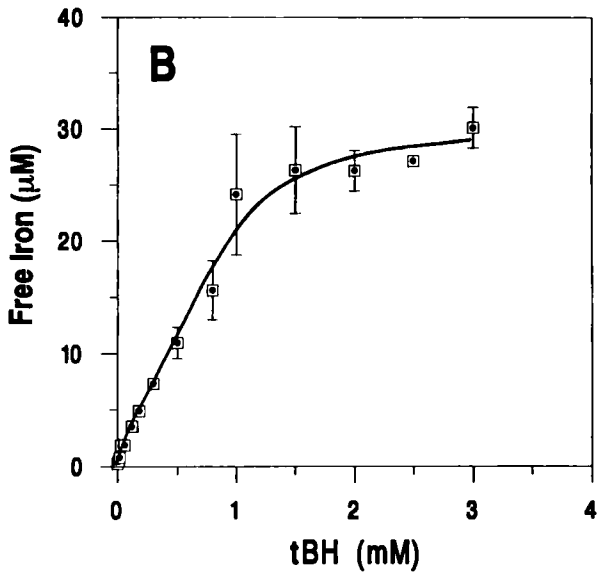


Figure 3 Iron release from 30 μM metMb treated with low concentrations of H_2O_2 aerobically or anaerobically (A) or with low and high concentrations of tBH (B). Each point shown in (B) represents the mean of at least three separate experiments.

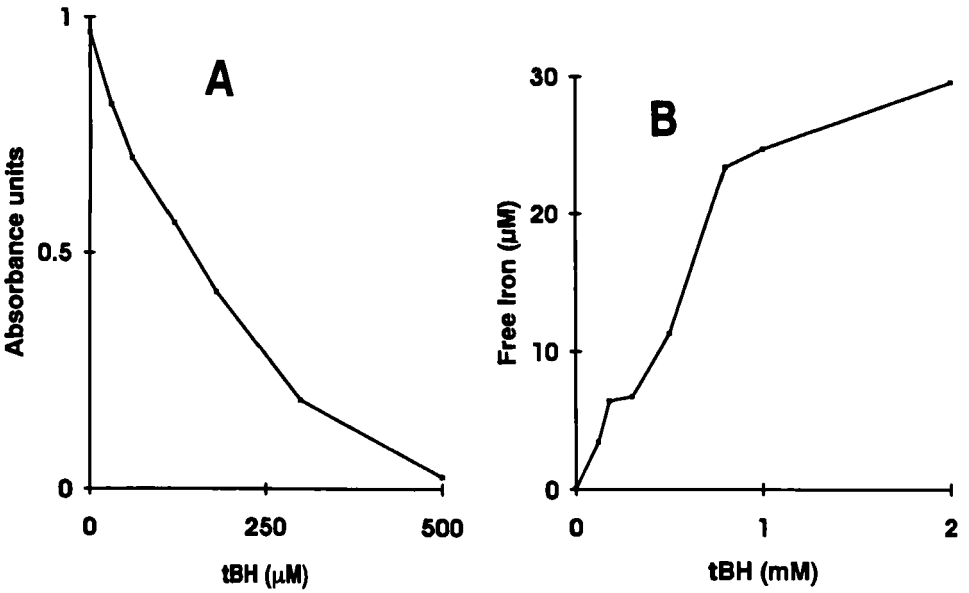


Figure 4 Comparison of metMb bleaching and iron release in one tBH-treated solution. metMb (30 μM) and 1 mM DTPA or 1 mM EDTA were treated with several additions of tBH and pre-incubated for at least 24 hours but not more than 40 hours at ambient temperature prior to the ESR and spectrophotometric assays. Bleaching, shown as the Soret band absorbance minus the residual absorbance at 410 nm after pre-incubation with 2 mM tBH (A), and iron release as determined by ESR (B).

required significantly more tBH exposure than did bleaching (Figure 4B), indicating that heme destruction leads to a significant pool of iron that is not chelatable by EDTA. Importantly the measurement of TOLH oxidation that was used for the iron assay indicated that sufficient time had elapsed after the tBH treatment to allow for a decay of persistent oxidizing species, demonstrating that the Soret band bleaching was not affected significantly by contributions from ferryl species.

Methyl Radical Detection

Methyl radicals arise from the decomposition of *tert*-butoxyl radicals and spin trapping them provides a means for analyzing the occurrence of the Fenton-type decomposition of tBH.[21] The characteristic ESR spectra of methyl radicals were detected when solutions of metMB were treated with tBH in the presence of excess ascorbate (relative to the tBH concentration, e.g., Figure 5). A major factor controlling the persistence of the methyl radical adducts was the protein concentration—the persistence of the ESR signals was approximately inversely proportional to this concentration. For example, only the first methyl radical peak during a spectral scan was detectable in reaction mixtures containing 4 mM metMb whereas the adduct spectra persisted for meanly an hour at 0.2 mM metMb (data not shown). Treatment of concentrated heme protein solutions with KCN concentrations of one or two equivalents per heme had little effect on the magnitudes of spin-trapped methyl radical ESR signals—at most a 50% reduction in peak heights was observed (data not shown).

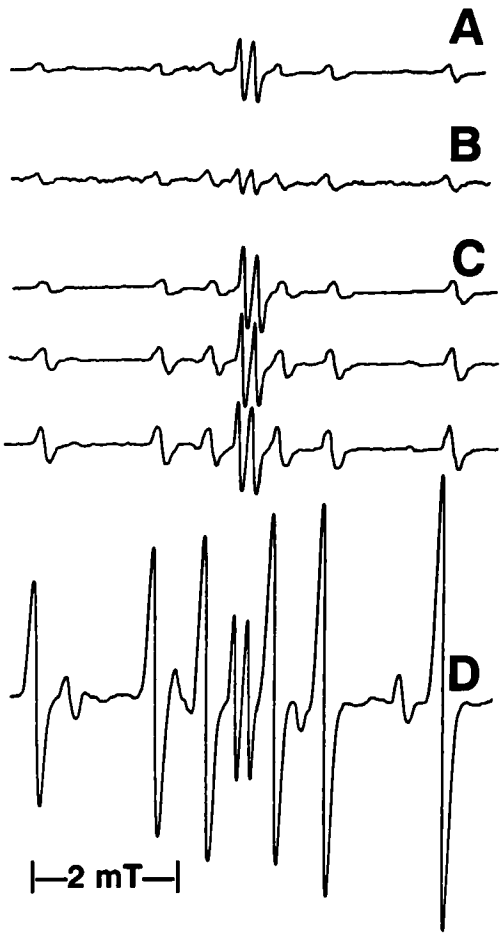


Figure 5 Spin trapping of free radicals derived from tBH in the presence of ascorbate in the same tBH-treated metMb preparation as was used for Figure 4. metMb (30 μM) and either 1 mM DTPA or 1 mM EDTA, incubated for more than 24 hr with or without 2 mM tBH, were assayed with 0.1 M DMPO, another 2 mM tBH and 2 mM ascorbate. (A) DTPA sample incubated without tBH; (B) DTPA sample incubated with tBH; (C) EDTA sample incubated without tBH—three sequential scans; (D) EDTA sample incubated with tBH. Scan rate: 1.25 mT/min.

The magnitudes of methyl and alkoxyl radicals were analyzed in the same samples that had been used for the comparison of iron release and bleaching (cf Figure 4). In samples that had been pretreated with 2 mM tBH, much larger ESR signals of DMPO adducts were observed in EDTA-treated metMb solutions than were detected in DTPA-treated metMb solutions (Figure 5). There was no signifiant enhancement of spin trap signals in tBH pre-treated DTPA samples relative to untreated controls (5A vs. 5B). In the EDTA-containing samples that were not pre-treated with tBH, a gradual increase in the ESR signal of methyl-DMPO adduct was evident, consistent with iron release. Some increase in methyl-DMPO adduct ESR signals was also seen in the DTPA samples, but the maximum signal intensity attained was only about

30% of that seen in the EDTA samples (data not shown); the former was substantially smaller than was the high-field peak observed in the tBH pre-treated sample, which had not yet reached its maximum intensity (Figure 5D). The magnitude of the ESR signal of the ascorbyl radical doublet (at the centre of the spectra) was larger in the DTPA sample that had not been pre-treated with tBH than in the control, consistent with little oxidizing activity in the former sample.

DISCUSSION

We have introduced a new ESR assay to analyze the formation of EDTA-chelatable iron. The assay has the following attributes: (1) protein-bound oxidants can be resolved from released, EDTA-chelatable iron, (2) it is applicable to optically opaque systems and does not require the use of protein precipitants, (3) it detects the effects of iron redox cycling and therefore does not introduce potential complications with exogenous reductants, and (4) it differs fundamentally from optical indicator assays, which is important because some previous work gave conflicting results with different methodologies.[5] Another distinction between our method and optical assays is that we require substantially smaller samples volumes than do the usual optical detection schemes; when this is taken into account our method is capable of detecting nearly an order-of-magnitude lower iron concentrations than is the ferrozine method.[22] We have developed the assay, in part, to set the stage for work with hemolysates and tissue homogenates, where the ability to analyze chelatable iron directly in unprocessed samples would be a distinct advantage.

The oxidation of TOLH by transition metal ions in aerobic solution is a multistep process, since many equivalents of TOLH are oxidized relative to the transition metal ion concentration. A likely sequence of reactions, which includes the rapid autoxidation of Fe^{2+}-EDTA is:

$$Fe^{3+}\text{-EDTA} + \text{TOLH} \leftrightarrow Fe^{2+}\text{-EDTA} + \text{Tempol} + H^+$$

$$Fe^{2+}\text{-EDTA} + O_2 \leftrightarrow Fe^{3+}\text{-EDTA} + O_2^-$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^{\cdot} + OH^-$$

$$OH^{\cdot} + \text{TOLH} \rightarrow H_2O + \text{Tempol}$$

Persistence of Oxidized MetMb Species

At the low H_2O_2 and metMb concentrations employed used in this study, significant H_2O_2 levels remained during the first hour of the reaction and newly oxidized metMb species were expected to arise during this initial period. Thereafter, the decay of the oxidized metMb products was a much slower process ($t_{1/2} = 4$ min for the H_2O_2 decay vs. $t_{1/2} = 3$ hrs for the decay of oxidized metMb species, see Figure 2). Thus iron release is expected to be a sluggish process whose accurate quantification requires that the released iron be accumulated in non-precipitable form throughout this reaction period, e.g., as the EDTA chelate as in the experiments reported here.

Effect of Peroxide Concentration on Iron Release

Since Hb and Mb and their ferric derivatives exhibit considerable catalatic activity towards an excess of H_2O_2[23] and since higher oxidation states, e.g., ferryl, of these proteins appear to be reduced by tBH,[15] it was expected that iron release would depend on the time course of peroxide treatment, e.g., iron release would depend on whether peroxide was added incrementally or as a single dose. In the work reported here peroxides were generally added at concentrations similar to those of the hemes. This treatment protocol led to relatively small fractional release of heme iron, of similar magnitude to that reported by Gutteridge.[5] On the other hand, Harel *et al.* found that treatment of heme proteins with an enzymic source of H_2O_2 released a substantially larger fraction of heme iron relative to the cumulative H_2O_2 dose.[6] These results suggest that the reactions involved in the catalatic activity of metMb do not contribute appreciably in the iron release.

Significance of Spin Trapping Experiments

The spin trapping of alkoxyl and methyl radicals in the presence of ascorbate (Figure 5) strongly suggests that tBH is reacting with reduced iron according to the Fenton-type reaction:

$$Fe^{2+} + (CH_3)_3COOH \rightarrow Fe^{3+} + (CH_3)_3CO\cdot + OH^-$$

$$(CH_3)_3CO\cdot \rightarrow (CH_3)_2C{=}O + CH_3\cdot$$

Where ascorbate serves to reduce ferric iron to the ferrous state, this allowing iron to act catalytically in alkoxyl and methyl radical production. Iron-DTPA is known to be a much less effective Fenton reagent than is iron-EDTA. (It is noteworthy that the former is also a less effective TOLH oxidant than is the latter, suggesting that TOLH may be useful for screening the effectiveness of transition metal chelates as Fenton catalysts). The low magnitudes of free radical adducts observed with DTPA relative to those observed with EDTA is consistent with a catalysis of hydroperoxide cleavage by ferrous-EDTA. The chelated iron is evidently derived from the hydroperoxide-oxidized heme proteins, judging by the relatively large free radical adduct signals observed in the tBH pre-treated metMb/EDTA preparation (Figure 5). However, significant iron release appears to occur with short-term hydroperoxide exposure, since appreciable spin trapped radicals were detected in metMb that was not pre-treated with tBH (Figure 5C).

The observation that the bleaching of metMb requires substantially less tBH treatment than does iron release from metMb (Figure 4), suggests that some iron remains strongly associated with the protein after heme destruction. However, with sufficient hydroperoxide treatment (about five times more than is required for bleaching), all iron appears to be released from the protein. Significantly, the spin trapping experiments (Figure 5) showed only weak ESR signals in any of the DTPA-treated samples, consistent with the interpretation that the protein-bound iron that is formed during the bleaching reaction is not an effective Fenton catalyst.

We attempted to gain more insight into the mechanism of iron release by testing the effects of cyanide. By binding to ferric heme, cyanide suppresses its reaction with hydroperoxides. Since cyanide was not effective in altering the spin trapping of methyl radicals it appears possible that it exerts at least two offsetting effects: (1) by binding

to ferric iron in metMb it substantially retards the rate of reaction of hemes with hydroperoxides and thus iron release and (2) by suppressing the formation of ferryl species it inhibits oxidation of spin adducts to non-paramagnetic products. We previously showed that nitroxides are readily oxidized by ferryl heme and suggested that this oxidation would suppress the magnitudes of spin trap adducts.[23]

Acknowledgements

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Abbreviations

DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriaminepentaacetic adic; EDTA, ethylenediaminetetraacetic acid; Mb, myoglobin; Hb, hemoglobin; Tempol, 4-hydroxy-2,2,6,6-tetramethyl piperidine-N-oxyl; TOLH, 1,4-dihydroxy-2,2,6,6-tetramethyl piperidine; tBH, tert-butyl hydroperoxide.

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