THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 27, Issue of July 7, pp. 16075–16081, 1995
Printed in U.S.A.

Self-peroxidation of Metmyoglobin Results in Formation of an Oxygen-reactive Tryptophan-centered Radical*

(Received for publication, March 20, 1995, and in revised form, May 2, 1995)

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In the reaction between hydrogen peroxide and metmyoglobin, the heme iron is oxidized to its ferryl-oxo form and the globin to protein radicals, at least one of which reacts with dioxygen to form a peroxyl radical. To identify the residue(s) that forms the oxygen-reactive radical, we utilized electron spin resonance (ESR) spectroscopy and the spin traps 2-methyl-2-nitrosopropane and 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS). Metmyoglobin radical adducts had spectra typical of immobilized nitroxides that provided little structural information, but subsequent nonspecific protease treatment resulted in the detection of isotropic three-line spectra, indicative of a radical adduct centered on a tertiary carbon with no bonds to nitrogen or hydrogen. Similar isotropic three-line ESR spectra were obtained by spin trapping the oxidation product of tryptophan reacting with catalytic metmyoglobin and hydrogen peroxide. High resolution ESR spectra of DBNBS/trp and of the protease-treated DBNBS/metMb were simulated using superhyperfine coupling to a nitrogen and three non-equivalent hydrogens, consistent with a radical adduct formed at C-3 of the indole ring. Oxidation of tryptophan by catalytic metMb and hydrogen peroxide resulted in spin trap-inhibitable oxygen consumption, consistent with formation of a peroxyl radical. The above results support self-peroxidation of a tryptophan residue in the reaction between metMb and hydrogen peroxide.

Oxidative damage to tissues has become a recurring theme as a mechanism for the induction of a variety of medical conditions (1) including myocardial ischemia (2), cancer (3), and aging (4). Myoglobin, a heme protein that is ubiquitous in aerobic muscle tissues, has been shown to acquire peroxidative activity (5) that can result in the oxidative damage to a variety of biological molecules, including proteins and membrane lipids (6–9).

Since the 1950's, metMb has been known to react with hydrogen peroxide, resulting in the one-electron oxidation of ferric heme to form ferryl heme (10, 11). The fate of the second oxidizing equivalent available from the reduction of hydrogen peroxide to water has been in question since the reaction was first reported (11). Free hydroxyl radical is one possibility, but this species has been detected only under conditions of excess hydrogen peroxide where heme damage has occurred (12, 13). No evidence has been obtained for formation of a porphyrin cation radical from metMb similar to compound I of horseradish peroxidase, and recent results support the formation of a protein-centered radical (14–31). However, the nature of the protein-centered radical is still under debate. Many previous investigations have concluded that the free radical is centered on a tyrosine residue (17–19, 21, 23, 31). Recently, however, site-directed mutagenesis studies, in which all of the tyrosine residues have been removed (26, 27), and ESR studies (24, 28) have demonstrated that another amino acid residue in addition to tyrosine must also be involved. The identity of the latter amino acid residue has not been determined.

In this investigation, we utilize the electron spin resonance (ESR) spectroscopy spin-trapping technique using the nitroso-based spin traps 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS)[1] and 2-methyl-2-nitrosopropane (MNP) to investigate the nature of the amino acid residue modified in the reaction between horse heart metMb and hydrogen peroxide. The spin trapping results indicate that the globin radical that has been spin trapped is centered on a tryptophan residue.

EXPERIMENTAL PROCEDURES

Materials—Horse heart metMb, proteinase K, tryptophan, tyrosine, and diethylenetriaminepentaacetic acid were acquired from Sigma. MNP dimer and sodium cyanide were obtained from Aldrich. 3,5-dibromo-4-nitrosobenzenesulfonic acid sodium salt was purchased from OMRF Spin Trap Source (Oklahoma City, OK). Pronase was obtained from Boehringer Mannheim. Hydrogen peroxide was obtained from Mallincrodt (St. Louis, MO). Chelex 100 was purchased from Bio-Rad. Glycylglycyltryptophan (gly-gly-trp), glycyltryptophylglycine (gly-trp-gly), and tryptophylglycylglycine (trp-gly-gly) were acquired from Accurate Chemical and Scientific (Westbury, NY). Tryptophan-indole-D_5 was obtained from Cambridge Isotope Laboratories (Cambridge, MA). 2,6-dideutero-3,5-dibromo-4-nitrosobenzenesulfonic acid was prepared by Robert Sik (NIEHS), following the published procedure (32).

Spin Trapping Experiments—All experiments were done in 50 mM sodium phosphate buffer, pH 7.4, treated with chelex by the batch method, and contained diethylenetriaminepentaacetic acid at a final concentration of 50 μM. When MNP was used as the spin trap, metMb, tryptophan, or peptides were dissolved into a solution of MNP (23 mM) prepared by stirring overnight at room temperature. In all experiments, hydrogen peroxide was added to the remaining reagents immediately before transfer of the solution to a quartz ESR flat cell, which was then positioned into the microwave cavity (TM_{110}) of a Bruker ESP 300 spectrometer. Instrument parameters are reported in the figure legends for each individual scan. Spectral simulations were calculated and optimized to a minimum sum of squared residuals using a program developed by David Duling (33) (available over the Internet). When samples were subjected to dialysis, several milliliters of sample were prepared, a small aliquot was scanned, and the remaining sample was placed in dialysis tubing (molecular weight cutoff 3,500) and dialyzed overnight against one 2-liter charge of 50 mM sodium phosphate buffer, pH 7.4. Following dialysis, a small aliquot was removed and scanned, and the remaining sample was subjected to protease treatment as described in the figure legends. Control experiments were performed in parallel with complete incubations.

Oxygen Consumption—All experiments were performed in chelex-

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[1] The abbreviations used are: DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; MNP, 2-methyl-2-nitrosopropane.

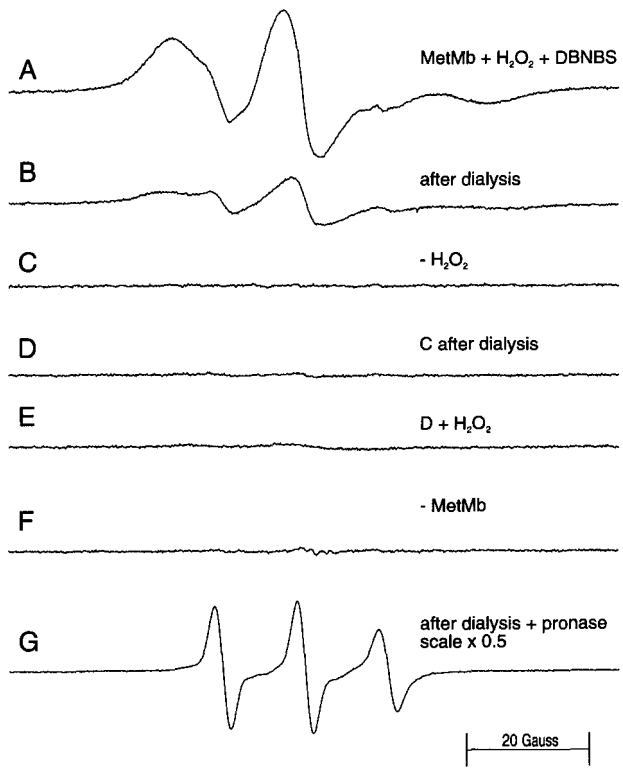


FIG. 1. **ESR spectra obtained from a mixture of metMb and H_2O_2 in the presence of DBNBS.** *A*, hydrogen peroxide (400 μM) was added to a solution of metMb (500 μM) in the presence of DBNBS (10 mM). *B*, an aliquot of the solution used in *spectrum A* after overnight dialysis into 50 mM sodium phosphate buffer. *C*, MetMb (500 μM) was added to a solution of DBNBS (10 mM). *D*, an aliquot of the solution used in *spectrum C* after overnight dialysis into 50 mM sodium phosphate buffer. *E*, hydrogen peroxide (400 μM) was added to an aliquot of the solution described in *scan C* after overnight dialysis. *F*, hydrogen peroxide (400 μM) was added to DBNBS (10 mM). *G*, an aliquot of the solution used to obtain *spectrum B* 20 min after addition of Pronase (final concentration, 2 mg/ml). Instrument parameters were as follows for all scans: modulation amplitude, 1 G; time constant, 0.33 s; scan time, 335 s; gain, 1×10^5; modulation frequency, 100 kHz; microwave frequency, 9.80 GHz; microwave power, 20 mW.

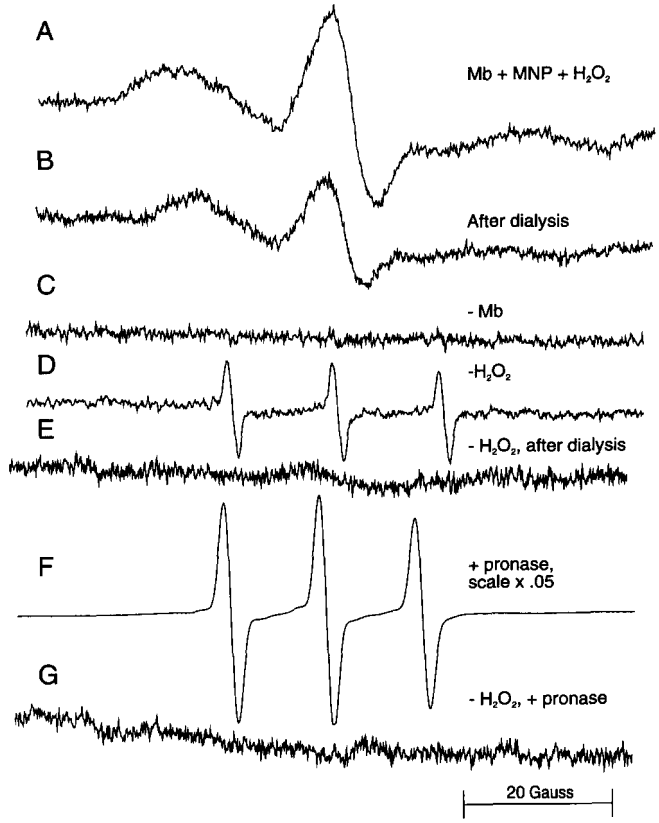


FIG. 2. **The ESR spectra obtained after addition of H_2O_2 to metMb in the presence of MNP.** *A*, hydrogen peroxide (400 μM) was added to metMb (500 μM) in the presence of MNP (23 mM). *B*, the ESR spectrum of an aliquot of the solution used to obtain *spectrum A* after dialysis for 3 h in 50 mM sodium phosphate buffer, pH 7.4. *C*, hydrogen peroxide (400 μM) was added to a solution of MNP (23 mM). *D*, MetMb (500 μM) was added to a solution of MNP (23 mM). The nitrogen hyperfine coupling constant (a^N = 17.2 G) is characteristic of di-*t*-butyl nitroxide. *E*, an aliquot of the solution used to obtain *spectrum D* was scanned after overnight dialysis in 50 mM sodium phosphate buffer. *F*, Pronase (final concentration, 2 mg/ml) was added to an aliquot of the solution used to obtain *spectrum B*. Note that the gain for this scan is 20-fold lower than in the other scans. The value for a^N for the observed radical adduct is 15.5 G. *G*, Pronase (2 mg/ml) was added to an aliquot of the solution used to obtain *spectrum E*. Instrument parameters for scans *A–C*, *E*, and *G* were as follows: modulation amplitude, 1 G; time constant, 0.33 s; scan time, 671 s; receiver gain, 5×10^5; all other parameters were as reported for Fig. 1. For *spectrum D*, the time constant was 0.66 s. For *spectrum F*, the receiver gain was 2.5×10^4.

treated 50 mM sodium phosphate buffer, pH 7.4, in the presence of 50 μM diethylenetriaminepentaacetic acid. When MNP was used, all further reagents were dissolved in prepared MNP solutions. MetMb was added after a base line had been established with no subsequent difference in the base line being observed. Hydrogen peroxide (final concentration, 100 μM unless otherwise noted) was then added. Data were collected using a YSI oxygraph equipped with a computer interface using a Clark oxygen electrode.

RESULTS

Spin Trapping a Free Radical Product of metMb and H_2O_2 with DBNBS—Addition of equimolar hydrogen peroxide to metMb in the presence of DBNBS resulted in the detection of an ESR spectrum characteristic of a highly immobilized nitroxide (Fig. 1*A*). Overnight dialysis of the product resulted in only slight changes in the shape of the ESR spectrum, indicating that the radical adduct has a molecular weight greater than 3,500 (Fig. 1*B*). No spectra could be detected in the absence of either metMb or hydrogen peroxide (Fig. 1, *C*, *D*, and *F*). Addition of hydrogen peroxide to the hydrogen peroxide-free control after dialysis did not result in detection of any ESR signal (Fig. 1*E*). Inclusion of sodium cyanide (10 mM) prevented detection of the immobilized nitroxide (data not shown). Treatment of the product with Pronase resulted in the detection of a nearly isotropic three-line spectrum with a hyperfine coupling constant of 13.6 G (Fig. 1*G*). Treatment of the product with proteinase K resulted in detection of a spectrum similar to that observed when Pronase was used (data not shown).

Spin Trapping the Product of metMb and H_2O_2 with MNP—When the reaction between metMb and hydrogen peroxide was investigated with MNP, the ESR spectrum of the resulting solution was similar to that observed in the presence of DBNBS (Fig. 2*A*). Only moderate decay of the immobilized nitroxide occurred upon dialysis (Fig. 2*B*). When hydrogen peroxide was excluded, a three-line spectrum with a nitrogen hyperfine coupling constant of 17.2 G was detected, characteristic of di-*t*-butylnitroxide, a decomposition product of MNP (Fig. 2*D*). The three-line spectrum was removed by dialysis, confirming that it was not a protein-centered radical adduct (Fig. 2*E*). Treatment of the product with Pronase (Fig. 2*F*) resulted in the detection of a three-line spectrum with a^N = 15.5 G, which was dependent upon hydrogen peroxide (Fig. 2*G*); the ESR spectra obtained when proteinase K was used in place of Pronase gave a similar spectrum, except that a significant contribution from immobilized nitroxide was also observed (data not shown).

Identification of the Amino Acid Residue Modified—The three-line spectra obtained after treatment of oxidized metMb

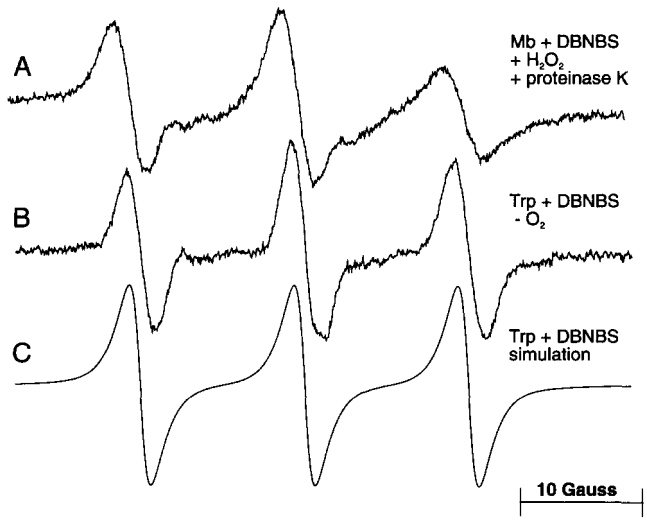


FIG. 3. **The ESR spectra obtained from the protease-treated DBNBS/•metMb radical and from free tryptophan and DBNBS.** *A*, a 50-G scan of the proteinase K-treated dialyzed sample obtained from the reaction of metMb and hydrogen peroxide in the presence of DBNBS. *B*, DBNBS (10 mM) was added to tryptophan (20 mM) in N_2-saturated solution under an atmosphere of N_2, and the solution was aspirated into a quartz ESR flat cell prepositioned in the microwave cavity of a Bruker ER300 ESR spectrometer. *C*, the optimized computer simulation of *spectrum B* with hyperfine coupling constant a^N = 13.6 G. The instrument conditions used for the experimental scans were the same as reported for Fig. 1.

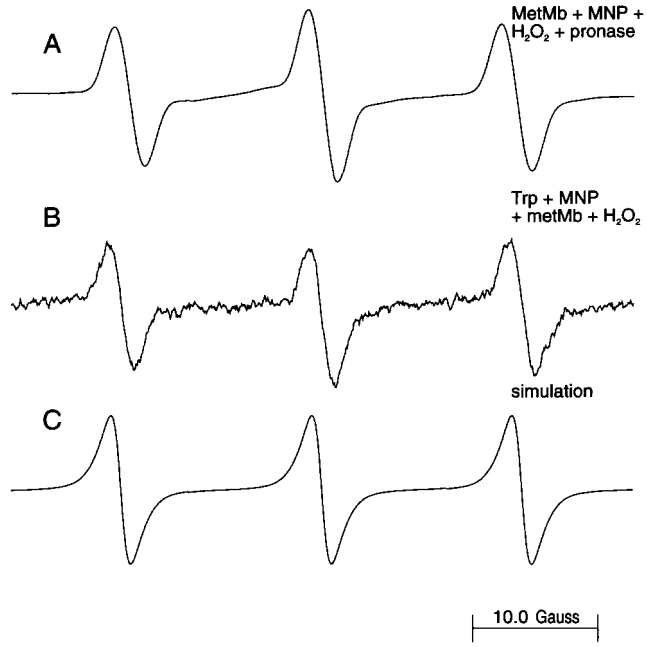


FIG. 4. **Comparison of the ESR spectra obtained from Pronase-treated MNP/•metMb radical adduct and from the oxidation of tryptophan in the presence of MNP.** *A*, a 50-G scan of the solution scanned in Fig. 2*F*. *B*, hydrogen peroxide (100 μM) was added to tryptophan (20 mM) in the presence of metMb (50 μM) and MNP (23 mM). *C*, the optimized computer simulation of the spectrum shown in *B*. Instrument settings: modulation amplitude, 1 G; time constant, 0.67 s; scan time, 671 s; receiver gain for *spectrum B*, 1×10^5; receiver gain for *spectrum A*, 2.5×10^4. All other spectrometer settings were the same as reported for Fig. 1.

with nonspecific proteases (Figs. 1*G* and 2*F*) are indicative of a tertiary carbon-centered radical adduct. The lack of additional hyperfine structure in the spectrum indicates that the tertiary carbon atom in the adduct has no bonds to atoms with nuclear spin such as nitrogen. The most reasonably oxidizable tertiary carbon atoms in proteins are C-3 of the indole ring of tryptophan and C-4 of the phenol ring of tyrosine. The peroxidase activity of metMb (5) was used to oxidize free amino acids to identify the type of amino acid residue modified. Free tryptophan (20 mM), free tyrosine (saturated, approximately 3 mM), or free histidine (20 mM) was added to a catalytic concentration of metMb in the presence of a spin trap, and hydrogen peroxide was added to the solution to initiate the reactions. No amino acid-dependent radical adducts were detected when either tyrosine or histidine was used with either spin trap. When tryptophan was studied using DBNBS as the spin trap, a very strong three-line signal (a^N = 13.6 G) nearly identical to that observed from metMb and hydrogen peroxide after proteinase treatment was detected (Fig. 3, *A* and *B*). The adduct was also observed in the absence of metMb, and further study demonstrated that the adduct formation was independent of hydrogen peroxide and oxygen as well (Fig. 3*B*). The spontaneous ene reaction between DBNBS and tryptophan to form a pseudo radical adduct has been reported (34). The broadening of the high field line in the triplet in Fig. 3*A* relative to that in Fig. 3*B* is the result of slower molecular motion, consistent with a higher molecular weight of the radical adduct(s) from protease-treated metMb.

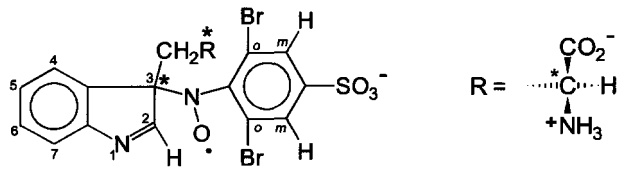
When tryptophan was incubated with MNP, hydrogen peroxide, and catalytic concentrations of metMb, a three-line ESR spectrum was detected with hyperfine coupling constant a^N = 16.1 G (Fig. 4*B*). The detected spectrum was different from the spectrum obtained from Pronase-treated metMb, which had a nitrogen hyperfine coupling constant a^N = 15.5 G (Fig. 4*A*). When either hydrogen peroxide or metMb was removed from the incubation, a three-line spectrum with a nitrogen hyperfine coupling constant characteristic of di-*t*-butylnitroxide was detected (data not shown). A radical adduct of *N*-acetyl-tryptophan with MNP has been reported with a nitrogen hyperfine coupling constant identical to that which we detected from the protease-treated MNP/•metMb (35). The presence of amide bonds at the amino terminus of the amino acid residue is expected to have similar effects on the nitrogen hyperfine coupling constant of the radical adduct (35). To investigate that possibility, we used metMb as a peroxidase to oxidize the tryptophan residues in the tripeptides trp-gly-gly, gly-trp-gly, and gly-gly-trp using MNP as a spin trap to detect the resulting radicals. The hyperfine coupling constants for the observed spectra range from 16.1 G for trp-gly-gly to 15.75 G for gly-gly-trp, indicating that the difference between free tryptophan and the protein-derived adduct could be due to the amino acid residues that retain amide bonds to the tryptophan residue during nonspecific protease treatment of oxidized metMb, particularly on its α-amine.

Resolution of the Superhyperfine Coupling of DBNBS/•Tryptophan Radical Adducts—The ene reaction between DBNBS and tryptophan results in the formation of a pseudo radical adduct that has an identical structure to the adduct formed through a radical pathway (34). The adduct is produced in sufficiently high concentration to resolve small hyperfine coupling constants from magnetic nuclei β to the carbon at which the radical adduct is formed (See Table I for the structure of the adduct). With the use of a smaller modulation amplitude (0.1 G), each broad line of the observed three-line spectrum was determined to be composed of a number of overlapping lines (Fig. 5*A*). The middle line of the high resolution spectrum of DBNBS/•trp was simulated using contributions from a nitrogen atom, three non-equivalent protons, and the expected two *meta*-protons of the spin trap, and the simulation was optimized using an iterative process in which each parameter is varied until the minimum residual is achieved. Details of the method and the program utilized have been published (33). A

TABLE I

Hyperfine coupling constants in Gauss used to simulate the ESR spectra from solutions of tryptophan and DBNBS

Asterisk indicates a chiral carbon.



Species	Linewidth	a^N	a^H	a^H	a^H	a^H_{meta} (2H)
trp	0.32	0.46	0.64	0.76	0.64	0.93
gly-trp-gly	0.39	0.37	0.21	0.89	0.62	0.90
trp-gly-gly	0.40	0.53	0.70	0.95	0.56	0.82
gly-gly-trp	0.38	0.42	0.24	0.79	0.78	0.80
2,6-D_2-DBNBS	0.37	0.39	0.85	0.99	0.85	0.14 (I = 1)
Indole-D_5-trp	0.35	0.39	0.70	1.06	0.13 (I = 1)	0.66
2,6-D_2-DBNBS, Indole-D_5-trp	0.29	0.38	0.79	0.99	0.16 (I = 1)	0.16 (I = 1)
metMb/H_2O_2, pronase	0.68	0.57	0.79	0.79	0.79	0.79

0.1-G modulation amplitude scan of the middle line of the radical adduct produced by Pronase treatment of DBNBS/ ·metMb gave rise to a similar, but not identical, superhyperfine structure (Fig. 5*E*). To account for effects of amide bonds to the tryptophan in the protease-treated metMb sample, the tripeptides trp-gly-gly, gly-trp-gly, and gly-gly-trp were studied in a similar manner (Fig. 5, *B–D*). The hyperfine coupling constants from the optimized simulation for each experimental spectrum are reported in Table I. To identify the atoms contributing hyperfine coupling in the various DBNBS adducts, indole-deuterated tryptophan and DBNBS deuterated on the *meta* positions were utilized. Deuteration of DBNBS removed the hyperfine interaction of two equivalent protons, which had contributed a 1:2:1 triplet to the superhyperfine structure, most notably in the outermost shoulders in the spectrum (Fig. 6, *B* compared to *A*), demonstrating the contribution of the *meta*-protons of the spin trap to the spectrum. When indole-deuterated tryptophan was used, a proton hyperfine coupling was replaced by a much smaller deuterium coupling, demonstrating hyperfine interaction with an indole ring hydrogen (Fig. 6*C*). The use of both deuterated tryptophan and deuterated spin trap greatly simplified the spectrum in the expected manner (Fig. 6*D*).

Oxygen Consumption by Tryptophan—Incubation of tryptophan (20 mM) with metMb (40 μM) and hydrogen peroxide (100 μM) resulted in the rapid consumption of oxygen (Fig. 7, *curve A*), which was inhibited by inclusion of 23 mM MNP (Fig. 7, *curve B*) and by 10 mM DBNBS (Fig. 7, *curve C*). The rate of oxygen consumption was dependent upon the concentration of metMb (data not shown). Slight oxygen evolution was detected when hydrogen peroxide was added to metMb in the absence of tryptophan, consistent with the known catalase activity of metMb (Fig. 7*D*) (5). No oxygen consumption was observed when tryptophan was replaced by either tyrosine or histidine (data not shown).

DISCUSSION

The initial spin-trapping data obtained with 5,5-dimethyl-1-pyrroline *N*-oxide to discern the nature of the electron-donating residue in metMb demonstrated that a free radical was formed (20, 21, 24, 29). The original assignment to a peroxyl radical adduct (21, 22) was subsequently demonstrated to be in error (24, 28), but no additional structural information was obtained. Likewise, the whole-protein data acquired in this study using the nitroso spin traps provided little structural information (Figs. 1 and 3). The high degree of rotational anisotropy observed in nitroso-spin trap/whole protein radical adducts can be removed by proteolysis, which results in fragmentation of the protein to form relatively freely rotating peptides that give isotropic spectra (37). Such was the case for both DBNBS/ ·metMb and MNP/·metMb, resulting in a great enhancement of the information content of the spectra (Figs. 1*G* and 2*G*). The similarity of the spectrum obtained by Pronase treatment of DBNBS/·metMb to that of DBNBS/·trp strongly suggests that the globin-centered radical resides on a tryptophan residue. The conclusion that the radical is centered on the indole ring, and in particular on C-3, is corroborated by the resolution of the superhyperfine coupling from each of the lines of the primary triplet, which were very convincingly simulated using coupling to a nitrogen, three non-equivalent hydrogens, and the two equivalent *meta*-hydrogens from the DBNBS (Fig. 5*A*, Table I). Previous reports of similar spectra acquired from tryptophan and DBNBS have assigned the radical to either C-3 of the indole ring (34) or to the α-carbon of the amino acid (37). The latter assignment seems unlikely due to the lack of the expected hyperfine coupling to the α-amine nitrogen. Previous studies using different techniques have demonstrated that tryptophan radicals centered on the indole ring are formed with significant free electron density residing on C-3 (35, 38, 39). Calculated electron densities of the indole ring-centered tryptophan radical have shown that the greatest electron density resides on C-3 (40).

The minor difference between the highly resolved spectrum obtained from free tryptophan and that obtained from the Pronase-treated DBNBS/·metMb can be accounted for by changes in the relative orientations of the methylene protons induced by the steric effects of additional amino acids on the amino and carboxyl groups of tryptophan that can increase or decrease the magnitudes of hyperfine coupling constants (41), as demonstrated with the spectra obtained using the trp-gly_2 tripeptides. In addition, the molecular inhomogeneity, resulting from nonspecific protease treatment of the intact DBNBS/ ·metMb and reflected in the increased linewidth used in the optimized simulation of the metMb-derived adduct (Table I), contributes to the deviation between the protein-derived and tryptophan spectra. Finally, the formation of the radical adduct at C-3 of the indole ring creates a chiral center that results in the possibility of the formation of a pair of diastereomers, which could themselves have non-identical spectra, thereby also contributing significant line broadening (41).

The hydrogen peroxide-independent addition of DBNBS to tryptophan, demonstrated in Fig. 3*B*, suggested that this tryptophan adduct might be formed by direct reaction of the spin

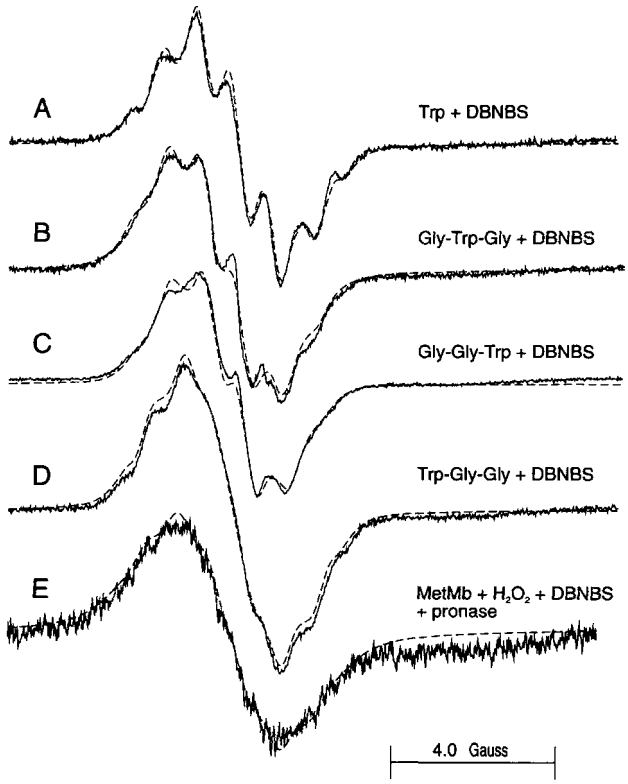


FIG. 5. **High resolution scans and computer simulations of the middle line of the spectra from the reaction of tryptophan derivatives with DBNBS.** Computer simulations were calculated from the hyperfine coupling constants reported in Table I and were the result of differential minimization optimization (30). Simulations (*dashed lines*) are shown superimposed on the experimental spectra (*solid lines*). *A*, hydrogen peroxide (100 μM) was added to a solution containing metMb (25 μM), tryptophan (20 mM), and DBNBS (10 mM). *B*, hydrogen peroxide (100 μM) was added to the tripeptide gly-trp-gly (saturated solution) in the presence of metMb (25 μM) and DBNBS (10 mM). *C*, hydrogen peroxide (100 μM) was added to the tripeptide gly-gly-trp (20 mM) in the presence of metMb (25 μM) and DBNBS (10 mM). *D*, hydrogen peroxide (100 μM) was added to the tripeptide trp-gly-gly (20 mM) in the presence of metMb (25 μM) and DBNBS (10 mM). *E*, Pronase (2 mg/ml, final concentration) was added to a fraction of a dialyzed solution prepared by addition of hydrogen peroxide (400 μM) to metMb (500 μM) in the presence of DBNBS (10 mM), and the spectrum was recorded after a 40-min incubation period to ensure complete proteolysis of the metMb. Instrument settings for scans *A–D* were as follows: modulation amplitude, 0.1 G; time constant, 0.65 s; scan range, 15 G; scan time, 5368 s; receiver gain, 5×10^5; microwave power, 0.2 mW. Instrument settings for *scan E* were: modulation amplitude, 0.1 G; time constant, 0.33 s; scan range, 15 G; scan time, 2684 s; receiver gain, 5×10^5; microwave power, 20 mW.

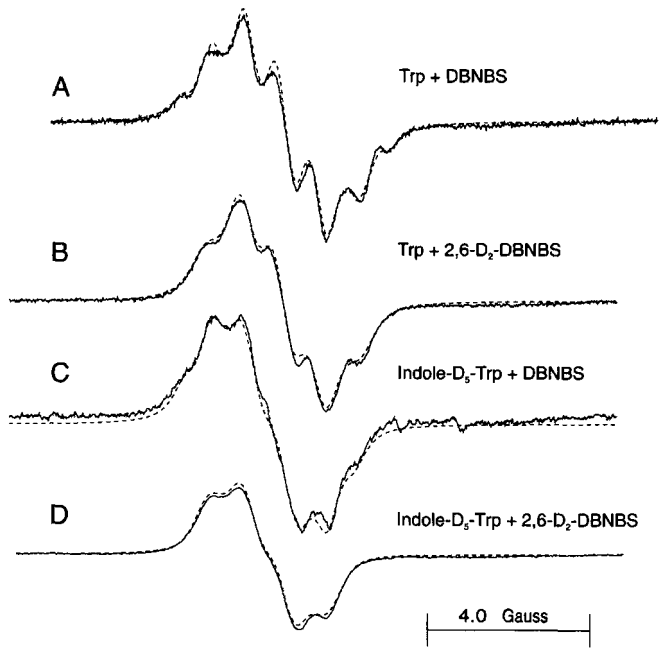


FIG. 6. **Effects of deuteration on the high resolution ESR scans of the reaction between tryptophan and DBNBS in the presence of catalytic metMb.** Computer simulations (*dashed lines*) were obtained as reported in Fig. 5. ESR parameters are the same as reported in Fig. 5. All reactions were initiated by the addition of hydrogen peroxide (100 μM) to solutions containing tryptophan (20 mM), metMb (25 μM), and DBNBS (10 mM). *A*, non-deuterated tryptophan and DBNBS. This spectrum is repeated from Fig. 5 to allow comparison. *B*, non-deuterated tryptophan and 2,6-D_2-DBNBS. *C*, indole-D_5-tryptophan and non-deuterated DBNBS. *D*, indole-D_5-tryptophan and 2,6-D_2-DBNBS.

trap with the tryptophan residues of the protein, either before or after proteolysis. DBNBS has been shown to react with biological compounds, including tryptophan, to give non-radical-derived adducts (34). The product of the ene reaction of DBNBS with an unsaturated system is a hydroxylamine that must then be oxidized to form an ESR-active nitroxide (34). No radical adduct spectrum was detected upon addition of hydrogen peroxide to the dialyzed control incubation between metMb and DBNBS (Fig. 1*E*), showing that the ene reaction using hydrogen peroxide to oxidize the proposed hydroxylamine intermediate is not the pathway for DBNBS/ferrylMb formation. The inhibition of adduct formation by cyanide, which coordinates heme iron and prevents access by the peroxide, thereby eliminating peroxidase chemistry, demonstrated the requirement for heme oxidation in the formation of the protein-centered radical adduct.

The results of the spin trapping experiments using MNP confirm the hydrogen peroxide dependence of globin-centered radical formation and also demonstrate that the radical adduct is centered on a tertiary carbon. The observed difference between the a^N for the Pronase-treated MNP/ferrylMb and for MNP/trp can be accounted for by the presence of amide(s) on the primary amine, the primary carboxylic acid, or both groups of tryptophan in the hydrolysate, as demonstrated by the observed a^N values for the tryptophan tripeptides. Effects of amidation, ionization, and acetylation of the functional groups of tryptophan similar to those observed here have been reported (37).

Previous studies have suggested that a tyrosine peroxyl radical is the spin-trapped radical in hydrogen peroxide-treated metMb (21, 22). A spin-trapping study of radicals formed from tyrosine by pulsed radiolysis using MNP did not report a spectrum at neutral pH, and spectra acquired in basic and acidic solutions exhibited different hyperfine coupling constants from those determined for the Pronase-treated metMb (42).

The present results provide strong evidence that the globin-centered radical that is spin trapped following the reaction of metMb with hydrogen peroxide is centered on a tryptophan residue. The formation of tryptophan radicals in intact proteins has been demonstrated (43–45). The identification of a tryptophan residue as an oxidized site is also consistent with the studies performed by Ortiz de Montellano and co-workers (26, 27), in which a direct ESR signal identical to that of the native protein is observed in the site-directed mutant protein, which lacks all tyrosine residues. However, the dityrosine cross-linking of sperm whale metMb (18) and the covalent binding of tyrosine to heme in horse metMb (6), as well as ESR studies (17, 23), all indicate that the oxidation of metMb by hydrogen peroxide forms one or more tyrosine radicals. Transfer of the oxidative equivalent between tryptophan and tyrosine residues is suggested by the similarity of

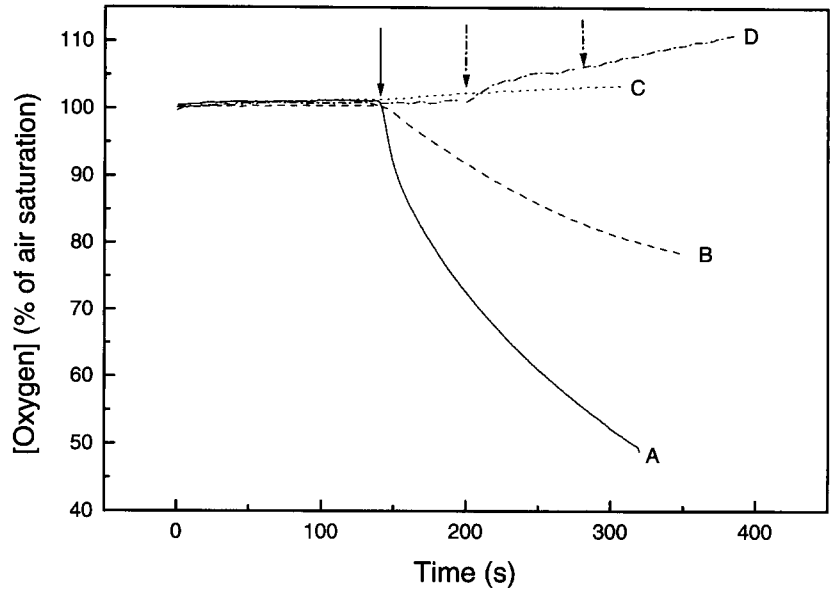
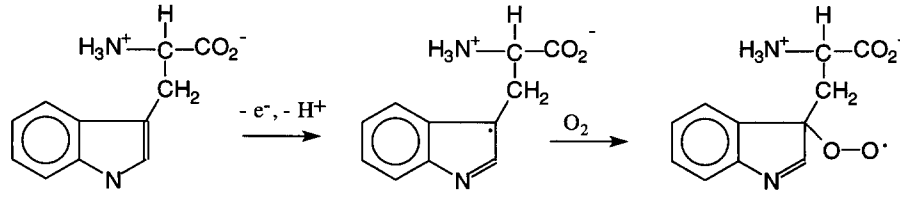


FIG. 7. **Oxygen consumption by solutions of tryptophan in the presence and absence of spin traps.** In each reaction, hydrogen peroxide (100 μM) was added to a solution that already contained metMb (50 μM) at the time indicated by the *solid arrow*. *A*, tryptophan (20 mM). *B*, tryptophan (20 mM) + MNP (23 mM). *C*, tryptophan (20 mM) + DBNBS (10 mM). *D*, the no tryptophan control. Additional hydrogen peroxide was added to demonstrate the catalase-like activity of metMb at the times indicated with the *broken arrows*.

SCHEME I.



their oxidation potentials (46, 47).

Previous spin-trapping studies have demonstrated that oxygen competes with the spin trap for the the primary radical, and direct ESR has demonstrated that the radical detected in frozen solution is a peroxyl radical (24, 28). Tryptophan radical formed photochemically has been shown to react with oxygen during its decomposition (48, 49). We found that tryptophan oxidation by the catalytic peroxidase activity of metMb consumed oxygen from the solution (Fig. 7), a reaction that was inhibited by the inclusion of the spin traps DBNBS and MNP. That result is consistent with the previously reported competition between the spin traps and oxygen for the globin radical formed in the metMb/hydrogen peroxide system (28). Since free tryptophan radical reacts with oxygen, it is likely that a tryptophan radical in a protein would also do so to form a peroxyl radical. We examined a computer-generated space-filling model calculated using theoretical van der Waals atomic radii from the crystal structure of horse metMb (50). Both tryptophan residues of metMb were found to be in contact with the solvent, with tryptophan 7 having more surface contact than either of the tyrosine residues. The generation of a protein-centered radical has been suggested to be necessary for the oxidation of styrene by metMb and hydrogen peroxide (51).

The present results provide strong evidence that the globin-centered radical that is detected by ESR and spin trapping is centered on a tryptophan residue. Spectral evidence strongly indicates that the radical adduct is formed at the C-3 carbon of the indole ring, which is also the most likely atom at which oxygen addition to form the peroxyl radical occurs, as is shown in Scheme I. Whether the radical adduct is formed on only one or both of the tryptophan residues in metMb, which a search of the metMb sequences in the PIR protein data base revealed to be rigidly conserved among terrestrial vertebrates, cannot be resolved at this time. The presence of the globin radical on a tryptophan residue that is near the surface of the protein provides a mechanism for the catalytic activity of metMb in the oxidation, peroxidation, or epoxidation of large substrates that do not have ready access to the heme iron.

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