Reaction of Mycobacterium tuberculosis Truncated Hemoglobin O with Hydrogen Peroxide

EVIDENCE FOR PEROXIDATIC ACTIVITY AND FORMATION OF PROTEIN-BASED RADICALS

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In this work, we investigated the reaction of ferric Mycobacterium tuberculosis truncated hemoglobin O (trHbO) with hydrogen peroxide. Stopped-flow spectrophotometric experiments under single turnover conditions showed that trHbO reacts with H2O2 to give transient intermediate(s), among which is an oxoferryl heme, different from a typical peroxidase Compound I (oxoferryl heme π-cation radical). EPR spectroscopy indicated evidence for both tryptophanyl and tyrosyl radicals, whereas redox titrations demonstrated that the peroxide-treated protein product retains 2 oxidizing eq. We propose that Compound I formed transiently is reduced with concomitant oxidation of Trp(G8) to give the detected oxoferryl heme and a radical on Trp(G8) (detected by EPR of the trHbO Tyr(CD1)Phe mutant). In the wild-type protein, the Trp(G8) radical is in turn reduced rapidly by Tyr(CD1). In a second cycle, Trp(G8) may be reoxidized by the ferryl heme to yield ferric heme and two protein radicals. In turn, these migrate to form tryrosyl radicals on Tyr55 and Tyr115, which lead, in the absence of a reducing substrate, to oligomerization of the protein. Steady-state kinetics in the presence of H2O2 and the one-electron donor 2,2′-oligomerization of the protein. Steady-state kinetics in the presence of H2O2 and the one-electron donor 2,2′-oligomerization of the protein. Steady-state kinetics in the presence of H2O2 and the one-electron donor 2,2′-oligomerization of the protein. Steady-state kinetics in the presence of H2O2 and the one-electron donor 2,2′-oligomerization of the protein.

In Mycobacterium tuberculosis, the glbO gene encodes truncated hemoglobin O (trHbO).3 The function of trHbO is unknown. Its very high affinity for O2 (nanomolar range) due to a very slow release of O2 (0.004 s⁻¹) makes it unlikely that its function is the delivery of O2. In addition, the slow oxidation of NO by oxy-trHbO (0.6 μM⁻¹ s⁻¹) in comparison with that observed for oxygenated truncated hemoglobin N (745 μM⁻¹ s⁻¹) and oxy-Mb (35–45 μM⁻¹ s⁻¹) also makes an NO detoxification role for trHbO unlikely (1, 2).

The x-ray structure of trHbO reveals the presence of three potentially oxidizable residues, Tyr35(B10), Tyr36(CD1), and Trp88(G8), in the vicinity of the heme, with Trp(CD1) and Trp(G8) with H-bonding distance from the bound ligand (Fig. 1) (3). Studies of trHbO variants suggest that Tyr(CD1) and Trp(G8) control O2 association and dissociation rates, with Tyr(B10) playing a minor role (1). Interestingly, O2, NO, and CO all combine with deoxyferrous trHbO at similar very slow rates (1). These slow ligand-independent combination rates indicate that the electronic factors that give rise to the large ligand-specific differences in most Mbs and Hbs are not dominant in trHbO. Instead, these observations are most consistent with limited access of ligands to the heme iron. Similarly, the comparable slow rate for the reaction of NO with heme-bound O2 of oxy-trHbO demonstrates that access of small molecules to heme-bound ligands is also limited.

Three observations suggest that trHbO may be designed to perform redox reactions. First, there is the presence of the electron-rich oxidizable residues Tyr(B10), Tyr(CD1), and Trp(G8) in the vicinity of the heme, which is quite unusual for a globin. Second, the crystal structure of the cyanomet derivative of M. tuberculosis trHbO revealed the presence of a presumably post-translational Tyr(B10)-Tyr(CD1) cross-link in the heme active site of one-half of the molecules (3). The Tyr-Tyr cross-link adds trHbO to a growing list of hemoproteins that have aromatic amino acids covalently modified in their active sites: cytochrome c oxidase (His240–Tyr244), catalase HP1 (His392–Tyr319), catalase-1 (Cys356–Tyr379), the catalase-peroxidases (KatG; Met255–Tyr229–Trp107), and a cytochrome c peroxidase.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7 and Tables S1 and S2.

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3 The abbreviations used are: trHbO, truncated hemoglobin O; Mb, myoglobin; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HRP, horseradish peroxidase.

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Reactor of a Hemoglobin from M. tuberculosis with \( \text{H}_2\text{O}_2 \)

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mutant (Trp\(^{51}\)-Tyr\(^{52}\)) (4–12). Third, oxy-trHbO is reduced by dithionite to deoxyferrous trHbO without prior dissociation of the oxygenous ligand (1). This is an unusual reaction for an oxyferrous globin, which has also been reported for trHbO of *Bacillus subtilis* (13). The ability of dithionite to reduce heme-bound \( \text{O}_2 \) without the prior dissociation of the \( \text{O}_2 \) and the presence of oxidizable residues in the immediate heme vicinity points to the possibility that highly sequestered ligands may be especially prone to redox reactions. In this regard, substitutions of Phe(CD1) with either Tyr or Trp in Mb have been shown to promote changes in redox properties (14, 15).

As a first approach to evaluate the redox properties of trHbO, we investigated its reactivity with \( \text{H}_2\text{O}_2 \) and its ability to catalyze oxidation reactions using \( \text{H}_2\text{O}_2 \). To obtain ferric hemeprations in a deprotonated form (16). In many cases, the latter clearly identified in the mutant Tyr(CD1)Phe. In the absence of a reducing substrate, the protein radicals lead to oligomerization of the protein involving Tyr\(^{55}\) and Tyr\(^{115}\). The steady-state kinetics in the presence of \( \text{H}_2\text{O}_2 \) and the one-electron donor ABTS indicate that trHbO has peroxidase activity. These findings may provide insights into the function of trHbO and other Group II trHbs.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification**—Amino acid substitutions were carried out using the QuikChange site-directed mutagenesis kit (Stratagene) following the recommended protocol. The cloned *M. tuberculosis* *glbO* gene was used as a template with the following complementary oligonucleotide primers: Tyr(B10)Phe, 5'-GTGGACCGTGTTCTTTGTGGCAGGTGCCGACCC-3' with 5'-GGCGACCTGGCAGCAAAGGCGCAAC-3'; Tyr(CD1)Phe, 5'-CTGGCCGGGGTGTTCCCCAGAAGATGAC-3' with 5'-GTCATCTTCCGGGGAAACACGGCAGGACCC-3'; and Trp(G8)Phe, 5'-GGAACGCCCGCAGGGTACGTGGCTGTCATG-3' with 5'-CATGCACCGCAGAGGCGGCGGTCCGAT-3'. The expression and purification of the recombinant proteins were performed in accordance with a previously published method (25).

**Chemicals**—\( \text{H}_2\text{O}_2 \) (30%, \( \nu/\nu \)) was obtained from BDH. The concentration of the stock solution was determined spectrophotometrically at 240 nm (\( \varepsilon = 43.5 \text{ M}^{-1} \text{ cm}^{-1} \)). Horseradish peroxidase (HRP), ferricytochrome \( \text{c} \), ABTS, (hexa)amine ruthenium III, sodium ascorbate, NADH and KCN were obtained from Sigma.

**Buffer**—Except when noted, all solutions were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing 50 \( \mu \text{M} \) EDTA.

**Optical Absorption Spectroscopy**—Optical absorption spectra were recorded using a Cary 3E spectrophotometer (Varian, Inc., Mississauga, Canada) equipped with a temperature-controlled multicell holder. Ferric trHbO samples were prepared in buffer. All spectra were recorded at 5 \( \mu \text{M} \) trHbO and 23 °C and analyzed using KaleidaGraph software (Synergy Software).

**Determination of \( \text{H}_2\text{O}_2 \) Concentration**—\( \text{H}_2\text{O}_2 \) concentration was determined using a peroxidase assay. \( \text{H}_2\text{O}_2 \) consumption was detected by the HRP-catalyzed formation of ABTS oxidation product at 414 nm in a mixture containing 1 \( \text{mM} \) ABTS, 10 \( \text{nM} \) HRP, and 2.5 \( \mu \text{M} \) trHbO that was reacted or not with \( \text{H}_2\text{O}_2 \) (1 and 2 eq). \( \text{H}_2\text{O}_2 \) concentration was calculated from the increase in the absorbance at 414 nm using a molar extinction coefficient of \( 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \). A standard curve was made using known concentrations of \( \text{H}_2\text{O}_2 \) (0–20 \( \mu \text{M} \)).

**Resonance Raman Spectroscopy**—Protein samples for the resonance Raman experiments were used at a concentration of 50 \( \mu \text{M} \) in buffer. The trHbO/\( \text{H}_2\text{O}_2 \) (1:1) reaction product was obtained by manually mixing ferric trHbO with 0.01 volume of 5 \( \text{mM} \) \( \text{H}_2\text{O}_2 \). trHbO Compound III was formed by reacting 100 \( \mu \text{M} \) ferric protein with 2 \( \text{mM} \) \( \text{H}_2\text{O}_2 \) on ice for 2 min. The excess \( \text{H}_2\text{O}_2 \) was rapidly removed by gel filtration on a P-6DG column equilibrated with buffer, and the protein concentration was then adjusted to 50 \( \mu \text{M} \). The oxyferrous form of trHbO was produced by the reduction of the ferric derivative with sodium ascorbate (1 \( \text{mM} \)) and the mediator (hexa)amine ruthenium III
(5 μM) and was directly transferred in a Raman quartz cell after 10 min. The resonance Raman spectra were obtained as described previously (27). Briefly, the 413-nm line of a krypton ion laser (Innova 302, Coherent Inc., Santa Clara, CA) was used to probe the ferric and H$_2$O$_2$-treated ferric and ferrous oxygenated forms of trHbO. The resonance Raman spectra were calibrated with the lines of indene in the 200–1700 cm$^{-1}$ range. All measurements were made at room temperature. Cosmic ray lines were removed from the spectra by a routine of WinSpec software (Roper Scientific, Trenton, NJ). Several 5-min spectra were acquired over a 30-min period and analyzed using GRAMS/AI software (Thermo Scientific).

**Titration with Ferrocytochrome c**—A stock solution of ferrocytochrome c was prepared by reducing ferrocytochrome c with 10 mM ascorbate. The progress of the reduction reaction was followed by optical absorption spectroscopy in the visible region. Excess ascorbate was then removed by gel filtration on a Bio-Gel P-6DG column (10 ml) equilibrated with buffer. The concentration of the stock solution was determined spectrophotometrically at 550 nm (ε$_{550} =$ 27.6 mm$^{-1}$ cm$^{-1}$) (28). The stock solution was kept frozen in liquid nitrogen until used. To determine the number of oxidizing equivalents in the product formed by the reaction of ferric trHbO with 1 eq of H$_2$O$_2$, increasing amounts of ferrocytochrome c were premixed with 5 μM ferric trHbO in 50 mM potassium phosphate buffer (pH 7.0). The reactions were started by the addition of H$_2$O$_2$ to a 5 μM final concentration and followed at 550 nm. The extinction coefficient Δε$_{550}$ (ferrocytochrome minus ferrocytochrome c = 19.6 mm$^{-1}$ cm$^{-1}$) was used to calculate the yield of oxidized cytochrome c (28). This experiment was performed twice with trHbO and once with HRP. The graph presented in Fig. 3 represents one set of data obtained with each of the two proteins. A control experiment in which 5 μM ferrocytochrome c was mixed with 5 μM H$_2$O$_2$ showed that <5% of the ferrocytochrome c was oxidized in the absence of trHbO.

**Oligomerization and SDS-PAGE of Protein Samples**—Cross-linking experiments were performed in buffer at 23 °C for 5 min. Ferric trHbO (20 μM) was reacted with 0, 0.25, 0.5, 1, 5, 10, 20, and 40 eq of H$_2$O$_2$ in a total volume of 75 μl. Following incubation, excess H$_2$O$_2$ and other low molecular weight substances were removed by gel filtration on a Microspin P-6 column (Bio-Rad) equilibrated with 30 mM Tris-Cl buffer (pH 6.8). SDS-PAGE sample buffer was added to the protein samples and heated at 65 °C for 5 min prior to loading onto 15% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue (ICN). The effect of NADH, ascorbate, mannitol, and KCN was checked by including these agents separately (1 mM) in the reaction mixture before the addition of H$_2$O$_2$ (H$_2$O$_2$/trHbO molar ratio of 5).

**Separation and Quantification of trHbO Cross-linked Products by Gel Filtration Chromatography**—Protein samples (100 μM) in buffer were exposed to 0, 1, and 3 molar eq of H$_2$O$_2$ for 5 min at 23 °C. Following incubation, excess H$_2$O$_2$ was removed by gel filtration on a Microspin P-6 column equilibrated with buffer containing 300 mM KCl. Protein samples were subjected to size exclusion chromatography at 23 °C on a Superdex 75 HR 10/30 column equilibrated in buffer containing 300 mM KCl. Gel filtration standards (Bio-Rad catalog no. 151-1901) were used to calibrate the column. The standards were dissolved in 100 μl of buffer containing 300 mM KCl. Elution of the protein was followed at 280 nm. The area under the peaks was determined and used to estimate the proportion of cross-linked proteins.

**Measurement of Catalytic Oxidation Activities**—The peroxidase catalytic pathway of trHbO and horse heart myoglobin was investigated by stopped-flow spectrophotometry (SX.18MV stopped-flow spectrophotometer, Applied Photophysics Ltd., Leatherhead, UK) at 23 °C in buffer. At least two experiments were performed for each experimental point. Steady-state kinetic constants for the oxidation of ABTS were determined by measuring the initial rates while varying the H$_2$O$_2$ concentration. V$_{max}$ and K$_m$ values were determined by fitting data with the Levenberg-Marquardt robust method (SoftZymics, Inc., Princeton, NJ). The formation rate of the ABTS oxidation product was determined from the increase in the absorbance at 414 nm using a molar extinction coefficient of 3.6 × 10$^4$ M$^{-1}$ cm$^{-1}$ (26). The reaction mixture contained 50 mM protein, 1 mM ABTS, and 0.1–100 mM H$_2$O$_2$.

**Kinetics Study of the Formation of trHbO Higher Oxidizing Intermediates**—Stopped-flow experiments were carried out with the SX.18MV stopped-flow spectrophotometer equipped with a photodiode-array detector. The integration time was 2.5 ms. Ferric proteins (5 μM) were reacted with 1 molar eq of H$_2$O$_2$. 1600 spectra were collected on time scales ranging from 4 to 524 s. Singular value decomposition and global analysis were performed using the ProK program (Applied Photophysics Ltd.). Kinetics constants obtained from fitting had uncertainties of ±5%. The results shown in Figs. 7–10 are representative of at least two experiments.

**EPR Spectroscopy**—X-band EPR spectra were recorded using a Bruker E500 EPR spectrometer with data acquisition and manipulation performed using XeprView and WinEPR software (Bruker Daltonics Inc., Billerica, MA). Low temperature spectra were recorded using an Oxford Instruments Spectrostat continuous-flow cryostat and ITC503 temperature controller for temperatures from 4 to 20 K. A finger Dewar flask inserted into the microwave cavity was used for recording spectra at liquid nitrogen temperature (77 K). The spectra of ferric proteins (200 μM) were recorded in 50 mM Tris-HCl buffer (pH 7.5) at 4 K. For spectra of intermediates, ferric proteins (167 μM final concentration) were reacted with 3 molar eq of hydrogen peroxide, both in 50 mM Tris buffer (pH 7.5) for 10 s and then rapidly frozen in precision bore EPR tubes immersed in liquid nitrogen. The samples were examined at 77 K (microwave frequency of 9.3910 GHz), 4 K, and 20 K (microwave frequency of 9.4940 GHz). Simulation of EPR data was performed using SimFonia software (Bruker Daltonics Inc.).

Electron transfer coupling factors were calculated by PATHWAYS analysis (29) and the HARLEM program based on coordinates from the wild-type trHbO crystal structure (Protein Data Bank code 1NGK) (30). This analysis provides the optimal donor-acceptor electron transfer pathway between tryptophan or tyrosine residues and the heme or between particular residues.
RESULTS

Spectroscopic Studies of the Reaction of trHbO and H2O2

Fig. 2A shows the UV-visible spectra of wild-type ferric trHbO and the products of its reaction with 1 and 2 eq of H2O2. The latter spectra recorded after 10 min had elapsed after the addition of peroxide are very similar to that of the initial ferric form, suggesting either that H2O2 did not react with the protein or that the heme iron atom of the product after turnover of peroxide had returned to the ferric state. The former possibility was ruled out because the addition of HRP and the peroxidase substrate ABTS after 10 min resulted in the barely detectable oxidation of the substrate (data not shown), consistent with the absence of unreacted peroxide in the solution. However, when the same reactions were performed in the absence of trHbO (but in the presence of HRP), 2 mol of oxidized ABTS were produced for each mole of added H2O2. The finding that all of the added H2O2 had been exhausted during the reaction indicated that trHbO was able to absorb at least 2 oxidizing eq and that a new product indeed was formed. In contrast, the spectra of the products of the reaction of HRP with H2O2 are those of Compounds I and II, as expected (Fig. 2B) (31).

The product of the reaction of trHbO with H2O2 was investigated further by resonance Raman spectroscopy. The high frequency region (1300–1700 cm⁻¹) of the resonance Raman spectra of hemeproteins is composed of several porphyrin in-plane vibrational modes, which are sensitive to the oxidation state, coordination number, and spin state (ν1, ν2, and ν3) of the heme iron (32). As shown in supplemental Fig. S1A, the resonance Raman spectra of untreated trHbO and H2O2-treated trHbO are nearly identical, consistent with the UV-visible spectroscopic observations above, suggesting that the heme iron is in the ferric state after incubation with peroxide.

To demonstrate that the reaction with hydrogen peroxide generates a new oxidation state and to evaluate how many oxidizing equivalents are stored, we titrated the protein product of the reaction of trHbO and 1 eq of H2O2 with the one-electron donor ferrocytochrome c. We chose ferrocytochrome c as reductant for two reasons: 1) it does not reduce ferric trHbO to ferrous trHbO, which avoids possible side reactions involving molecular O2; and 2) it does not react with H2O2 under the conditions used. When 5 μM ferric trHbO was incubated simultaneously with 1 eq of H2O2 and ferrocytochrome c, a maximum of 10 μM ferrocytochrome c could be oxidized to ferricytochrome c (Fig. 3). Thus, both oxidizing equivalents from H2O2 were retained in the trHbO product, although, as shown above, the iron was found in the ferric state when observed after manual mixing with peroxide. Titration of HRP under these conditions indicated that the expected 2 oxidizing eq were retained in the product.

Most peroxidases and many Hbs and Mbs form Compound III at high H2O2 concentration (33–36). This species is similar to oxy-Mb. As shown in Fig. 2A, mixing ferric trHbO with 100 molar eq of H2O2 gave a stable species with an absorption spectrum identical to that of oxy-trHbO. To confirm the identity of the trHbO species, we used resonance Raman spectroscopy. As shown in supplemental Fig. S1B, the spectrum of H2O2-treated trHbO is nearly identical to that of oxy-trHbO, with ν1, ν2, and ν3 mode lines at 1379, 1506, and 1582 cm⁻¹, respectively, indicating a reaction with heme iron, but no modification or breakdown of the macrocycle. These results demonstrate that, in common with many Hbs and peroxidases, trHbO forms Compound III readily at high H2O2 concentrations.

Oligomerization of H2O2-treated trHbO

The preceding results led us to hypothesize that 2 oxidizing eq, initially resident on the heme and its ligand, may have been transferred to amino acid residues near the heme to form radicals. These primary protein-based radicals may have then led, via internal electron transfer, to the formation of surface-ex-
posed amino acid radical(s) with subsequent cross-linking of the protein. Such reactions have been observed in several heme proteins, including sperm whale Mb, when reacted with 
H$_2$O$_2$ in the absence of an exogenous electron donor (37–43). In most cases reported to date, the process involves the formation of tyrosine-tyrosine cross-links. In trHbO, there are six tyrosines, Tyr$^6$, Tyr$^{23}$(B10), Tyr$^{36}$(CD1), Tyr$^{55}$, Tyr$^{62}$, and Tyr$^{115}$ (Fig. 1), potentially available for conversion to tyrosyl radicals and for quenching by radical combination. Of these, Tyr$^6$ and Tyr$^{55}$ are exposed to the solvent. Accordingly, we reacted ferric trHbO with increasing amounts of H$_2$O$_2$, and analyzed the samples by SDS-PAGE to detect the formation of cross-linked products. As shown in Fig. 4A, trHbO dimers were detected with as little as 0.25 molar eq of H$_2$O$_2$. The amount of the cross-linked dimers increased with the H$_2$O$_2$ concentration, reaching a maximum with 10 molar eq of H$_2$O$_2$. Above 5 molar eq of H$_2$O$_2$, small amounts of trimer and tetramer were observed, suggesting the existence of more than one surface-exposed radical site. It should be noted that the amount of trimers and higher oligomers varied from one experiment to the other.

To determine the number of sites available for cross-linking, we determined the relative proportion of the cross-linked products formed upon reaction of trHbO with 1 or 3 eq of H$_2$O$_2$. For this, the reaction products were separated by gel filtration chromatography, and the peak areas corresponding to the different species were calculated from the elution profiles. The fraction of the total area attributed to dimers was 44 or 65% for the protein that had reacted with either 1 or 3 eq of H$_2$O$_2$, respectively (supplemental Fig. S2). The content of trimers, which was very low (~0.6%) at 1:1, increased to ~5% at 3:1, consistent with the presence of at least two surface-exposed protein-based radicals (supplemental Fig. S2).

To determine whether trHbO oligomerization requires reaction of H$_2$O$_2$ at the heme, we repeated this experiment with the cyanomet derivative of trHbO. Blocking the heme by bound cyanide inhibited the oligomerization reaction, confirming that the initial reaction of trHbO with H$_2$O$_2$ must involve the heme group and, accordingly, that H$_2$O$_2$ did not directly oxidize surface-exposed residues (Fig. 4B).

In another set of experiments, we examined the effects of two reductants, NADH and ascorbate, on the oligomerization of trHbO. As shown in Fig. 4B, the addition of excess NADH or ascorbate to the reaction mixture completely inhibited the formation of cross-linked products. These observations suggest either that both agents reduced an initial product in which the 2 oxidizing eq resided on the peroxide-treated protein or that they both directly reduced the surface-exposed radicals.

We also tested the effect of mannitol, a specific quencher of the hydroxyl (OH$^-$) radical (44). The OH$^-$ radical is a freely diffusible oxidant potentially generated by homolitic cleavage of heme-bound H$_2$O$_2$, which can diffuse outside the active site.
and attack surface-exposed residues to create radicals. As shown in Fig. 4B, mannitol did not inhibit the oligomerization reaction.

Additional species migrating slightly faster or slower than the monomer and dimer species were also observed (Fig. 4C). These species may have arisen from either intramolecular cross-linking or oxidative cleavage of the amino acid chain backbone (45).

Identification of the Residue(s) Involved in Protein Oligomerization

To identify the site(s) of dimerization, we produced six mutants in which a single tyrosine was replaced with phenylalanine. Each of these mutants was incubated with 5 mM H2O2 and analyzed by SDS-PAGE. As shown in Fig. 5, all of the mutants formed cross-linked products to about the same extent as did wild-type trHbO. Interestingly, in both the Y55F and Y115F variants, the slower and faster migrating species were no longer observed. Because Tyr55 and Tyr115 are close to each other in the folded protein, with Tyr55 exposed to solvent, we produced the double mutant Y55F/Y115F and checked for the formation of cross-linked products. As demonstrated in Fig. 5, Y55F/Y115F no longer formed dimers.

Peroxidase Activity of trHbO

We examined whether trHbO possesses peroxidase activity. For this, the one-electron oxidation of ABTS to its correspond-
shown). The difference spectrum shown in Fig. 7B (spectrum B minus spectrum A of Fig. 7A) shows peaks at 427, 528, 561, and 600 nm. The 427, 528, and 561 nm peaks are very similar to the 424, 530, and 563 nm peaks seen in yeast cytochrome c peroxidase Compound I (18, 19, 23). The 600 nm peak is unique to the spectrum of trHbO and will be discussed below. The peaks at 528 and 561 nm are reminiscent of an oxoferryl heme. The difference spectrum thus indicates that the intermediate that we labeled SP-427, similar to yeast cytochrome c peroxidase Compound I, is formed transiently during the reaction. The evolution of SP-427 over the time course of the reaction revealed that SP-427 accumulated over the first 115 s and then decayed slowly to species C (supplemental Fig. S4C). The most likely pathway to SP-427 is through a rapid one-electron reduction of a classical Compound I intermediate by an amino acid.

Tyr(B10)Phe Mutant—As noted for the wild-type protein, spectra A and B are almost identical and indicate a six-coordinated low spin ferric heme (Fig. 8A) (47). The difference spectrum shown in Fig. 8B (spectrum B minus spectrum A of Fig. 8A) of the Tyr(B10)Phe protein shows peaks at 427, 528, and 561 nm but lacks one at 600 nm. This implies that the 600 nm peak is related to Tyr(B10). In Tyr(B10)Phe, the abundance of SP-427 increased and declined more rapidly than in the wild-type protein, leading to a fast accumulation of the final product, species C (Table 1 and supplemental Fig. S5C). Spectrum C is not that of a pure species, but represents part of a heme-bleaching process evidenced by a loss of absorbance at the Soret band and a greater absorbance in the 600–700 nm region. The behavior in this mutant is therefore suggested to result from a more efficient electron transfer process compared with the wild-type protein but also from a greater sensitivity of the heme to oxidant.

Tyr(CD1)Phe Mutant—The spectral changes of the Tyr(CD1)Phe mutant were more significant. Spectrum A (corresponding to the initial form) shows a high and sharp Soret band at 405 nm and a well developed CT1 band at 634 nm, indicating that the Tyr(CD1)Phe mutant is enriched in six-coordinated high spin heme compared with the wild-type protein (Fig. 9A) (47). Spectrum B shows a Soret band at 410 nm with a broad absorbance centered at 530 nm. Spectrum C is similar to that of the initial form. The difference spectrum shown in Fig. 9B (spectrum B minus spectrum A of Fig. 9A) shows the same SP-427 peaks as observed in the wild-type protein except that the difference in absorbance is 6-fold higher. These data suggest that Tyr(CD1) is involved in the formation and/or decay of SP-427.

Trp(G8)Phe Mutant—Fig. 10A presents the singular value decomposition and global analysis of the reaction of the ferric Trp(G8)Phe mutant with H2O2. Spectrum B is representative of a six-coordinated high spin complex with a more intense Soret band at 405 nm and a CT1 band at 630 nm (47). Spectrum C is similar to species B except for a small decrease in intensity at the Soret band. The difference spectrum shown in Fig. 10B was measured at 414 nm. B, plot of the initial velocities (Vi) versus H2O2 concentration for trHbO. C, plot of the initial velocities versus H2O2 concentration for horse heart Mb. The data were fitted to the Michaelis-Menten equation using the Levenberg-Marquardt method.
A Hemoglobin from M. tuberculosis with H2O2

**TABLE 1**

| trHbO   | Soret | Visible | Soret | Visible | Soret | Visible |
|---------|-------|---------|-------|---------|-------|---------|
| Wild-type | 405  | 500, 537, 577, 636 | 405  | 507, 537, 577, 634 | 405  | 537, 577, 636 |
| Tyr(B10)Phe | 411  | 541, 580  | 411  | 541, 580  | 410  | 541, 580  |
| Tyr(CD1)Phe | 405  | 496, 539, 581, 636 | 410  | 530, 581  | 405  | 496, 539, 581, 636 |
| Trp(G8)Phe | 406  | 496, 541, 580, 632 | 405  | 496, 541, 580, 632 | 405  | 496, 541, 580, 632 |

| Wild-type | 1.9E-02 ± 4.8E-05 | 3.3E-02 ± 7.2E-04 | 6.5E-02 ± 3.8E-05 | 7.8E-03 ± 3.1E-05 |
| Tyr(B10)Phe | 4.3E-04 ± 9.3E-06 | 1.2E-03 ± 3.8E-05 | 1.9E-03 ± 2.7E-06 | 4.0E-03 ± 1.9E-05 |

**TABLE 2**

| trHbO | Soret | Visible | Soret | Visible | Soret | Visible |
|--------|-------|---------|-------|---------|-------|---------|
| Wild-type | 2.004 | 2.005 (Fig. 11A) | 5.80 and 2.16–2.20, | 1.69–1.79 (supplemental Table S1). The low spin species in the mutants compared with the wild-type protein are likely due to small structural differences in the distal side residues within the heme pocket. trHbO Reacted with H2O2—Resting (ferric) protein was mixed with a small excess (3-fold) of hydrogen peroxide and frozen after 10 s of incubation. These conditions are expected first to produce hypervalent heme iron and then to generate amino acid-based radicals according to the observations reported above. EPR spectra were recorded at 4, 20, and 77 K in attempts to identify the intermediates and/or radical species. A signal at g = 2.004 with a line width of ~21 G was detected at 77 K in all samples (Fig. 11A) with no or poorly resolved hyperfine splitting. In addition, a small proportion of ferric iron was still present as evidenced by g = 6 signals visible in the low field region of spectra recorded at 4 K (data not shown). Preliminary simulation of the g = 2.004 signal for wild-type trHbO suggested that it arises from a tyrosyl radical, as hyperfine interactions for two nonequivalent β-methylene protons with coupling constants of ~12 and ~1 G, similar to protein-based tyrosyl radical species formed in other enzymes (50–52), gave an adequate fit to the data. The hyperfine coupling parameters in tyrosyl radicals depend on the orientation of the phenolic ring plane relative to the position of β-methylene protons (24, 53). Examination of the three-dimensional crystal structure of trHbO suggested that Tyr(B10), Tyr(CD1), or Tyr115 could give rise to tyrosyl radicals with EPR signals similar to those observed here. However, no further analysis or detailed simulation was performed because of the heterogeneous nature of the structures of Tyr(B10) and Tyr(CD1), which are covalently linked in some subunits of the enzyme according to the three-dimensional crystal structure (3). Such linkage would remove the contribution of some ring protons from the total hyperfine coupling in the spectra of the radicals. Furthermore, neutral tryptophanyl radicals can give signals that may overlap with tyrosyl radical signals in X-band EPR spectra. Rapid freeze-quench EPR experiments were also performed to examine radical formation at time scales <1 s, but the low intensity of the signals did not provide useful information (data not shown).

EPR spectra were also recorded at liquid helium temperatures (on the same samples as described above) in an attempt to identify other heme or radical intermediates formed in the protein upon treatment with peroxide. No new features were found at 20 K for any of the samples (Fig. 11B, upper spectrum shown as an example), whereas at 4 K, the Tyr(CD1)Phe mutant exhibited a new axial signal with effective g-values of g∥ = 2.026 and g⊥ = 2.005 (Fig. 11B, lower spectrum). This spectrum is very similar to that of the exchange-coupled tryptophanyl π-cation radical species found in cytochrome c peroxidase Compound I (54, 55). Therefore, the signal found at low temperature in this mutant is reasonably assigned to the same type of tryptophan radical exchange-coupled to oxoferryl heme (S = 1). A small component due to the signal from a tyrosyl radical is present with diminished intensity because of power saturation at this temperature (20-milliwatt microwave power) (Fig. 11B, lower spectrum). No evidence for a classical oxoferryl heme π-cation radical (classical peroxidase Compound I) was found in the EPR spectra of any of the proteins, consistent with rapid electron transfer(s) from the nearby amino acids that quench this intermediate. Although an EPR signal arising from a Trp cation radical coupled to oxoferryl heme as in cytochrome c peroxidase Compound I could be present along with a tyrosyl radical in the same protein molecule (according to the fact that 2 oxidizing eq...
are stored), its contribution at X-band is not apparent in the spectra of the wild-type protein at 77 and 4 K.

An analysis of potential redox partners using PATHWAYS analysis helped to confirm the idea that Trp(G8) and Tyr(CD1) are optimally coupled to the heme and to each other (see “Discussion” and supplemental Table S2) (30) for electron transfers. Although Tyr62 can also be a candidate for electron transfer and tyrosyl radical formation, the apparent involvement of Trp(G8), the poor coupling between Tyr62 and Trp68, and the good coupling between Tyr(CD1) and Trp(G8) suggest that Tyr62 may be only a secondary site of radical formation. Furthermore, a Tyr62 radical is predicted based on the x-ray crystal structure of trHbO to give an EPR signal with a line width narrower than that observed here.

DISCUSSION

UV-visible spectroscopy and titration experiments demonstrated that the reaction of ferric trHbO with 1 eq of H₂O₂ produces a novel intermediate containing ferric heme iron and 2 oxidizing eq resident on the protein. Resonance Raman data confirmed that the electronic properties of the resting protein and the peroxide-reacted protein observed minutes after mixing are nearly identical. However, the latter species contains 2 oxidizing eq titratable with ferrocytochrome c. In contrast, when ferric Mb, which is structurally and evolutionary related
to trHbO, is reacted with 1 eq of H\textsubscript{2}O\textsubscript{2}, a “putative” Compound I is transiently formed and converted spontaneously to an oxoferryl species and a protein radical (56, 57). The oxoferryl species auto-returns slowly over several minutes or hours to the ferric state.

The lack of evidence for a classical Compound I in trHbO could be explained if the reaction pathway initiated by peroxide involves rapid formation and immediate reduction of this intermediate. Our EPR experiments demonstrating both Trp and Tyr radical formation are consistent with such a pathway. Under the conditions of the optical stopped-flow experiments and the rapid freeze-quench EPR experiments, both of which operate on the millisecond time scale, Compound I remained undetected. These observations suggest that Compound I is formed slowly (under the conditions using either 1 eq or only a small excess of peroxide) and is rapidly reduced by the electron transfer processes, leading to amino acid radicals. To our knowledge, trHbO represents a first case for a hemeprotein in which the accumulated hydrogen peroxide product has a ferric heme with 2 oxidizing eq on the protein.

**Oxidation Activity of trHbO**—Steady-state kinetic analyses of the H\textsubscript{2}O\textsubscript{2}-dependent oxidation of ABTS by trHbO revealed that it has peroxidase activity (1350 m\textsuperscript{-1} s\textsuperscript{-1}), ~3-fold higher than that of horse heart Mb but 100–1000-fold lower than that reported for class III peroxidases such as HRP (16, 58–60).
However, the turnover rate for ABTS peroxidation (50 s⁻¹ at 40 mM H₂O₂) is much faster than that in horse heart Mb (k_{cat} = 0.45 s⁻¹). The latter observation suggests that the active site of trHbO may have evolved to perform oxidation reactions. In this regard, dehaloperoxidase from the polychaete worm Amphitrite ornata provides strong evidence for a peroxidase having evolved from an oxygen carrier (61–63). Dehaloperoxidase catalyzes the dehalogenation of halometabolites in the presence of H₂O₂, allowing A. ornata to inhabit sediments contaminated by halometabolites.

Kinetic and EPR Studies of the Reaction of H₂O₂ with Wild-type trHbO and the Heme-distal Mutants under Single Turnover Conditions—Kinetic analyses of the reaction of H₂O₂ with wild-type trHbO and its mutants did not reveal evidence for the formation of a classical Compound I. However, kinetic difference spectra revealed the existence of an intermediate species (SP-427) in wild-type trHbO and the Tyr(B10)Phe and Tyr(CD1)Phe mutants. SP-427 has characteristics very similar to those reported for yeast cytochrome c peroxidase Compound I except for an additional band at 600 nm. The 600 nm band is lost in mutant Tyr(B10)Phe (Fig. 8B), suggesting that it may arise from Tyr-Tyr radical interactions. Although it represents only a rough estimate of SP-427 concentration, the absorbance at 427 nm of the difference spectra indicates that SP-427 levels are ~6-fold higher in Tyr(CD1)Phe than in the wild-type protein, implying that Tyr(CD1) is involved in the decay of SP-427.

EPR analyses performed at liquid helium temperatures revealed a transient tryptophanyl radical signal, similar to the cytochrome c peroxidase Compound I species, in the Tyr(CD1) Phe mutant reacted with H₂O₂. The absence of this signal in the wild-type protein is consistent with Tyr(CD1) being intimately involved in the rapid quenching of the tryptophanyl radical. The proximity of Tyr(G8) to the heme makes it a likely candidate for the tryptophanyl radical cation. There is an additional tryptophan (Trp⁵⁶) in trHbO, but the latter is located 14 Å from the heme iron. SP-427 could not be detected in the Trp(G8)Phe mutant, which underscores the participation of Trp(G8) in the formation of SP-427. However, a tyrosyl radical is still found in the EPR spectra of this mutant, which indicates that, in addition to electron transfer involving Trp(G8) and a Tyr residue, there is also direct oxidation of Tyr residue(s) without any Trp intermediary. Interestingly, the crystal structures of both yeast ferric cytochrome c peroxidase and the cyanomet derivative of trHbO show a distal Trp residue in close proximity to the heme. In both proteins, the indole ring is oriented parallel to the heme plane, and the nitrogen of the indole is positioned within H-bonding distance of their respective ligands to iron (3, 64).

Figure 11 shows EPR spectra of radicals formed in wild-type trHbO and its mutants recorded at 77 K. Resting enzymes (167 μM final concentration) were mixed with a 3-fold molar excess of H₂O₂ at 25 °C in precision bore EPR tubes. Reaction mixtures were quenched after 10 s by immersion of tubes in liquid nitrogen. Spectrometer conditions were as follows: modulation amplitude, 4 G; microwave power, 1 milliwatt (mW), and modulation frequency, 100 kHz. A, EPR spectra of tyrosyl (upper spectrum) and tryptophanyl radical cations in the trHbO Tyr(CD1)Phe mutant recorded at 4 and 20 K. The arrows indicate the axial signal similar to that observed for Compound I of cytochrome c peroxidase (SP-427 in trHbO).
with $\text{H}_2\text{O}_2$, pointing to different functions for these two residues (23).

We propose that Trp(G8) is very rapidly oxidized in trHbO by the porphyrin radical cation of a postulated Compound I (Fig. 12, Reaction 1) to generate oxoferryl heme and a Trp(G8)-centered radical (Reaction 2). The Trp(G8) radical is in turn rapidly reduced by Tyr(CD1) (as only the tyrosine radical appears in the EPR spectra of the wild-type protein) (Reaction 3). Although speculative, Trp(G8) may then be reoxidized, this time by the oxoferryl heme, leaving a ferric heme and two protein radicals (Reaction 4). The Trp(G8) radical must again propagate to other residues, as the EPR spectra of the wild-type protein does not reveal the Trp radical cation found in the Tyr(CD1) mutant. Direct oxidation of other residues (by oxoferryl heme) could also lead to the formation of other tyrosyl radicals.

PATHWAYS analysis (29, 30), which provides the optimal donor-acceptor electron transfer pathway between particular residues, was used to calculate the optimal paths and to predict the electron coupling factors for tyrosines and Trp(G8) (supplemental Table S2) near the heme. The electron transfer coupling constants decrease in the order $\text{Tyr}^6^{2} > \text{Trp(G8)} > \text{Tyr(CD1)} > \text{Tyr}^{115} > \text{Trp(B10)} > \text{Tyr}^{55} > \text{Trp}^{56}$, whereas the order for the donor-acceptor coupling to Trp(G8) is $\text{Tyr(CD1)} > \text{Trp(B10)} > \text{Tyr}^{62}$, confirming Trp(G8) and Tyr(CD1) as primary candidates (if Trp(G8) is always first to become a radical) and Tyr(B10) among secondary sites for radical formation. Even though Tyr$^{62}$ is predicted to be more efficiently coupled to the heme compared with Trp(G8) and Tyr(CD1), it is not the principal tyrosyl radical detected by EPR as stated above. This residue may not be susceptible to radical formation because of its weaker coupling to Trp(G8), the latter being the preferred site for electron transfer to heme intermediates in all cases. A tyrosine at the CD1 position is unique to the Group II trHbs from actinomycetes (2). In all 3/3 globins and in most trHbs, a Phe residue occupies this position. The retention of Tyr in Group II trHbs may thus reflect an ancient and functional adaptation. By contrast, Trp(G8) is an invariant residue found in both Group II and III trHbs.

**Conclusion**—The presence of Tyr(B10), Tyr(CD1), and Trp(G8) makes the active site of trHbO unique within the globin family. trHbO does not form a stable Compound I and/or II upon reaction with $\text{H}_2\text{O}_2$. This is in contrast to heme peroxidases, which form a stable Compound I and/or II plus a protein radical upon reaction with $\text{H}_2\text{O}_2$. The reaction of $\text{H}_2\text{O}_2$ with trHbO involves formation of a transient oxoferryl species (SP-427) with a short-lived radical likely on Trp(G8) that is reduced by Tyr(CD1). Overall, our data indicate that trHbs constitute a new enzymatic system to study how heme proteins perform oxidation reactions.

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Reaction of a Hemoglobin from M. tuberculosis with H₂O₂