Horse Heart Myoglobin Catalyzes the H_2O_2-Dependent Oxidative Dehalogenation of Chlorophenols to DNA-Binding Radicals and Quinones†

Robert L. Osborne,‡ Michael K. Coggins,‡ Mike Walla,‡ and John H. Dawson*,‡,§

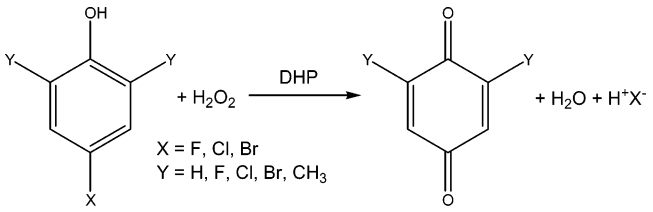
Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, and School of Medicine, University of South Carolina, Columbia, South Carolina 29208

Received April 11, 2007; Revised Manuscript Received May 22, 2007

ABSTRACT: The heme-containing respiratory protein, myoglobin (Mb), best known for oxygen storage, can exhibit peroxidase-like activity under conditions of oxidative stress. Under such circumstances, the initially formed ferric state can react with H_2O_2 (or other peroxides) to generate a long-lived ferryl [Fe(IV)═O] Compound II (Cpd II) heme intermediate that is capable of oxidizing a variety of biomolecules. In this study, the ability of Mb Cpd II to catalyze the oxidation of carcinogenic halophenols is demonstrated. Specifically, 2,4,6-trichlorophenol (TCP) is converted to 2,6-dichloro-1,4-benzoquinone in a H_2O_2-dependent process. The fact that Mb Cpd II is an active oxidant in halophenol dehalogenation is consistent with a traditional peroxidase order of addition of H_2O_2 followed by TCP. With 4-chlorophenol, a dimerized product is formed, consistent with a mechanism involving generation of a reactive phenoxy radical intermediate by an electron transfer process. The radical nature of this process may be physiologically relevant since recent studies have revealed that phenoxy radicals and electrophilic quinones, specifically of the type described herein, covalently bind to DNA [Dai, J., Sloat, A. L., Wright, M. W., and Manderville, R. A. (2005) *Chem. Res. Toxicol. 18*, 771−779]. Thus, the stability of Mb Cpd II and its ability to oxidize TCP may explain why such compounds are carcinogenic. Furthermore, the initial rate of dehalogenation catalyzed by Mb Cpd II is nearly comparable to that of the same reaction carried out by turnover of the ferric state, demonstrating the potential physiological danger of this long-lived, high-valent intermediate.

Myoglobin (Mb)[1] is an intracellular, heme-containing oxygen storage protein found in skeletal, cardiac (*1*), and smooth muscles (*2*). Its primary function as an O_2 storage reservoir in the ferrous [Fe(II)] oxidation state is well-established. However, under conditions of oxidative stress, oxygen-bound Mb can autoxidize to ferric [Fe(III)] Mb, the reaction of which with peroxides in the presence and absence of substrates has been thoroughly investigated (*3*−*12*). Fe(III) Mb reacts with H_2O_2 by heterolytic O−O bond cleavage to form a transient two-electron ferryl [Fe(IV)═O] intermediate known as Compound I (Cpd I) [Fe(IV)═O/porphyrin π-cation radical] (*6*−*8*). Compound ES (Cpd ES) [Fe(IV)═O/amino acid radical] forms rapidly as a result of transfer of the radical to an adjacent amino acid residue before being reduced to Compound II (Cpd II) [Fe(IV)═O] (*6*, *7*, *13*). Herein, we demonstrate the ability of Fe(IV)═O Mb intermediates to catalyze the oxidative dehalogenation of halophenol substrates.

The first enzymatic globin discovered, dehaloperoxidase (DHP) from the terebellid polychaete *Amphitrite ornata*, catalyzes the H_2O_2-dependent oxidative dehalogenation of halogenated phenols to yield the corresponding quinones (*14*−*17*) (eq 1).



The three-dimensional fold of DHP is nearly isomorphic with Mb (*18*), and this fact motivated us to examine whether Mb will also oxidatively dehalogenate halophenols. The DHP dehalogenation mechanism has been investigated, yet remains unresolved (*15*, *17*, *19*). Enzymatic assays suggest that the mechanism is similar to that for the same reaction catalyzed by *Caldariomyces fumago* chloroperoxidase (CCPO) (*20*), horseradish peroxidase (HRP) (*21*), and lignin peroxidases (*22*, *23*), which has been proposed to involve two, one-electron transfer steps mediated successively by Cpd I and Cpd II (*20*). In this mechanism, the new oxygen atom in the quinone product is derived from a solvent water molecule. Interestingly, an obligatory order of addition involving substrate binding before peroxide that is contradictory to

† Financial support was provided by the University of South Carolina Environmental Research Initiative, the National Science Foundation (Grant MCB 964004), and the National Institutes of Health (Grant GM 26730).

* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, 631 Sumter St., University of South Carolina, Columbia, SC 29208. Phone: (803) 777-7234. Fax: (803) 777-9521. E-mail: dawson@sc.edu.

‡ Department of Chemistry and Biochemistry.

§ School of Medicine.

[1] Abbreviations: Mb, myoglobin; Hb, hemoglobin; DHP, dehaloperoxidase; HRP, horseradish peroxidase; Cpd I, Compound I; Cpd II, Compound II; Cpd ES, Compound ES; TCP, 2,4,6-trichlorophenol; DBQ, 2,6-dichloro-1,4-benzoquinone; UV−vis, UV−visible; CCPO, *Caldariomyces fumago* chloroperoxidase; GC−MS, gas chromatography−mass spectrometry.

10.1021/bi700684u CCC: $37.00
Published on Web 08/04/2007

those of all other known peroxidases (*24*) has recently been proposed for DHP-catalyzed reactions (*17*).

Mb Cpd II is a relatively long-lived intermediate in the absence of substrate, and oxidative stress-induced formation of Fe(IV)═O globins has been implicated in a variety of diseases. For example, an increased amount of Fe(III) Mb is observed in blood under conditions of oxidative stress (*25*). It will react with peroxides to initiate the oxidation of lipids and can also consume lipid hydroperoxides, leading to a cascade of additional oxidative events, including the formation of isoprostanes (*26*−*29*). Additionally, rhabdomyolysis is the consequence of kidney damage caused in part by Fe(IV)═O Mb, which is released into the blood as a result of muscle injury (*30*). Also, subarachnoid hemorrhaging results in the discharge of cerebrospinal fluid in the brain containing hemoglobin significantly damaged by peroxide (*31*).

Chlorinated phenols are common in the pesticide industry and can be found in domestic wastewater (*32*). They are considered toxic, and many exhibit carcinogenic traits. More specifically, 2,4,6-trichlorophenol (TCP) is used as an antiseptic, in pesticides, and as a preservative in wood, leather, and fabric industries. It is also a synthetic precursor of pentachlorophenol, another suspected carcinogen. Additionally, mice exposed to TCP developed leukemia, malignant lymphoma, and soft tissue sarcomas. Consequently, the EPA has classified TCP as a Group B2 or probable human carcinogen (*33*, *34*). Phenols can undergo a one-electron oxidation to form highly reactive phenoxy radicals, and such radicals have been implicated in glutathione oxidation (*35*, *36*) as well as in lipid, protein, and DNA damage (*37*−*39*). Recent studies by Manderville and co-workers have shown that phenoxy radicals and 1,4-benzoquinones can covalently modify DNA following activation by heme-containing peroxidase enzymes or redox-active transition metals (*40*, *41*). Specifically, phenoxy radicals have been shown to bind covalently with the C8 position of deoxyguanosine, yielding both oxygen- and carbon-bound adducts (*41*).

Herein, we demonstrate the catalytic ability of horse heart Mb to oxidatively dehalogenate halophenols to the corresponding quinone products by way of a radical intermediate. 4-Chlorophenol has been used as a mechanistic probe, and the dimeric product that is formed supports an electron transfer mechanism. Additionally, the Fe(IV)═O moiety of Mb Cpd II catalyzes the same oxidative dehalogenation reaction, which suggests that it is an efficient and active oxidant. The potential health risks from toxic chlorinated phenols entering the body through water or food intake are explained here by the formation of reactive phenoxy radicals and quinone products in the presence of Mb Cpd II.

EXPERIMENTAL PROCEDURES

Materials and Instrumentation. Reagent-grade chemicals (Aldrich, ACROS, or Fisher) were used without further purification except for potassium ferricyanide, which was recrystallized from water. H_2O_2 was taken from a 30% stock solution. Myoglobin (Mb) (horse heart type III) was purchased from Sigma. UV−vis absorption spectra were measured using a Cary 400 spectrophotometer interfaced with a Dell personal computer.

Preparation of Samples. Mb was completely oxidized to the ferric state by treatment with potassium ferricyanide, followed by desalting with a P-6 DG (Bio-Rad) column in 100 mM potassium phosphate buffer at pH 7.0 and 4 °C (no glycerol or EDTA present). Mb concentrations were determined using published molar absorptivities ($\epsilon_{409} = 170$ mM^{-1} cm^{-1}) (*42*). Fresh 10 mM H_2O_2 stocks in deionized water were made daily, and H_2O_2 concentrations were confirmed spectrophotometrically using an ϵ_{240} of 39.4 M^{-1} cm^{-1}. Halophenol stocks (10 mM) were made in a 50/50 ethanol/deionized H_2O mixture. 4-Chloro-, 2,4,6-trichloro-, and 4-chloro-3,5-dimethylphenol were the substrates used for this study.

Dehaloperoxidase Activity Assay. UV−vis absorption spectroscopy was used to assay for dehaloperoxidase activity using H_2O_2 to initiate the reaction. The ability of Mb to dehalogenate 2,4,6-trichlorophenol (TCP) was followed by addition of four equal aliquots (one total equivalent) of H_2O_2 (250 μM) to a cuvette containing protein (20 μM) and TCP (250 μM). UV−vis absorption spectra were recorded before the addition of H_2O_2 and 45 min after the addition of each H_2O_2 aliquot. The reactions were carried out in a 0.5 cm cuvette in 100 mM potassium phosphate buffer at pH 7.0 and 4 °C.

Gas Chromatography−*Mass Spectrometry Analyses.* A HP-5890 Restek RTX-5 GC capillary column (30 m × 0.32 mm) was used for detection of the product(s). The initial temperature of the column was set at 50 °C for 2 min, and the temperature was subsequently increased at a rate of 10 °C/min up to a final temperature of 300 °C and held for 10 min. MS detection was in the electron impact (EI) ionization mode, and a VG70S mass spectrometer scanning from 50 to 450 amu was used for product identification. Authentic samples of 2,6-dichloro-1,4-benzoquinone and 2,6-dimethyl-1,4-benzoquinone gave essentially identical GC retention times and mass spectral fragmentation patterns.

Peroxidase Activity Assay. Turnover measurements ([enzyme] = 5−40.0 μM) were carried out directly in a 0.5 cm cuvette by monitoring the change in absorbance at 272 nm for the appearance of quinone products ($\epsilon_{272} = 14$ mM^{-1} cm^{-1}). The turnover number was determined by calculating the velocity from the initial linear portion of the trace at 272 nm as a function of H_2O_2 concentration (0−2 mM). For each set of reaction conditions, 10 individual reactions were performed and then averaged to determine the velocity at the varying H_2O_2 concentrations.

Single-Turnover Assay. To determine the rate of dehalogenation catalyzed by Mb Cpd II, the Fe(IV)═O state was formed by addition of a near-equimolar amount of ferric Mb (60 μM) to H_2O_2 (65 μM) (delay time ∼ 10 min) before the reaction was quenched with TCP (150 μM). Ten individual reactions were performed and then averaged to determine the initial rate which was calculated from the initial linear portion of the trace at 272 nm.

Myoglobin Compound II Activity and Stability Assays. The reaction of ferric Mb (50 μM) with a 1.2-fold molar excess of H_2O_2 (60 μM) was monitored spectrophotometrically at 422 nm versus time to illustrate the stability of Mb Cpd II. Mb Cpd II was generated as described above and reacted with 4-chloro-3,5-dimethylphenol. The organic products were identified by gas chromatography and mass spectrometry.

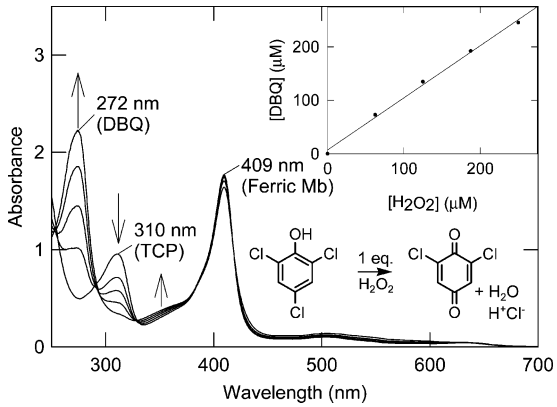


FIGURE 1: UV−visible absorption spectra of the reaction of ferric horse heart myoglobin (20 μM) with 2,4,6-trichlorophenol (TCP) (250 μM) initiated by H_2O_2 (four 62.5 μL aliquots; 250 μM total) in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. Each spectrum was recorded 45 min after H_2O_2 addition. The inset shows the formation of 2,6-dichloro-1,4-benzoquinone (micromoles) plotted as a function of the amount of H_2O_2 consumed (micromoles).

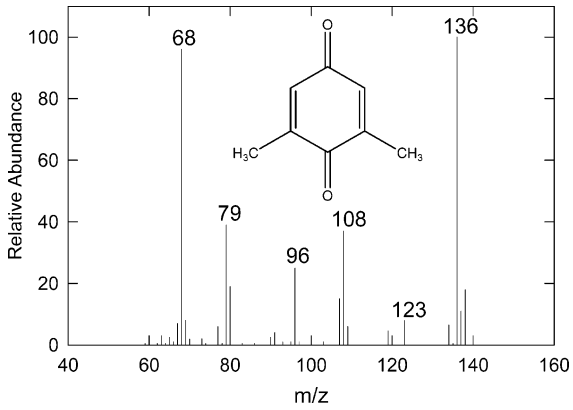


FIGURE 2: Mass spectrum of 2,6-dimethyl-1,4-benzoquinone extracted with ethyl acetate from the ferric horse heart myoglobin (5 μM)/4-chloro-3,5-dimethylphenol (250 μM)/H_2O_2 (500 μM) reaction mixture in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C; the reaction time was 1 h. The same product was identified when myoglobin Compound II was initially formed (ferric myoglobin reacted with H_2O_2 for 30 min) and reacted with 4-chloro-3,5-dimethylphenol.

RESULTS AND DISCUSSION

The ability of horse heart myoglobin (Mb) to catalyze the oxidative dehalogenation of halophenol substrates under conditions of oxidative stress has been investigated. The results herein show that the long-lived, stable Mb Compound II (Cpd II) is potentially responsible for oxidizing halophenols into DNA-alkylating radicals and quinones. Additionally, the order of addition of substrate [2,4,6-trichlorophenol (TCP)] versus cosubstrate (H_2O_2) has been examined. Functional analyses for the dehalogenation activity of Mb include UV−visible (UV−vis) absorption spectrophotometry, gas chromatography−mass spectrometry (GC−MS), and kinetic assays for probing how Mb catalyzes the same reactions as traditional peroxidases (*20*−*24*) and the enzymatic globin, dehaloperoxidase (DHP) (*14*). UV−vis absorption spectra collected during manual mixing experiments for the Mb-catalyzed oxidative dehalogenation of TCP (250 μM) initiated by H_2O_2 are displayed in Figure 1. A higher concentration of Mb was used in this assay to more accurately represent physiological conditions and to monitor the Soret band of Mb upon reaction with H_2O_2. One total equivalent of H_2O_2 was added to the reaction mixture in four equal aliquots (4 × 62.5 μM aliquots). Upon addition of H_2O_2, the magnitude of the absorption band for TCP (310 nm) decreased and those for 2,6-dichloro-1,4-benzoquinone (DBQ) (272 nm, 342 nm) increased. UV−vis absorption spectra were collected ~45 min after addition of each H_2O_2 aliquot, resulting in the regeneration of ferric [Fe(III)] Mb and the gradual conversion of TCP to DBQ. The formation of the product required almost exactly 1 equiv of H_2O_2 per TCP molecule that is oxidized. Since this is a two-electron process, the observed stoichiometry strongly suggested that both Mb ferryl [Fe(IV)═O] species (i.e., Cpd I/ES and II) participate in the reaction. The participation of a peroxyl radical in the first step of the oxidative dehalogenation of TCP is posible (*43*). A near-linear correlation of DBQ formed to hydrogen peroxide consumed is displayed (Figure 1, inset). As a control, TCP was incubated with excess H_2O_2 overnight, and no DBQ was detected (data not shown).

The formation of DBQ in Figure 1 is based on the appearance of its characteristic UV−vis absorption spectrum (*15*, *20*, *21*). At physiological pH, DBQ is insufficiently stable for GC−MS analyses. However, reaction of 4-chloro-3,5-dimethylphenol with Fe(III) Mb in the presence of H_2O_2 led to formation of the more stable 2,6-dimethyl-1,4-benzoquinone, which could be extracted and identified by GC−MS analysis (Figure 2). Additionally, the same product was identified when Mb, pre-reacted with H_2O_2 for 30 min to ensure homogeneous Cpd II formation, was treated with 4-chloro-3,5-dimethylphenol (Figure 2). This clearly demonstrates that Mb Cpd II can catalyze the oxidative dehalogenation of halophenol substrates. The mass spectra of the observed product are in agreement with those of authentic standards, and the spectral characteristics are similar to those previously reported for the quinone product identified by GC−MS for similar reactions catalyzed by HRP (*21*).

The mechanism of oxidative halophenol dehalogenation by several peroxidases is currently under investigation (vide supra). The data support an electron transport mechanism involving both Cpds I and II, i.e., two consecutive one-electron steps. This conclusion is based on the use of *p*-halophenols as mechanistic probes of the dehaloperoxidase activity of *C. fumago* chloroperoxidase (CCPO) (*20*). Both 1,4-benzoquinone and a dimeric product were detected after reaction of CCPO with 4-chlorophenol; formation of the dimeric product is consistent with the involvement of radicals during the reaction. The same products were identified for the Mb-catalyzed reaction (Figure 3). The structure of the dimer shown (Figure 3A) is based on the proposed halophenol radical intermediate (*20*).

The formation of DBQ can be monitored spectrophotometrically at 272 nm as a function of time (Figure 4). The rate of Mb dehalogenation is 270-fold slower than that for the same reaction catalyzed by DHP (*15*) and even slower than that catalyzed by CCPO (Table S1 of the Supporting Information) (*20*) and horseradish peroxidase (HRP) (*15*, *21*). Furthermore, examination of the spectrum during the linear phase of turnover experiments reveals that the predominant species is Mb Cpd II (Figure S1 of the Supporting Information). The variation in rates is likely due to differences in the catalytically important active site residues. For example, the proximal histidine ligands of Mb and DHP do not contain

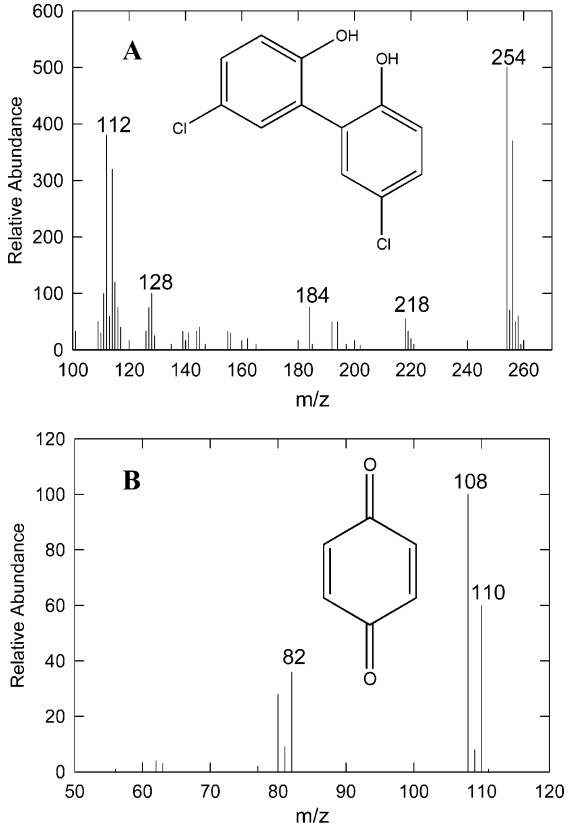


FIGURE 3: (A) Mass spectrum of the proposed structure of the *p*-chlorophenol dimer extracted with ethyl acetate from the horse heart myoglobin (5 μM)/4-chlorophenol (250 μM)/H_2O_2 (500 μM) reaction mixture in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. The pH of the reaction mixture was lowered with 100 mM hydrochloric acid before extraction. (B) Mass spectrum of 1,4-benzoquinone extracted with ethyl acetate from the horse heart myoglobin (5 μM)/4-chlorophenol (250 μM)/H_2O_2 (500 μM) reaction mixture in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C; the reaction time was 1 h.

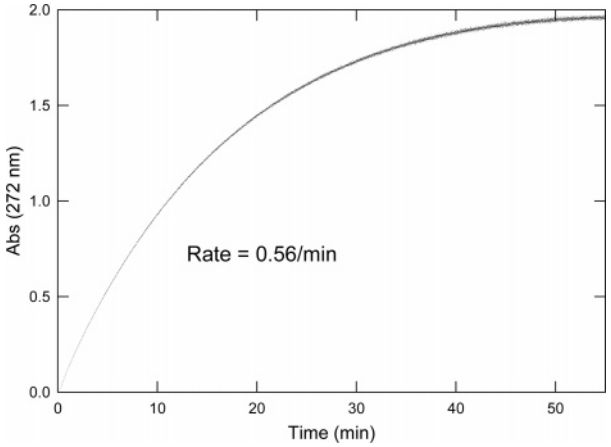


FIGURE 4: UV−visible absorption kinetic assay monitored at 272 nm of formation of 2,6-dichloro-1,4-benzoquinone as a result of the myoglobin (30 μM)/2,4,6-trichlorophenol (286 μM)/H_2O_2 (500 μM) reaction mixture in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. The reaction was designed so that an absorbance of ~2 corresponded to complete conversion of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-benzoquinone.

a hydrogen bond to an aspartate residue, as found in most traditional histidine-ligated peroxidases such as HRP, and they are thought to impart the "push" of electron density through the heme iron to facilitate O−O bond cleavage upon

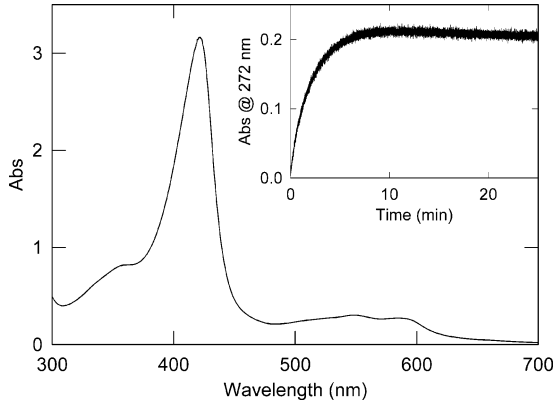


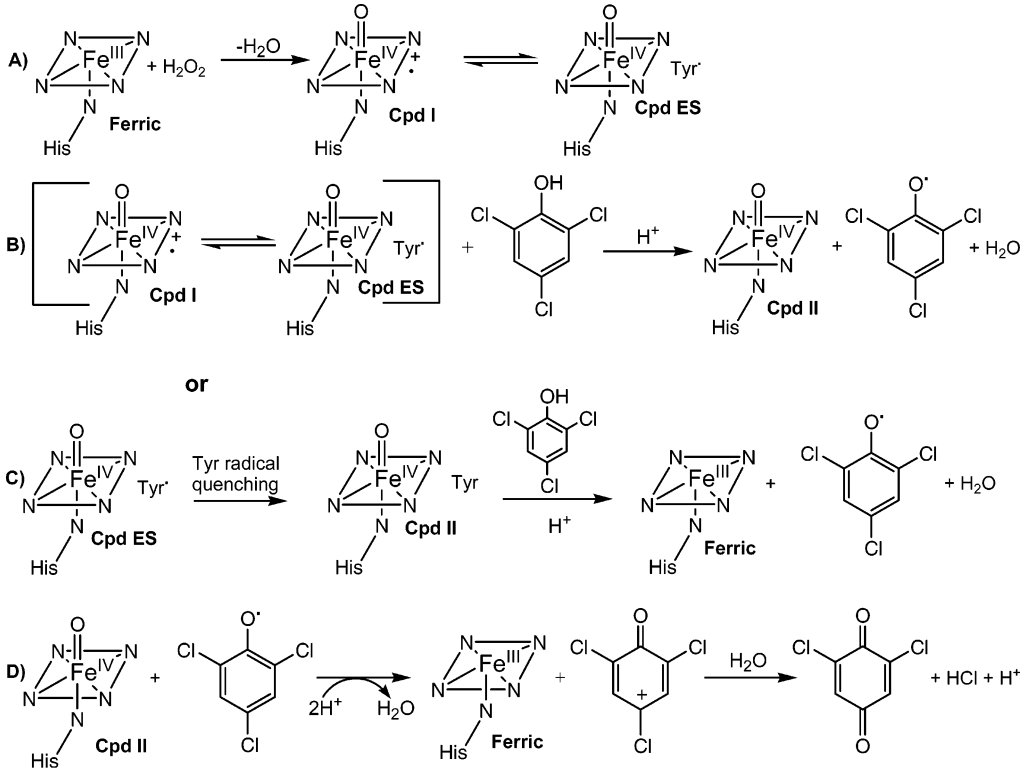
FIGURE 5: UV−visible absorption spectrum of myoglobin Compound II formed as a result of ferric myoglobin (60 μM) being incubated with H_2O_2 (65 μM) for 10 min in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C (black trace). The inset shows the results of an UV−visible absorption kinetic assay monitored at 272 nm and 4 °C for formation of 2,6-dichloro-1,4-benzoquinone as a result of addition of 2,4,6-trichlorophenol (150 μM) to myoglobin Compound II generated by addition of H_2O_2 (65 μM) to myoglobin (60 μM) in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C.

reaction with H_2O_2 (*24*). Additionally, the distal histidine of Mb is in position to stabilize O_2, whereas the distal histidine of DHP is in the typical "peroxidase" position to activate H_2O_2 (*6*−*8*, *18*). Mb and DHP also lack the conserved distal arginine residue thought to stabilize the developing negative charge during O−O bond cleavage. These active site structural differences likely explain the slow catalytic activity of Mb (*24*).

Mb is known to form a two-electron oxidized Fe(IV)═O intermediate over the Fe(III) heme with the radical located on an amino acid residue (*43*−*47*). The fate of the amino acid radical depends on the particular Mb being studied. The radical species of sperm whale Mb has been shown to result in a tryptophanyl radical, a peroxyl radical, two distinct tyrosyl radicals, and a dimerized protein product formed between two tyrosine residues (*45*). Human ferryl Mb undergoes an intramolecular electron transfer process, resulting in a cysteine thiyl radical (*46*). The formation of a tyrosyl radical and a peroxyl radical as a result of reaction of horse heart Mb with H_2O_2 and O_2 has also been reported (*43*, *46*). The tyrosyl radical of horse heart Mb is essentially gone 10 min after the addition of H_2O_2 (*47*). Thus, formation of homogeneous Mb Cpd II can be achieved by mixing Fe(III) Mb with H_2O_2 for at least 10 min before reacting it with substrate.

Mb Cpd II is poised to carry out transformations characteristic of peroxidases if the nature of the substrate is suitable for donating electrons to reduce the Mb Cpd II intermediate. Furthermore, it is remarkably stable. Monitoring the Soret absorption peak (λ_{422}) versus time (i.e., 1 h) did not result in any noticeable spectral changes (data not shown). Thus, the stability of this Fe(IV)═O intermediate could be potentially responsible for adverse physiologically relevant reactions. To test this idea, Fe(III) Mb was reacted with a slight excess of H_2O_2 for 10 min to generate homogeneous Mb Cpd II (vide supra), followed by substrate addition. After addition of TCP, the formation of DBQ was monitored at 272 nm as a function of time (Figure 5, inset). The increase in absorbance at 272 nm reveals that Mb Cpd II itself is an

Scheme 1 : (A) Reaction of Ferric Myoglobin with H_2O_2 Resulting in a Very Short-Lived Compound I State, Which Quickly Oxidizes an Adjacent Amino Acid (Tyr) To Generate Myoglobin Compound ES, (B) Reaction of either Myoglobin Compound I or Compound ES with 2,4,6-Trichlorophenol Generating Ferric Myoglobin and a 2,4,6-Trichlorophenoxy Radical, (C) Formation of Myoglobin Compound II as a Result of Reduction of Compound ES and Reaction of Compound II with 2,4,6-Trichlorophenol Resulting in Ferric Myoglobin and a 2,4,6-Trichlorophenoxy Radical, and (D) Reaction of a Second Equivalent of Myoglobin Compound II with a 2,4,6-Trichlorophenoxy Radical Yielding Ferric Myoglobin and the Proposed 2,4,6-Trichlorocyclohexadienone Cation and Reaction of the Proposed 2,4,6-Trichlorocyclohexadienone Cation with a Solvent Water Molecule To Yield 2,6-Dichloro-1,4-benzoquinone



active oxidant for conversion of TCP to DBQ. Consistent with this proposal, we find that the initial rate of the reaction of Mb Cpd II with TCP is nearly comparable to that of the ferric protein under turnover conditions (Table S1 of the Supporting Information). Single-turnover experiments resulted in formation of nearly 0.5 equiv of DBQ after reaction of 1 equiv of TCP with 1 equiv of Mb Cpd II (Figure 5, inset). Furthermore, ferric Mb is regenerated upon reduction of Mb Cpd II by TCP. The ability of Mb Cpd II to catalyze the oxidative dehalogenation of halophenols is consistent with a mechanism involving two one-electron steps.

Presumably, Fe(III) Mb undergoes a two-electron oxidation upon reaction with H_2O_2 to transiently form Mb Cpd I, which rapidly oxidizes a nearby amino acid residue (i.e., tyrosine) to form Mb Cpd ES [Fe(IV)═O/Tyr•] (Scheme 1A) (*44*), in a manner similar to the mechanism observed for cytochrome *c* peroxidase (*24*). The data in Figure 1 show that Mb Cpd I (or ES) is capable of carrying out the first of the two one-electron oxidations of the dehalogenation mechanism (Scheme 1B). Alternatively, the tyrosine radical is quenched by solvent to yield the Mb Cpd II state (*43*, *46*, *47*), which is reduced back to the Fe(III) state with concomitant oxidation of TCP, presumably while generating a trichlorophenoxy radical (Scheme 1C). A second equivalent of Mb Cpd II reacts with the radical intermediate, yielding Fe(III) Mb and the proposed 2,4,6-trichlorocyclohexadienone cation (Scheme 1D). A solvent water molecule is likely the source of the second oxygen atom in the quinone product (Scheme 1D), which has been suggested when the reaction is catalyzed by CCPO or HRP (*20*, *21*).

Interestingly, the mechanism of Mb-catalyzed dehalogenation reactions appears to be similar to that of the same reactions catalyzed by the enzymatic globin, DHP. However, the active site of Mb is designed to bind O_2, while a substrate binding pocket exists near the active site of DHP (*18*). Thus, Mb most likely catalyzes this reaction by an outer sphere electron transfer process. Substrate binding prior to H_2O_2 has been proposed to be obligatory for DHP catalysis (*17*). In contrast, the fact that Mb Cpd II is an active oxidant in halophenol dehalogenation (Figure 5, inset) is consistent with a traditional peroxidase order of addition (*24*) of H_2O_2 followed by TCP.

In summary, Mb catalyzes the oxidative dehalogenation of halophenol substrates under conditions representative of oxidative stress. We have reported results that implicate higher valent states of Mb (i.e., Cpd I or ES and Cpd II), although only Cpd II is stable enough to be analyzed spectrophotometrically. Furthermore, TCP can be completely converted to 2,6-dichloro-1,4-benzoquinone (DBQ) by 2 equiv of Mb Cpd II, a result consistent with a mechanism involving two one-electron steps and H_2O_2 addition first. The dehalogenation of TCP therefore likely involves trichlorophenoxy radical formation en route to the DBQ product (Scheme 1). The generation of phenoxy radicals and quinones as a result of peroxidase catalysis demonstrates the potential danger of these reactive compounds as they have been shown

to react covalently with DNA (*40*). The same radical intermediates and quinone products form as the result of reaction of TCP with Mb in the presence of H_2O_2. Furthermore, while the active oxidizing intermediates of peroxidase enzymes are typically short-lived, Mb Cpd II is very stable. Thus, the stability of Mb Cpd II and its reactivity in forming phenoxy radicals and quinones known to alkylate DNA reveal the potential human health hazards this species poses when exposed to toxic compounds like TCP, especially in the absence of antioxidants.

ACKNOWLEDGMENT

We thank Drs. Masanori Sono and Paul R. Thompson for many helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Additional UV−vis absorption spectra and a table comparing the rates of dehalogenation. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wittenberg, B. A., and Wittenberg, J. B. (1989) Transport of oxygen in muscle, *Annu. Rev. Physiol. 51*, 857−878.
2. Qiu, Y., Sutton, L., and Riggs, A. F. (1998) Identification of myoglobin in human smooth muscle, *J. Biol. Chem. 273*, 23426−23432.
3. Kobert, R. (1900) Beitrage zurKenntniss der Methamoglobine, *Pfluegers Arch. Gesamte Physiol. Menschen Tiere 82*, 603−630.
4. Keilin, D., and Hartree, E. F. (1950) Reaction of Methaemoglobin with Hydrogen Peroxide, *Nature 166*, 513−514.
5. Shiga, T., and Imaizumi, K. (1975) Electron spin resonance study on peroxidase and oxidase reactions of horseradish peroxidase and methemoglobin, *Arch. Biochem. Biophys. 167*, 469−479.
6. Egawa, T., Yoshioka, S., Takahashi, S., Hori, H., Nagano, S., Shimada, H., Ishimori, K., Morishima, I., Suematsu, M., and Ishimura, Y. (2003) Kinetic and spectroscopic characterization of a hydroperoxy compound in the reaction of native myoglobin with hydrogen peroxide, *J. Biol. Chem. 278*, 41597−41606.
7. Matsui, T., Ozaki, S.-I., and Watanabe, Y. (1997) On the formation and reactivity of compound I of the His-64 myoglobin mutants, *J. Biol. Chem. 272*, 32735−32738.
8. Kato, S., Ueno, T., Fukuzumi, S., and Watanabe, Y. (2004) Catalase reaction by myoglobin mutants and native catalase: Mechanistic investigation by kinetic isotope effect, *J. Biol. Chem. 279*, 52376−52381.
9. Augusto, O., Kunze, K. L., and Ortiz de Montellano, P. R. (1982) N-Phenylprotoporphyrin IX formation in the hemoglobin-phenylhydrazine reaction, *J. Biol. Chem. 257*, 6231−6241.
10. Rice, R. H., Lee, Y. M., and Brown, W. D. (1983) Interactions of heme proteins with hydrogen peroxide: Protein crosslinking and covalent binding of benzo[a]pyrene and 17β-estradiol, *Arch. Biochem. Biophys. 221*, 417−427.
11. Ortiz de Montellano, P. R., and Catalano, C. E. (1985) Epoxidation of styrene by hemoglobin and myoglobin. Transfer of oxidizing equivalents to the protein surface, *J. Biol. Chem. 260*, 9265−9271.
12. Reeder, B. J., and Wilson, M. T. (2005) Hemoglobin and myoglobin associated oxidative stress: From molecular mechanism to disease states, *Curr. Med. Chem. 12*, 2741−2751.
13. Pond, A. E., Roach, M. P., Thomas, M. R., Boxer, S. G., and Dawson, J. H. (2000) The H93G myoglobin cavity mutant as a versatile template for modeling heme proteins: Ferrous, ferric, and ferryl mixed-ligand complexes with imidazole in the cavity, *Inorg. Chem. 39*, 6061−6066.
14. Chen, Y.-P., Woodin, S. A., Lincoln, D. E., and Lovell, C. R. (1996) An unusual dehalogenating peroxidase from the marine terebellid polychaete *Amphitrite ornata*, *J. Biol. Chem. 271*, 4609−4612.
15. Osborne, R. L., Taylor, L. O., Han, K. P., Ely, B., and Dawson, J. H. (2004) *Amphitrite ornata* dehaloperoxidase: Enhanced activity for the catalytically active globin using MCPBA, *Biochem. Biophys. Res. Commun. 324*, 1194−1198.
16. Osborne, R. L., Sumithran, S., Coggins, M. K., Chen, Y.-P., Lincoln, D. E., and Dawson, J. H. (2006) Spectroscopic characterization of the ferric states of *Amphitrite ornata* dehaloperoxidase and *Notomastus lobatus* chloroperoxidase: His-ligated peroxidases with globin-like proximal and distal properties, *J. Inorg. Biochem. 100*, 1100−1108.
17. Belyea, J., Gilvey, L. B., Davis, M. F., Godek, M., Sit, T. L., Lommel, S. A., and Franzen, S. (2005) Enzyme function of the globin dehaloperoxidase from *Amphitrite ornata* is activated by substrate binding, *Biochemistry 44*, 15637−15644.
18. LaCount, M. W., Zhang, E. L., Chen, Y.-P., Han, K., Whitton, M. M., Lincoln, D. E., Woodin, S. A., and Lebioda, L. (2000) The crystal structure and amino acid sequence of dehaloperoxidase from *Amphitrite ornata* indicate common ancestry with globins, *J. Biol. Chem. 275*, 536−540.
19. Franzen, S., Roach, M. P., Chen, Y.-P., Dyer, R. B., Woodruff, W. H., and Dawson, J. H. (1998) The unusual reactivities of *Amphitrite ornata* dehaloperoxidase and *Notomastus lobatus* chloroperoxidase do not arise from a histidine imidazolate proximal heme iron ligand, *J. Am. Chem. Soc. 120*, 4658−4661.
20. Osborne, R. L., Raner, G. M., Hager, L. P., and Dawson, J. H. (2006) *C. fumago* chloroperoxidase is also a dehaloperoxidase: Oxidative dehalogenation of halophenols, *J. Am. Chem. Soc. 128*, 1036−1037.
21. Ferrari, R. P., Laurenti, E., and Trotta, F. (1999) Oxidative 4-dechlorination of 2,4,6-trichlorophenol catalyzed by horseradish peroxidase, *J. Biol. Inorg. Chem. 4*, 232−237.
22. Ferrari, R. P., Laurenti, E., Cecchimi, P. I., Gambino, O., and Sondergaard, I. (1995) Spectroscopic investigations on the highly purified lactoperoxidase Fe(III) heme catalytic site, *J. Inorg. Biochem. 58*, 109−127.
23. Hammel, K. E., and Tardone, P. J. (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular lignin peroxidases, *Biochemistry 27*, 6563−6568.
24. Pond, A. E., Ledbetter, A. P., Sono, M., Goodin, D. B., and Dawson, J. H. (2001) Redox Enzymes: Correlation of Three-dimensional Structure and Mechanism for Heme-containing Oxygenases and Peroxidases, in Part I, Biological Systems (Gray, H. B., and Winkler, J. R., Eds.) in *Handbook on Electron Transfer* (Balzani, V., Ed.) Vol. III, pp 56−104 Wiley VCH Publishers, New York.
25. Vollaard, N. B. J., Shearman, J. P., and Cooper, C. E. (2005) Exercise-induced oxidative stress: Myths, realities and physiological relevance, *Sports Med. 35*, 1045−1062.
26. Kanner, J., and Harel, S. (1985) Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin, *Arch. Biochem. Biophys. 237*, 314−321.
27. Holt, S., Reeder, B., Wilson, M., Harvey, S., Morrow, J. D., Roberts, L. J., and Moore, K. (1999) Increased lipid peroxidation in patients with rhabdomyolysis, *Lancet 353*, 1241.
28. Rodriguez-Malaver, A. J., Leake, D. S., and Rice-Evans, C. A. (1997) The effects of pH on the oxidation of low-density lipoprotein by copper and metmyoglobin are different, *FEBS Lett. 406*, 37−41.
29. Reeder, B. J., and Wilson, M. T. (2001) The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin: A role for the protonated ferryl species, *Free Radical Biol. Med. 30*, 1311−1318.
30. Moore, K. P., Holt, S., Patel, R. P., Svistunenko, A., Zackert, W., Goodier, D., Reeder, B. J., Clozel, M., Anand, R., Cooper, C. E., Harvey, S., Morrow, J. D., Wilson, M. T., Darley-Usmar, V., and Roberts, L. J. (1998) A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure, *J. Biol. Chem. 273*, 31731−31737.
31. Reeder, B. J., Sharpe, M. A., Kay, A. D., Kerr, M., Moore, K., and Wilson, M. T. (2002) Toxicity of myoglobin and haemoglobin: Oxidative stress in patients with rhabdomyolysis and subarachnoid haemorrhage, *Biochem. Soc. Trans. 30*, 745−748.
32. Quan, X., Shi, H., Liu, H., Wang, J., and Qian, Y. (2004) Removal of 2,4-dichlorophenol in a conventional activated sludge system through bioaugmentation, *Process Biochemistry 39*, 1701−1707.
33. Agency for Toxic Substances and Disease Registry (1990) *Toxicological Profile for 2,4,6-Trichlorophenol*, U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta.
34. U.S. Environmental Protection Agency (1999) *Integrated Risk Information System (IRIS) on 2,4,6-Trichlorophenol*, Office of Research and Development, National Center for Environmental Assessment, Washington, DC.

35. Bolton, J. L. (2002) Quinoids, quinoid radicals, and phenoxyl radicals formed from estrogens and antiestrogens, *Toxicology 177*, 55−65.
36. Goldman, R., Claycamp, G. H., Sweetland, M. A., Sedlov, A. V., Tyurin, V. A., Kisin, E. R., Tyurina, Y. Y., Ritov, V. B., Wenger, S. L., Grant, S. G., and Kagan, K. (1999) Myeloperoxidase-catalyzed redox-cycling of phenol promotes lipid peroxidation and thiol oxidation in HL-60 cells, *Free Radical Biol. Med. 27*, 1050−1063.
37. Dix, T. A., and Aikens, J. (1993) Mechanisms and biological relevance of lipid peroxidation initiation, *Chem. Res. Toxicol. 6*, 2−18.
38. Stadtman, E. R. (1992) Protein oxidation and aging, *Science 257*, 1220−1224.
39. Burrows, C. J., and Muller, J. G. (1998) Oxidative nucleobase modifications leading to strand scission, *Chem. Rev. 98*, 1109−1151.
40. Dai, J., Sloat, A. L., Wright, M. W., and Manderville, R. A. (2005) Role of phenoxyl radicals in DNA adduction by chlorophenol xenobiotics following peroxidase activation, *Chem. Res. Toxicol. 18*, 771−779.
41. Dai, J., Wright, M. W., and Manderville, R. A. (2003) Ochratoxin A forms a carbon-bonded C8-deoxyguanosine nucleoside adduct: Implications for C8 reactivity by a phenolic radical, *J. Am. Chem. Soc. 125*, 3716−3717.
42. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, p 44, North-Holland Publishing Co., Ámsterdam.
43. Kelman, D. J., DeGray, J. A., and Mason, R. P. (1994) Reaction of myoglobin with hydrogen peroxide forms a peroxyl radical which oxidizes substrates, *J. Biol. Chem. 269*, 7458−7463.
44. Witting, P. K., Mauk, A. G., and Lay, P. A. (2002) Role of tyrosine-103 in myoglobin peroxidase activity: Kinetic and steady-state studies on the reaction of wild-type and variant recombinant human myoglobins with H_2O_2, *Biochemistry 41*, 11495−11503.
45. Detweiler, C. D., Lardinois, O. M., Deterding, L. J., Ortiz de Montellano, P. R., Tomer, K. B., and Mason, R. P. (2005) Identification of the myoglobin tyrosyl radical by immuno-spin trapping and its dimerization, *Free Radical Biol. Med. 38*, 969−976.
46. Gunther, M. R. (2004) Probing the free radicals formed in the metmyoglobin hydrogen peroxide reaction, *Free Radical Biol. Med. 36*, 1345−1354.
47. Gunther, M. R., Sturgeon, B. E., and Mason, R. P. (2000) A long-lived tyrosyl radical from the reaction between horse metmyoglobin and hydrogen peroxide, *Free Radical Biol. Med. 28*, 709−719.

BI700684U