Heterolytic versus Homolytic Peroxide Bond Cleavage by Sperm Whale Myoglobin and Myoglobin Mutants

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Abstract: Despite extensive work on homolytic versus heterolytic peroxide bond cleavage by iron porphyrins, the nature of the corresponding reactions catalyzed by myoglobin (Mb) and hemoglobin (Hb) remains unclear. These hemoproteins react with peroxides to give a ferryl (Fe^{IV}=O) complex and a protein radical, but the coupling of dioxygen bond cleavage to protein radical formation is obscure. This process is examined here with the help of 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dien-1-one (BHTOOH), a compound that is converted heterolytically to an alcohol and homolytically to several rearranged products. The homolytic/heterolytic product ratio for recombinant sperm whale Mb (0.53) is the same as for native sperm whale Mb (0.57) and slightly higher than that for bovine Hb (0.44). Reaction of the hydroperoxide with Fe^{2+} and low concentrations of ascorbate results in a much lower rate of peroxide cleavage and overwhelming production of homolytic products. High concentrations of ascorbate prevent the formation of rearranged homolytic products by reducing the alkoxy radical to the alkoxide anion. Reaction of [^{18}O]BHTOOH with Mb or Hb yields rearranged products that retain >95% of the ^{18}O-labeled peroxide oxygen. This suggests that oxidation of the hydroperoxide to the hydroperoxy radical (ROOH → ROO•) is not a major reaction because the peroxy radical is expected to undergo exchange with molecular oxygen. The extent of doubly (^{2}H/^{18}O) labeled products formed in incubations of [^{18}O]BHTOOH and [^{2}H]BHTOOH with Mb suggests that 92% of the radical products arise by peroxide homolysis and 8% via the hydroperoxy radical route. Similar homolytic/heterolytic product ratios for the Tyr-151 → Phe (0.55), Tyr-103 → Phe/Lys-102 → Gln (0.63), and His-64 → Val (0.57) mutants of sperm whale Mb show that partitioning between the two pathways is insensitive to proximal hydrogen bonding or electron-donating residues. Peroxide cleavage is doubled in the His-64 → Val mutant due to conversion of the iron to the pentacoordinated state without significantly altering the homolytic/heterolytic ratio. In sum, the homolytic/heterolytic ratio for Mb of approximately 0.5 is apparently determined by the identity of the fifth iron ligand and the general physical properties of the heme site rather than by identifiable acid–base or electron transfer interactions with specific amino acid residues.

The reactions of alkyl hydroperoxides with iron porphyrins have been proposed to involve both homolytic and heterolytic dioxygen bond cleavage.[1-4] The published studies suggest that partitioning between these two reaction pathways is sensitive to the reaction medium as well as other reaction conditions. The available evidence suggests that reactions in organic media involve heterolysis of the peroxide bond and are subject to general acid catalysis,[2-4] although it has been suggested that very nonbasic hydroperoxides may cleave homolytically.[5] Coordination of an imidazole to the iron accelerates cleavage of the peroxide bond,[5,6] and hydrogen bonding or deprotonation of the coordinated imidazole by a second base further enhances peroxide cleavage.[7] In contrast, reactions of alkyl hydroperoxides with water-soluble iron porphyrins in aqueous media have been reported to result primarily in homolytic bond scission, as evidenced by the formation of methanol and acetone in high yield in the aqueous reactions of metalloporphyrins with *tert*-butyl hydroperoxide.[2a] It has been argued, furthermore, that aqueous homolytic reactions are relatively insensitive to general acid or general base catalysis.[2a]

The reactions of peroxides with classical peroxidases result exclusively in heterolytic cleavage of the dioxygen bond. Heterolytic cleavage gives the corresponding hydroxyl derivative (water from H_2O_2) and a ferryl (Fe^{IV}=O) species coupled to a porphyrin or protein radical.[8-11] The crystal structure of cytochrome *c* peroxidase, and conservation of the imidazole iron ligand and the distal histidine and arginine in the sequences of other peroxidases, suggests that heme peroxidases catalyze heterolytic cleavage of the oxygen–oxygen bond by a push–pull mechanism.[12] In this mechanism, transfer of electrons from the prosthetic group to the oxygen is facilitated by electron release from the proximal histidine iron ligand and by proton transfer to the departing oxygen from the protonated distal histidine.[12] Heterolysis is also facilitated by a positively charged arginine in the distal pocket that helps to stabilize the charge-separated heterolytic transition state.[12] NMR studies suggest that electron donation by the proximal histidine is enhanced by its deprotonation during the catalytic cycle,[13] in agreement with the results of metalloporphyrin studies.[7] The reaction in the peroxidase active site is thus subject to general acid–general base catalysis and occurs exclusively by a heterolytic mechanism, in accord with the reactions of peroxides with metalloporphyrins in organic media.

Hb and Mb are oxygen-transport proteins but their reactions with oxidizing agents, including alkyl hydroperoxides,[14] may be important in pathological processes such as hemolytic anemia and reperfusion injury.[15-17] The reaction of Mb with H_2O_2 gives rise

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(1) Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation and ligation state of the iron; BHT, 2,6-di-*tert*-butyl-4-methylphenol; BHTOOH, 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dien-1-one; Mb, myoglobin; Hb, hemoglobin; metMb, metmyoglobin; metHb, methemoglobin; DETAPAC, diethylenetriaminepentaacetic acid.

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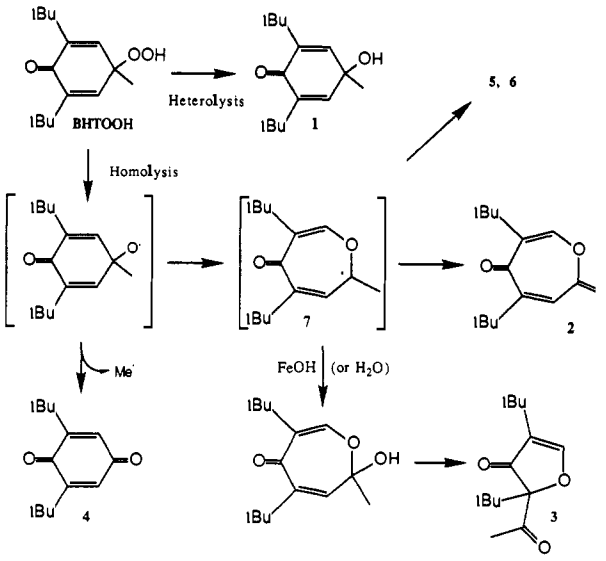

Figure 1. Reaction products from the reaction of BHTOOH with iron catalysts and mechanisms proposed for their formation.

to a ferryl complex and a transient protein radical.[18-22] The protein radical is quenched by still obscure mechanisms, including protein–protein[23] and heme–protein[24] cross-linking reactions, although the ferryl species, as indicated by its chromophore, is stable for a period of several minutes.[19,21] The precise location of the protein radical is also unknown, but a fraction of the unpaired electron density must reside on tyrosines 103 and 151 to account for the residue specificity of the cross-linking reactions.[23-25] Studies of sperm whale Mb mutants in which each of the tyrosines has been site-specifically replaced by a phenylalanine demonstrate, however, that the protein radical is formed in the absence of all tyrosines.[25] The unpaired electron density therefore must readily redistribute among oxidizable residues in the myoglobin structure. The mechanism of the formation of the protein radical is unclear. One alternative is heterolytic cleavage of the peroxide to give a ferryl/porphyrin radical cation, as in horseradish peroxidase, although electron transfer from the protein would have to rapidly quench the porphyrin radical cation because it is not a detectable intermediate. The other alternative is homolytic cleavage to give the ferryl species and an oxygen radical (HO· or RO·) that directly abstracts an electron from the protein.

The heme crevice in Mb is less conducive to heterolysis than that in horseradish peroxidase because Mb retains the histidine but not the polar arginine group in the distal cavity.[8] NMR studies suggest, furthermore, that the imidazole iron ligand in myoglobin, contrary to that in horseradish peroxidase, is not deprotonated by a vicinal base and is therefore a weaker electron donor.[13] Little is known about the extent of homolysis versus heterolysis in the reactions of peroxides with Mb, although some information is available on the corresponding Hb reactions. Homolytic formation of the hydroxyl radical from H_2O_2 by Hb has been reported, but

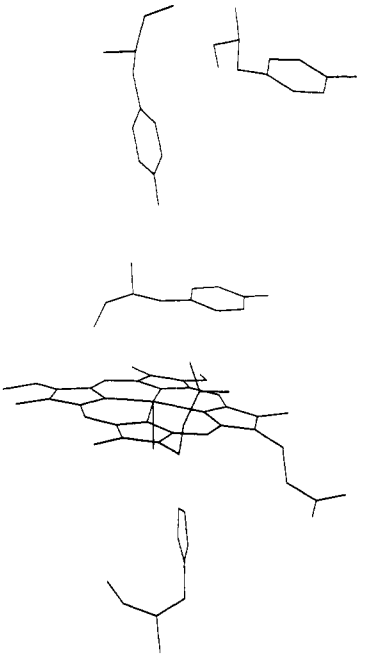

Figure 2. Disposition of Tyr-103, Tyr-151, and His-64 about the heme crevice of sperm whale Mb from the crystal structure of this protein.[31] Only the heme group and the relevant residues are shown. The tyrosine residues, in order of increasing distance from the heme, are Tyr-103, Tyr-146, and Tyr-151. The tyrosines are on the proximal side, and His-64 on the distal side, of the heme. As shown, His-64 blocks the access route from the medium into the heme site.

subsequent work suggests that the *detectable* hydroxyl radical is formed by secondary reactions of the peroxide with iron released from the oxidatively damaged heme group.[26,27] Analysis of the products formed in the reaction of Hb with 10-hydroperoxy-8,12-octadecadienoic acid also led to the inference that peroxide homolysis occurred four times more frequently than heterolysis, although formation of homolytic products by secondary reactions of the hydroperoxide with the oxidized hemoprotein was not excluded.[28] In order to clarify the nature of dioxygen bond cleavage by Mb and Hb, we have characterized the reactions of these hemoproteins with 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dien-1-one (BHTOOH), a reporter hydroperoxide that is reduced heterolytically to the 4-hydroxy derivative and homolytically to multiple rearranged products (Figure 1).[29,30] The possible roles of key active site residues in determining the mechanism of the dioxygen bond cleavage have been examined with the help of the His-64 → Val, Tyr-103 → Phe, and Tyr-151 → Phe site specific mutants of sperm whale Mb. The relationships of these residues to the heme iron atom in the crystal structure of the hemoprotein are shown in Figure 2.

Experimental Section

Hemoproteins. Sperm whale myoglobin and bovine methemoglobin were purchased from Sigma (St. Louis, MO). Recombinant sperm whale myoglobin and its Tyr-151 → Phe, Tyr-103 → Phe/Lys-102 → Gln, and His-64 → Val mutants were expressed in *E. coli* and purified as previously described.[25]

Substrates. Unlabeled BHTOOH, [^{2}H]BHTOOH (>98% trideuterated on the 4-methyl group), and [^{18}O]BHTOOH (97.6% ^{18}O labeled on the two hydroperoxide oxygens) were prepared as previously described.[30] [^{14}C]BHTOOH (label on the *tert*-butyl groups) was obtained from the correspondingly labeled [^{14}C]BHT, which was synthesized by Friedel–Crafts alkylation of 2-*tert*-butyl-4-methylphenol with [^{14}C]*tert*-butyl alcohol (J. A. Thompson, unpublished). Radiolabeled BHT was converted to the hydroperoxide by exposure to molecular ox-

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Table I. Products from the Reduction of BHTOOH by Bovine Hb, Sperm Whale Mb, and Mb Mutants

	products (nmol/nmol of hemoprotein)						
	heterolysis	homolysis					
hemoprotein	1	2	3	4	5	6	homo/het
wild type[a]	20.45	1.74	2.61	0.68	5.21	0.62	0.53
Tyr-151 → Phe	32.10	2.52	5.75	1.05	7.28	1.16	0.55
Tyr-103 → Phe[b]	27.55	2.74	5.31	1.23	7.33	0.61	0.63
His-64 → Val	50.45	5.07	11.56	2.76	7.33	1.91	0.57
Mb[c]	32.70	2.34	2.82	1.06	11.60	0.94	0.57
Mb[c] + 1 mM ascorbate	36.60	0.67	2.98	0.47	9.30	0.15	0.37
Hb	64.82	5.52	14.30	3.25	4.23	1.23	0.44

[a] Recombinant sperm whale myoglobin. [b] All Tyr-103 → Phe mutants carry a stabilizing Lys-102 → Gln mutation.[25] [c] Native sperm whale myoglobin.

ygen in ethanolic base. To a stirred solution of [^{14}C]BHT (7 mg, 0.032 mmol) in ethanol (1 mL) was added a solution of KOH (3.2 mg) in 32 μL of water and oxygen was bubbled through the solution for 3 h at 25 °C. The reaction solution was then poured over ice water, acidified with glacial acetic acid, and extracted three times with diethyl ether. Concentration of the combined organic layers in vacuo and purification of the crude hydroperoxide by silica gel flash chromatography (5:95 ethyl acetate–hexane) gave 5.6 mg of pure [^{14}C]BHTOOH. A 100 mM solution in DMSO of this material, which had a specific activity of 1.3 mCi/mmol, was prepared.

Incubations. To a solution of the hemoprotein (20 nmol in heme) in 50 mM potassium phosphate buffer (pH 7.4) containing 1.5 mM DETAPAC was added 5 μL of a 100 mM solution (500 nmol) of [^{14}C]BHTOOH in DMSO (the final volume was 1 mL). The mixture was incubated for 10 min at 25 °C and was then quenched by adding 5 mL of cold, distilled diethyl ether. After 5 μL of a 7.3 mM DMSO solution of 2,4-di-*tert*-butylphenol (the internal standard) was added, the metabolites were isolated by extraction with two 5-mL portions of diethyl ether, the combined extracts were concentrated under nitrogen, and the residue was dissolved in 100 μL of isopropyl alcohol. The isopropyl alcohol solution of the metabolites was analyzed by HPLC on a C-18 reverse phase column (4.6 × 250 mm Ultrasphere, Beckman) eluted first with 60% acetonitrile in water for 20 min and then with 80% acetonitrile in water for 15 min at a flow rate of 1 mL/min. The eluent, monitored at 236 and 270 nm with an LKB Model 2141 UV detector, was collected on an LKB Model 2112 fraction collector (3 fractions/min). Each fraction was diluted with 4 mL of scintillation fluid (Safety-Solve, Research Products International, Mount Prospect, IL) and the ^{14}C content was determined by liquid scintillation counting. The metabolites were then quantitated from the counts associated with each metabolite in the radiochromatogram by subtracting the background counts, converting to nanocuries, and dividing by the specific activity of BHTOOH. The incubations and analyses were performed in duplicate. In some experiments, metabolites obtained with unlabeled, ^{2}H-labeled, or ^{18}O-labeled substrate were isolated by HPLC and were analyzed by selected ion GC/MS on an HP 5988A instrument.

Isotopic Analysis of Labeled Metabolite 3. Product **3** isolated by HPLC from incubations containing [^{2}H]BHTOOH and/or [^{18}O]BHTOOH was analyzed by GC/MS on an HP-1 capillary column (12 m × 0.2 mm, Hewlett Packard) heated from 120 to 200 °C at 3 °C/min on a Hewlett Packard 5988A instrument. The fragment ion corresponding to *m/z* 181 in unlabeled **3** was monitored. This ion is 4-fold more abundant than the molecular ion and is formed by the loss of *tert*-butyl-derived hydrocarbon fragments.[29] The ion intensities corresponding to *m/z* 181 (unlabeled), 183 (1 × ^{18}O), 184 (3 × ^{2}H), 185 (2 × ^{18}O), and 186 (1 × ^{18}O, 3 × ^{2}H) were integrated and corrected for ^{13}C isotope contributions.

Results

Sperm Whale Mb and Bovine Hb. The sequence of the recombinant sperm whale Mb employed in these studies is identical to that of the native hemoprotein except that the initiating methionine is not processed off in the *E. coli* expression system and is retained in the recombinant protein.[25,32,33] Comparison of the crystal structures of the native protein and a recombinant version differing from the one used here only by the presence of an additional Asn-122 → Asp mutation shows that the methionine causes very little alteration in the protein structure.[33] Incubation of BHTOOH with recombinant Mb results in the formation of quinol (**1**), oxacycloheptadienone (**2**), dihydrofuranone (**3**), quinone (**4**), and two incompletely characterized products (**5** and **6**) (Figures 1 and 3). Product **5** has been shown to arise by addition of dioxygen to radical intermediate **7** (Figure 1; J. A. Thompson, unpublished). Compound **6** also appears to be formed via the free radical pathway because its formation is inhibited in the presence of reducing radical traps such as ascorbate. As reported earlier, quinol (**1**) is produced by heterolysis and the other products by homolysis of the BHTOOH peroxide bond.[29,30,34] The product distribution, which was quantitated with radiolabeled BHTOOH to optimize the accuracy of the analysis, indicates that recombinant sperm whale Mb turns over BHTOOH to the extent of approximately 32 nmol/10 min at 25 °C with a homolytic:heterolytic product ratio of 0.53 (Table I). This product ratio is essentially the same as the homolytic:heterolytic ratio of 0.57 obtained with native sperm whale Mb but is slightly larger than the ratio of 0.44 obtained with bovine Hb (Table I). The same five products are formed by the three hemoproteins, although there are small, difficultly interpretable differences in the ratio of the five homolytic products.

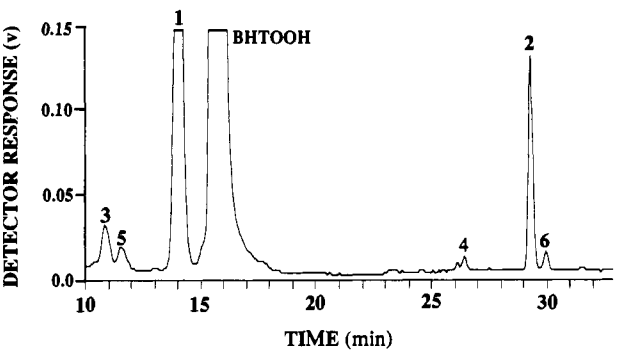

Figure 3. HPLC analysis of the products formed in the reaction of native sperm whale Mb with BHTOOH. The detector response at 236 nm is given on the ordinate. Peak numbers correspond to the compounds described in Figure 1.

Table II. Products from the Reduction of BHTOOH by Iron plus Ascorbic Acid

	products (nmol/nmol of Fe^{2+})					
	heterolysis	homolysis				
catalyst[a]	1	2	3	4	5	homo/het
Fe^{2+}/ascorbate (1 mM)	0.1	0.2	1.4	*b*	1.2	28.0
Fe^{2+}/ascorbate (10 mM)	2.8		0.3		0.6	0.32

[a] Reactions were conducted with 0.5 mM BHTOOH and 0.1 mM $FeCl_2$ plus ascorbate in aqueous phosphate buffer (pH 7.4) for 5 min at 25 °C. Product formation was linear with time to 5 min. [b] Blanks denote <0.05 nmol of product/nmol of Fe.

Reaction with $FeCl_2$. Product formation in incubations of $FeCl_2$ with BHTOOH was investigated in the presence of low and high

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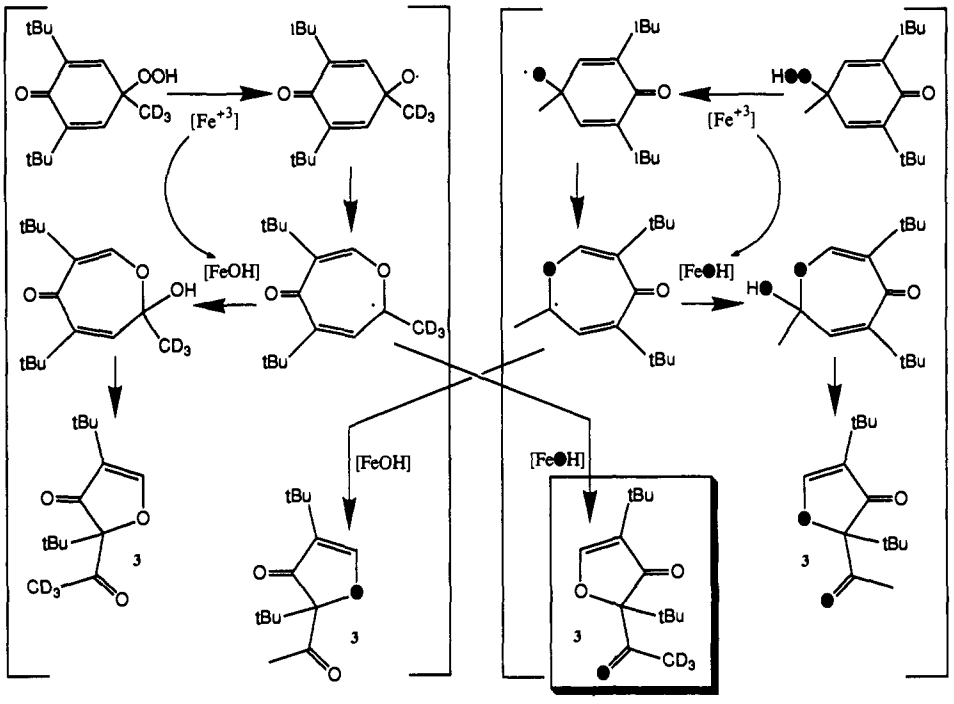

Figure 4. Scheme illustrating isotope labeling patterns expected for product **3** in co-incubations of [^{18}O]BHTOOH and [^{2}H]BHTOOH with sperm whale Mb. Product **3** is expected to have the same label as the parent peroxide if conversion of BHTOOH to **3** occurs within a single active site. Diffusion of the alkoxy radical from the active site, however, followed by reaction with the ferryl oxygen in a second active site yields a statistical distribution of labeled and unlabeled oxygen incorporation. A statistical distribution of oxygen label incorporation is also expected if the alkoxy radical is formed by peroxy radical dimerization/fragmentation outside the active site, followed by diffusion of the alkoxy radical into the active site.

concentrations of ascorbate to evaluate the effect of adventitious iron on product formation and to determine the affect of electron donors on the ratio of homolytic to heterolytic products. A low yield (3–4 nmol) of BHTOOH products is generated by 0.1 mM $FeCl_2$ in the presence of either 1 or 10 mM ascorbate under conditions otherwise comparable to those employed for the hemoprotein incubations (Table II). The product distributions in the two incubations are very different, however. Homolytic:heterolytic product ratios of 28 and 0.32 are obtained in the presence of 1 and 10 mM ascorbate, respectively. Incubation of BHTOOH with 10 mM ascorbate in the absence of an iron catalyst only produces 0.3 nmol of the quinol product. This concentration of ascorbate, however, clearly alters the product distribution obtained when iron is present, presumably by reducing the homolytically generated alkoxy radical to the alkoxy anion at a rate that competes effectively with skeletal rearrangement of the alkoxy radical. Addition of ascorbate to incubations of BHTOOH with sperm whale Mb also decreases the proportion of homolytic products (Table I), presumably by reducing the alkoxy radical to the alkoxy anion before it rearranges to radical **7** (Figure 1).

Peroxide Homolysis versus Hydroperoxide Oxidation. The reaction of BHTOOH with sperm whale Mb yields both **1** and homolytic products **2–6** (Figure 1, Table I). The alkoxy radical that gives rise to the homolytic products could be produced by simple peroxide bond homolysis (Figure 1) or, according to the mechanism outlined by Traylor and co-workers,[3,4] by oxidation of the hydroperoxide to the hydroperoxy radical (eq 1), dimerization of the hydroperoxy radical (eq 2), and elimination of molecular oxygen (eq 3) (R = non-hydroperoxide framework of BHTOOH). To evaluate the importance of reactions 1–3, we

$$[Fe^{V}{=}O] + ROOH \rightarrow [Fe^{IV}{=}O] + ROO^{\bullet} \quad (1)$$

$$2ROO^{\bullet} \rightarrow ROO{-}OOR \quad (2)$$

$$RO{-}OO{-}OR \rightarrow 2RO^{\bullet} + O_2 \quad (3)$$

have determined the content of labeled oxygen in **3** produced in incubations of Hb with BHTOOH ^{18}O labeled on both oxygens of the peroxide. The purpose of this experiment is to detect the exchange of alkyl hydroperoxy radicals with molecular oxygen according to the equilibrium of eq 4 to obtain an estimate of hydroperoxy radical formation.[35] Since the vinyl ether oxygen

$$ROO^{\bullet} \rightleftarrows R^{\bullet} + O_2 \quad (4)$$

in product **3** derives from the internal oxygen of the hydroperoxide group (Figure 1), exchange according to eq 4 should decrease the ^{18}O label at the vinyl ether position in **3** and provide a minimum measure of the extent of the product generated by reactions 1–3. Mass spectrometric analysis of dihydrofuranone **3** from the reduction of [^{18}O]BHTOOH by Mb shows that the vinyl ether and side chain carbonyl oxygens retain 95 ± 2 and 62 ± 2%, respectively, of the ^{18}O label. The corresponding values for Hb are 95% and 60%, respectively. In agreement with a low degree of oxygen exchange, only 1.5% of the vinyl ether oxygen was labeled in a converse experiment in which unlabeled BHTOOH was incubated with Mb under an atmosphere of $^{18}O_2$. High (>95%) retention of the original peroxide oxygen in the vinyl ether group rules out appreciable oxygen exchange via reaction 4 between the peroxy radical and molecular oxygen. The results provide little support for significant formation of homolytic products via oxidation of ROOH to the ROO• radical, although the contribution of the hydroperoxy radical pathway could still be significant if the oxygen exchange of eq 4 is slow relative to the hydroperoxy radical dimerization of eq 2. The labeling data also confirm the earlier finding that a substantial fraction of the ketone oxygen in **3** arises, as shown in Figure 1, by recombination of the ferryl oxygen with rearranged radical **7**.[29] The unlabeled oxygen incorporated into the carbonyl group of **3** in incubations with [^{18}O]BHTOOH is introduced by oxidation of the radical to a cation that is trapped by water.[29]

To further define the role of the processes described by eq 1–3, the formation of doubly (^{2}H and ^{18}O) labeled **3** in incubations of sperm whale Mb with a 39:61 mixture of [^{18}O]BHTOOH and [^{2}H]BHTOOH was determined. If compound **3** is produced by peroxide homolysis, it would be expected to retain the isotopic

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composition of the parent molecule if homolysis of the dioxygen bond, rearrangement of the alkoxy radical to **7**, and trapping of the radical by the ferryl oxygen occur within the same active site (Figure 4). On the other hand, the formation of doubly ^2H- and ^{18}O-labeled **3** is expected if the alkoxy radical diffuses out of the active site (Figure 4), or is formed via the peroxy radical dimerization process of eq 1–3. In the latter case, alkoxy radical **7** produced by peroxy dimer fragmentation must diffuse to some extent into a myoglobin active site to react with the ferryl oxygen to account for the 62% of **3** formed by radical recombination. The probability that **7** reacts with an ^{18}O-labeled ferryl oxygen would then be determined by the ratio of the ^{18}O-labeled to unlabeled BHTOOH in the reaction mixture. Thus, if 62% of the carbonyl oxygen of deuterated **3** is generated by recombination of deuterated radical **7** with the ferryl oxygen, and 39% of the ferryl oxygen is ^{18}O labeled, the theoretical maximum yield of **3** containing both ^2H and ^{18}O labels is 24.2% (39% of 62%). The observed value for the double labeled material is 1.9%, or 8% of the theoretical maximum. It therefore appears that 92% of the compound **3** that is generated by recombination of **7** and the ferryl oxygen is produced by homolytic scission of the BHTOOH dioxygen bond and recombination within the same active site rather than by diffusion of the alkoxy radical to a second active site or oxidation of BHTOOH to the hydroperoxy radical. These results strongly indicate that peroxide homolysis is the dominant mechanism for the production of **7** in the reaction of Mb and Hb with BHTOOH, although a fraction of the radical products can be accounted for by sequences such as that represented by reactions 1–3.

Reaction with the Tyr-103 → Phe, Tyr-151 → Phe, and His-64 → Val Sperm Whale Mb Mutants. To assess the influence of oxidizable and/or hydrogen bonding active site residues on the nature of the peroxide bond cleavage, we have examined the reaction of BHTOOH with three site specific mutants of sperm whale Mb: Tyr-103 → Phe, Tyr-151 → Phe, and His-64 → Val. The Tyr-103 → Phe mutant was investigated because Tyr-103 is in direct contact with the heme group (Figure 2) and, as shown by its involvement in heme and protein cross-linking reactions, is a locus of unpaired electron density in the oxidized protein.[23,24] The Tyr-103 → Phe mutant carries a second mutation (Lys-102 → Gln) because the Tyr-103 mutant is unstable unless the vicinal lysine is simultaneously converted to a glutamine. The same two amino acid changes are coupled in kangaroo myoglobin, one of the few myoglobins without a tyrosine at position 103.[25] Tyr-151, which is some distance (~14 Å) from the heme iron (Figure 2), has also been implicated by protein–protein cross-linking experiments as a locus of unpaired electron density in the oxidized protein.[23] The Tyr-151 → Phe mutation has been examined here to determine if a distant residue that is oxidized by the reaction with peroxides influences the peroxide bond cleavage. Finally, the His-64 → Val mutant has been examined because His-64 (Figure 2) corresponds to the catalytic distal histidine in the active site of cytochrome *c* peroxidase and other classical peroxidases.[8,12] The histidine in the peroxidases is believed to promote heterolysis of the peroxide bond by accepting the terminal proton from the hydroperoxide and transferring it to the internal oxygen in the dioxygen bond cleavage step. The histidine could play a similar role in Mb or could modulate peroxide bond cleavage by serving as an electron donor due to its proximity in the heme iron atom.

Analysis of the product mixtures obtained in the reactions of [^{14}C]BHTOOH with the Tyr-151 → Phe, Tyr-103 → Phe, and His-64 → Val sperm whale mutants demonstrates that the same products are obtained, and in similar proportions, as are obtained with the parent hemoprotein (Table I). The ratio of homolytic to heterolytic products is thus 0.55 for Tyr-151 → Phe, 0.63 for Tyr-103 → Phe, and 0.57 for His-64 → Val. These values differ little from that for the parent recombinant protein (0.53). A difference is seen in the rates of product formation, however, in that BHTOOH is turned over by the His-64 → Val mutant roughly twice as fast as it is by the native or wild type protein or by the other mutants (Table I).

Discussion

The results indicate that native and recombinant sperm whale metmyoglobin and bovine methemoglobin catalyze approximately twice as much heterolytic as homolytic scission of the BHTOOH peroxide bond. The resulting product distributions and the homolytic:heterolytic product ratios, which are more remarkable for their similarities than their differences, indicate that BHTOOH is similarly turned over by the three hemoproteins. The heterolytic process is readily rationalized by oxidation of the ferric heme to the ferryl complex with concomitant release of the BHTOOH-derived alcohol **1**, although it remains ambiguous whether the second electron required for the reaction is provided initially by the porphyrin and subsequently by the protein or directly by the protein. Two quite different mechanisms are possible, however, for the formation of homolytic products. One of these is direct homolysis of the peroxide bond of BHTOOH to give the corresponding alkoxy radical, as shown in Figure 1. The second mechanism involves oxidation of BHTOOH to the hydroperoxy radical (BHTOO$^\bullet$) by the ferryl species formed in an earlier heterolytic reaction of Mb with BHTOOH. Dimerization of the peroxy radical and oxygen extrusion (reactions 1–3) could then produce the alkoxy radical and thus give rise to the observed radical products. Two labeling experiments have been used to establish that the radical products are predominantly produced by direct homolytic scission of the peroxide bond rather than by the sequence triggered by initial formation of the peroxy radical. First, the dihydrofuran product **3** obtained in incubations of [^{18}O]BHTOOH under an atmosphere of normal oxygen, or of BHTOOH under an atmosphere of $^{18}O_2$, shows that there is little incorporation of atmospheric oxygen into the product, contrary to what would be expected for the peroxy radical if the equilibrium of eq 4 is in operation. In the second and more definitive experiment, we have found that there is little crossover of the labels when a mixture of [^{18}O]BHTOOH and [^2H]BHTOOH is incubated with Mb, as expected if the alkoxy radical rearranges and is trapped by the ferryl oxygen within the same active site in which it is formed. In contrast, a statistical mixture of the two labels would be expected by the peroxy radical mechanism because dimerization and alkoxy radical formation according to eq 1–3 must occur outside of the active site. The alkoxy radical from a given BHTOOH molecule therefore has a statistical chance of recombining, after diffusion into an active site, with the ferryl species produced by earlier reaction of the hemoprotein with either ^{16}O or ^{18}O peroxide. The reactions catalyzed by myoglobin and hemoglobin thus fall between that catalyzed by horseradish peroxidase, which converts BHTOOH exclusively to the heterolytic product **1**,[30] and rat liver microsomal cytochrome P450, which appears to catalyze nearly equivalent amounts of homolysis (47%) and heterolysis (53%).[34]

The reaction of BHTOOH with ferrous iron in the presence of a low concentration of ascorbate, in contrast to the reaction with myoglobin, yields primarily homolytic products (Table II). High concentrations of ascorbate, however, appear to quench the alkoxy radical, causing the predominant formation of what appear to be heterolysis products. These experiments confirm that the products formed in the myoglobin reactions are not due to adventitious iron and indicate that electron transfer to the alkoxy radical can compete with the rearrangement reaction. Inclusion of ascorbate in the Mb reactions decreases the homolytic:heterolytic ratio from 0.57 to 0.37, indicating partial suppression of radical product formation. This is likely to involve electron transfer to the alkoxy radical either in the active site or, to the extent that the alkoxy radical diffuses out of the active site, in the medium. Indeed, it could be argued that the peroxide bond is exclusively cleaved by a homolytic mechanism, yielding RO$^\bullet$ radicals of different oxidation potentials. Rapid electron transfer to the high oxidation potential radicals would give the unrearranged alcohol normally characteristic of peroxide heterolysis, whereas more sluggish electron transfer to radicals with lower oxidation potentials would give mixtures of rearranged and unrearranged products.

Partitioning between the homolytic and heterolytic pathways appears to be insensitive to the presence of electron-donating

(Tyr-103, His-64) or hydrogen-bonding (His-64) groups in the active site, although these groups may not be optimally placed to influence the course of the reaction. Thus, the observation that the homolytic:heterolytic product ratio is not significantly altered when Tyr-103 and Tyr-151 is replaced by a phenylalanine indicates that electron transfer from these residues, one of which is in direct contact with the heme (Figure 2), does not influence the nature of the peroxide bond cleavage. This finding argues against the formation of unrearranged ("heterolytic") products via homolysis and rapid transfer of a second electron to the alkoxy radical because such a mechanism should be sensitive to the nature of the electron donating residues in the active site. The data also show that the partition between homolytic and heterolytic pathways is not sensitive to replacement of His-64 by a valine (Table I). His-64 therefore does not facilitate heterolysis by acid–base catalysis or by serving as an electron donor. Either the histidine imidazole is inappropriately located with respect in BHTOOH bound in the heme crevice or the bond cleavage reaction is intrinsically insensitive to general acid–general base catalysis. The lack of influence of His-64 on the peroxide bond cleavage reaction is not due to an inherent inability to form a hydrogen bond to an iron-coordinated oxygen atom because precisely such a hydrogen bond is required to stabilize the water molecule that is normally coordinated to the iron in metmyoglobin.[25,36-40] Indeed, the higher product yields obtained with the His-64 → Val mutant are almost certainly due to faster reaction of the peroxide with the pentacoordinated heme group of the mutant hemoprotein than with the hexacoordinated heme group of native Mb.[38] We[41] and others[42] have shown, however, that His-64 is readily displaced to make room for bulky iron ligands. The displacement of His-64 required to allow BHTOOH to bind in the heme crevice could decrease its ability to participate in acid–base reactions with the iron-bound dioxygen moiety of the peroxide. BHTOOH serves as a good model in this regard for the reactions of myoglobin and hemoglobin with lipid hydroperoxides[43,44] and *tert*-butyl hydroperoxide, the latter of which has been shown by spin trapping studies to give rise to both the *tert*-butoxy and *tert*-butylhydroperoxy radicals,[45,46] but possibly not for H_2O_2, which is likely to be accommodated in the myoglobin active site with no more than a relatively small displacement of His-64.

The observation of both heterolytic and homolytic dioxygen bond cleavage indicates that the reaction of BHTOOH with Mb does not parallel the reaction of alkylperoxides with metalloporphyrins in organic solvents, in which heterolytic cleavage prevails,[2-4] nor in aqueous media, in which it has been argued that homolysis predominates.[2a] The failure of His-64 to alter the ratio of homolysis to heterolysis more closely parallels the absence of general acid–general base catalysis claimed for peroxide cleavage in water[2a] rather than the observation of such catalysis in organic solvents.[2-4] However, the geometry of His-64 with respect to the bond-breaking, bond-making centers is fixed in the protein but not in solution and may be suboptimal, particularly when an alkyl peroxide is bound. The failure of Tyr-103 to influence the ratio is more surprising because it is well placed for electron transfer to the heme, albeit not directly to the dioxygen bond. Allowance must clearly be made for the uniquely structured environment of proteins in extrapolating conclusions from model studies to enzyme mechanisms, but other factors may contribute to the differences between the model and enzymatic results.

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