Monoxygenation of an Aromatic Ring by F43W/H64D/V68I Myoglobin Mutant and Hydrogen Peroxide

MYOglobin MUTANTS AS A MODEL FOR P450 HYDROXYLATION CHEMISTRY*

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Myoglobin (Mb) is used as a model system for other heme proteins and the reactions they catalyze. The latest novel function to be proposed for myoglobin is a P450 type hydroxylation activity of aromatic carbons (Watanabe, Y., and Ueno, T. (2003) Bull. Chem. Soc. Jpn. 76, 1309–1322). Because Mb does not contain a specific substrate binding site for aromatic compounds near the heme, an engineered tryptophan in the heme pocket was used to model P450 hydroxylation of aromatic compounds. The monoxygenation product was not previously isolated and characterized as 6-hydroxytryptophan (Hara, I., Ueno, T., Ozaki, S., Itoh, S., Lee, K., Ueyama, N., and Watanabe, Y. (2001) J. Biol. Chem. 276, 36067-36070). In this work, a Mb variant (F43W/H64D/V68I) is used to characterize the monoxygenated intermediate. A modified (+16 Da) species forms upon the addition of 1 eq of H2O2. This product was digested with chymotrypsin, and the modified peptide fragments were isolated and characterized as 6-hydroxytryptophan using matrix-assisted laser desorption ionization time-of-flight tandem mass spectroscopy and 1H NMR. This engineered Mb variant represents the first enzyme to preferentially hydroxylate the indole side chain of Trp at the C6 position. Finally, heme extraction was used to demonstrate that both the formation of the 6-hydroxytryptophan intermediate (+16 Da) and subsequent oxidation to form the +30 Da final product are catalyzed by the heme cofactor, most probably via the compound I intermediate. These results provide insight into the mechanism of hydroxylation of aromatic carbons by heme proteins, demonstrating that non-thiolate-ligated heme enzymes can perform this function. This establishes Mb compound I as a model for P450 type aromatic hydroxylation chemistry.

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The abbreviations used are: Mb, myoglobin; Mb WDI, Mb F43W/H64D/V68I mutant; Mb WL, Mb F43W/H64L mutant; mCPBA, meta-chloroperoxybenzoic acid; OH-Trp, hydroxytryptophan; P450, cytochrome P450; WT Mb, recombinant sperm whale Mb; COSY, two-dimensional correlated spectroscopy; ROESY, rotational nuclear Overhauser effect spectroscopy; MS, mass spectroscopy; ESI-TOF, electrospray ionization-mass spectrometry; LC, liquid chromatograph; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
the study of P450s requires specialized low temperature techniques to trap its intermediates (5), other systems such as myoglobin could provide useful insight once suitable substrate binding sites are engineered.

WhenMb reacts with peroxide it forms a two-electron oxidized species called compound I that consists of an iron ferryl species and a porphyrin σ cation radical (Scheme 1). Compound I can perform either two-electron oxidation chemistry (catalasase and peroxide activity), regenerating the ferric resting state, or one-electron oxidation chemistry (peroxidase activity), generating compound II, which consists of a ferryl heme species. Compound II can then perform a second one-electron oxidation which returns the enzyme to its ferric resting state.

Theporphyrin radical species of compound I in WTMb is very unstable and cannot be detected. It can, however, be detected in certain variants where His-64 has been mutated, for example in the Mb F43W/H64D variant (19). His-64 has been proposed to donate an electron to the porphyrin σ cation radical, destabilizing compound I. Subsequently Trp and Tyr residues donate an electron forming more stable Trp or Tyr radical, destabilizing compound I. Subsequently Trp and Tyr radicals (20–28). Mutation of this distal His-64 prevents leakage of the oxidizing equivalents vital for two-electron oxidation chemistry away from the catalytic heme center. Wong and co-workers (29) have engineered a Tyr at position 43 of Mb to retain the oxidizing equivalents close to the heme catalytic site. This increased the two-electron, epoxidation rate 25- and peroxygenase activity, regenerating the ferric resting state. Compound II can then perform a second one-electron oxidation which returns the enzyme to its ferric resting state.

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In the process of studying Mb with an engineered Trp at the position 43, it was discovered that the protein was oxidatively modified, resulting in an increase in molecular mass of 30 Da (13). Proteolytic digestion with Lys-C indicated the modification was on the peptide fragment consisting of residues 43–47, which includes the engineered Trp (12). Its formation was determined to be heme-dependent, and peroxygenase activity, regenerating the ferric resting state. Compound II can then perform a second one-electron oxidation which returns the enzyme to its ferric resting state.

The proposed 6-OH-Trp intermediate was isolated and characterized by mass spectrometry and NMR, which provided data in support of the mechanism proposed by Hara et al. (12). Its formation was determined to be heme-dependent, which establishes Mb compound I as a model for P450 aromatic carbon hydroxylation chemistry.

**EXPERIMENTAL PROCEDURES**

**Materials**—H$_3^16$O$_2$ was prepared from $^{16}$O$_2$ as described by Sawaki and Foote (30), and the $^{18}$O content of the peroxide was determined to be 90% by oxidation of triphenylphosphine and gas chromatography/MS analysis (31). All other chemicals were obtained from Wako, Nakalai Tesque, and Sigma-Aldrich and used without further purification.

**UV-Visible Spectroscopy**—The UV-visible spectra of ferric Mb WDI was recorded on a Shimadzu UV-2400 spectrophotometer. The measurements were carried out with 10 μm protein in 50 mM potassium phosphate buffer (pH 7.0).

**Preparation of Myoglobin Variants**—The H64D and V68I mutants was performed as previously reported (32, 33). Protein concentrations were determined by the pyridine hemochromogen method (34). The Mb WL variant was previously prepared (13). Protein concentrations were determined by the ESI-TOF MS (Micromass).

**Purification of Peptide Fragments Bearing Trp-43**—Protein digestion was performed with Lys-C (1/50 w/w) in 100 mM Tris-HCl buffer (pH 9.0) containing 2 μM urea incubated at 37 °C for 12 h or chymotrypsin (1/50 w/w) in 50 mM potassium phosphate buffer (pH 8.0) incubated at 37 °C for 1.5 h. Digestion was stopped by flash-freezing in liquid N$_2$. The digested proteins were analyzed on a Q-STAR hybrid LC/MS system (PerkinElmer Life Sciences). Flow rates were 0.7 ml/min for analytical scale with a Vydac C-18 reverse phase column (0.46 × 25 cm) or 2.0 ml/min on a LC-10ADVP HPLC system (Shimadzu) with a Vydac C-18 reverse phase column (1.0 × 25 cm) for preparative scale. Peptides were eluted with a gradient of solvent A (0.1% trifluoroacetic acid in water) to solvent B (90% acetonitrile and 0.1% trifluoroacetic acid in water) over 55 min. The eluent was monitored either at 215 nm for amide bonds or 280 nm for aromatic residues in peptide fragments. The isolated fragments containing modified Trp-43 (+16 Da) were further purified with a gradient over 120 min. Isolated fragments containing modified Trp-43 (mass ± 30 Da) were not further purified.

**MS/MS Analysis of the Peptide Fragments**—The fragments, containing modified Trp-43, were analyzed on a Q-STAR hybrid LC/MS or Voyager DE-PRO (PerSeptive Biosystems) for MALDI-TOF MS. The spectrometer was calibrated with angiotensin I (molecular mass 1296.48 Da). 1 μl of concentrated sample was mixed with 1 μl of saturated α-cyano-4-hydroxycinnamic acid in water/acetonitrile (7:3) and applied to the sample plate by the dried-droplet method. Product ions were obtained using a post-source-decay method. Theoretical ionization spectra were predicted using Sherpa-lite 4.0.

**NMR Spectroscopy**—$^1$H NMR spectra of the fragments containing modified Trp-43 were obtained using an ECA 800 MHz (JEOL) spectrometer or on an INOVA 500 MHz (Varian) spectrometer. $^1$H NMR spectra of 5-OH-1-Trp (Wako) was measured on a 270 MHz G5X-270 NMR (JEOL). $^1$H NMR measurements were undertaken in D$_2$O at 30 °C, and chemical shifts were referenced to HDO. Complete proton resonance assignments were made using double quantum filtered-COSY, two-dimensional total correlation spectroscopy (TOCSY), and ROESY experiments.

**Preparation of Apo-unmodified and -modified Mb WDI and Their Reactions with Peroxides**—ApoMb WDI was prepared by heme extraction with butanone as previously described (35). To investigate whether...
heme was involved in the Trp-43 oxidation. 1–20 eq of H$_2$O$_2$ or mCPBA were added to apoMb WDI as described above. To determine whether the insertion of the second oxygen atom was heme-dependent, 1 eq of H$_2$O$_2$ was added to holo-Mb WDI to generate the +16-Da intermediate. Heme was then removed, and oxidation reactions were carried out. ESI-TOF MS of the reaction mixtures were measured before and after the reaction to determine the mass change.

**Stopped-flow UV-Visible Spectroscopy—** The spectral changes were monitored on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG6000 diode array spectrophotometer for a multiwavelength scan or on a Unisoku RSP-601 stopped-flow apparatus for a single-wavelength scan. The reaction with H$_2$O$_2$ was carried out in the presence of 5 μM protein in potassium phosphate buffer (pH 7.4) at 5.0 °C. The kinetic constants were determined by varying the H$_2$O$_2$ concentration under the pseudo-first-order conditions.

**Catalase Activity Measurement of Mb Variants—** Catalase activities of Mb WDI, Mb WL, and WT Mb were determined from the formation of molecular oxygen measured with a DO METER TD-51 oxygen electrode (TOKO) at 25 °C. The reaction mixture contained 10 μM Mb in 50 mM potassium phosphate buffer (pH 7.0) and 1 mM H$_2$O$_2$.

**Determination of Oxygen Source in the Modified Trp-43—** In previous studies, Trp-43 was engineered Trp at position 43 (12, 13), which were not modified by mCPBA— possible rearrangement of 2,6-dioxoindole to 2-imino-6-oxoindole. It had been proposed that many heme proteins other than P450s (and chloroperoxidase) could perform the hydroxylation of aromatic carbons (4, 12, 13), including those lacking thiolate ligation, which was long thought to be the key factor in conferring hydroxylation activity to P450 enzymes (7). A primary factor regulating the hydroxylation is likely the presence of an appropriate binding site that positions the substrate at the correct distance and orientation from the reactive compound I intermediate. We have, thus, used a covalently attached Trp (at position 43) in the distal heme pocket of a myoglobin variant to test this hypothesis (Fig. 1). In previous studies, Trp-43 was oxidized to 2,6-dioxoindole and 2-imino-6-oxoindole derivatives in a 6-electron oxidation process (12). The use of a strong oxidant (mCPBA) precluded the detection of two-electron-oxidized intermediate species. We hypothesized that a different mutant that could be modified by a milder oxidant such as H$_2$O$_2$ may allow the reaction to stop at the two-electron-oxidized intermediate. Substitution of the Val-68 in H64D variants of Mb has been shown to be a key in regulating peroxidase and peroxygenase rates as well as stereoselectivity of the products (36, 37). The combination of H64D/V68I mutations in Mb increased peroxygenase activity 1600-fold over WT Mb and altered the dominant product from (R) to the (S) isomer (36). The addition of H64D/V68I mutations to Mb F43W allowed for the oxidation of Trp by a single equivalent of H$_2$O$_2$ and characterization of this key intermediate.

**UV-Visible Spectroscopy of Mb WDI—** The UV-visible spectra of ferric Mb WDI in 50 mM potassium phosphate buffer (pH 7.0) is shown in Fig. 2. Ferric Mb WDI displays a Soret band at 408 nm and a visible band at 505 nm with a shoulder at 540 nm assignable to α and β bands, respectively, and a band at 636 nm assignable to a charge transfer band. The ratio of A$_{505}$/A$_{280}$ (RZ) is 3.2, which is lower than WT Mb but consistent with the addition of an extra Trp residue at position 43 in close proximity to the heme. This spectrum contains a broad shoulder at 380 nm, typical of penta-coordinated high spin heme found in WT Mb. The UV-visible data suggest no major structural changes affecting the heme of the Mb WDI variant have occurred.

**Oxidation of Mb F43W/H64D/V68I with Hydrogen Peroxide and mCPBA—** In contrast to the previous studies Mb F43W and Mb F43W/H64L (called Mb WL) variants with an engineered Trp at position 43 (12, 13), which were not modified by H$_2$O$_2$, Mb WDI was modified by H$_2$O$_2$. ESI-TOF MS data shows that the addition of 1 eq of H$_2$O$_2$ to Mb WDI resulted in an increase in mass of 16 Da from 17,360 to 17,376 Da (Fig. 3). In the previous studies the strong oxidant (mCPBA) precluded the detection of this +16-Da species. Although it is not possible to determine the nature of the modification from the MS data, these results are consistent with the formation of a 6-OH-Trp intermediate formed with 1 eq of H$_2$O$_2$. 

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**Scheme 2.** 1, proposed intermediates in tryptophan oxidation. 2, possible rearrangement of 2,6-dioxoindole to 2-imino-6-oxoindole.
The addition of 3 eq of H$_2$O$_2$ (Fig. 3) resulted in the almost complete conversion to a species with an increase in mass of 30 Da. The ESI-TOF MS spectrum after the addition of 5 eq of H$_2$O$_2$ showed a clear conversion to the +30-Da species had occurred (Fig. 3). Formation of 2,6-dioxoindole and 2-imino-6-oxoindole, tryptophan derivatives identified by Hara et al. (12) for Mb F43W/H64L, is consistent with the increase of 30 Da.

The reaction of Mb WDI with mCPBA revealed that more equivalents of mCPBA than of H$_2$O$_2$ were required to increase the mass of the enzyme (Fig. 3). These results are interesting because mCPBA is a stronger oxidant than H$_2$O$_2$; therefore, less should be required. Because mCPBA is bulkier, its access to the heme iron may be more restricted than H$_2$O$_2$. A crystal structure may aid to confirm this hypothesis. The possibility that mCPBA oxidizes the easily oxidizable amino acid residues such as Met on the surface of the protein is consistent with the formation of higher molecular weight species observed in the ESI-TOF MS spectrum (38).

Proteolytic Digestion and Purification of Modified Products—To determine the location of modification, the protein was digested with chymotrypsin or Lys-C after reaction with 1 or 5 eq of H$_2$O$_2$, and the resulting peptides were separated by reverse phase HPLC as described under “Experimental Procedures.” LC/MS and MALDI-TOF MS analysis were used to identify the peptides in each peak and/or fraction. Peaks corresponding to the unmodified fragment WDRFK (751 Da) of residues 43–47 containing the engineered Trp-43, as predicted from a theoretical digest with Sherpa Lite 4.0 and a +30-Da product (781 Da), could be detected in the Lys C-digested sample. This corresponds to the insertion of two oxygens and the loss of two protons as shown by Hara et al. (12); however, a +16-Da product from single oxygen insertion was not detected. Either the +16-Da product is unstable under the digestion conditions (pH 9.0 at 37 °C for 12 h), or it does not ionize well, or it is destroyed upon ionization. The inability to detect a Lys-C-digested fragment with an increase in mass of 16 Da (767 Da) proposed to be due to a 6-OH-Trp intermediate prompted a switch to chymotrypsin. Chymotrypsin has a milder optimum for digestion (pH 8.0 for 6 h at 37 °C) than Lys-C (pH 9.0 for 12 h at 37 °C). Analysis of the sample digested with chymotrypsin after the reaction with 1 eq of H$_2$O$_2$ by LC/MS resulted in the detection of a peak at 896 Da. This peak was consistent with the molecular mass of the modified (16+Da) EKWDRF fragment if chymotrypsin did not cleave at the modified tryptophan. The missed cleavage provides support for the modification occurring on Trp-43 since modification with oxygen(s) would make this residue more hydrophilic and bulkier to the extent that it could possibly no longer bind in the hydrophobic active site of chymotrypsin. This 896 Da (16+Da)
peak was purified by HPLC, and LC/MS/MS and MALDI-TOF MS (Data not shown) were obtained. The purified fragment was further characterized by MALDI-TOF MS/MS and NMR.

**MALDI-TOF MS/MS Analysis of Chymotrypsin and Lys-C-digested Fragments—**
Analysis of the MALDI-TOF MS/MS data for the modified (+16 and +30 Da) fragments shown in Fig. 4 identified the 781-Da (+30 Da) fragment as modified WDRFK (43–47) and the 896-Da (+16 Da) fragment as EKWDRF (41–46). A number of product ions were detected by MALDI-TOF MS/MS using post-source decay, which could be assigned to the \( b \) and \( y \) ion systems (predicted by Sherpa lite 4.0). The results indicate that both the +16 and +30 Da modifications are on the Trp-43 residue. Because MS/MS data, however, cannot determine the nature of the modification, the fragments were further characterized by \(^1\)H NMR.

**NMR Characterization of Digested Modified Samples—**
HPLC purified +16-Da (see Fig. 6) and +30-Da peptide fragments were characterized by one-dimensional and two-dimensional (COSY and ROESY) \(^1\)H NMR. First, it was important to confirm the nature of the +30-Da product. The +30-Da product was expected to be identical to the product identified by Hara et al. (12), however it was not infeasible that the product could be different since a different Mb variant and different oxidant were used. Peaks at 6.61 (\( s \)), 6.65 (\( d \)), and 7.21 (\( d \)) (Table I) can be assigned to C7, C5, and C4 protons, respectively. Comparison of the 781 Da (+30 Da) fragment NMR spectra with data from Hara et al. (12) clearly indicates this product is identical to the 2-imino-6-oxoindole obtained by Hara et al. (12) (Table I). Coupling between C4 and C5 protons in the COSY spectrum also supports this assignment. That the final products obtained in the Mb WL and the Mb WDI variants are identical provides strong evidence that the variants share a common mechanism.

In the 896-Da (+16 Da) fragment spectrum the aromatic region between 6.5 and 7.5 ppm contains 7 peaks (Fig. 5). Table I lists the chemical shifts and coupling constants. They have been assigned to a modified 6-OH-Trp-43 and Phe-46. Coupling between the C4 and \( \alpha \) and \( \beta \) protons in the ROESY spectra (Fig. 5B) assigns the C4 proton to the peak at 7.43 ppm. Based on this assignment, the resonances at 6.70 (\( dd \)), 6.86 (\( d \)), 7.07 (\( s \)), and 7.43 (\( d \)) ppm are unambiguously assigned to C5, C7, C2, and C4 protons of 6-OH-Trp-43, respectively. Furthermore, these shifts (and coupling constants) are consistent with those previously reported for 5-OH-Trp (39). Coupling between the C4 and C5 protons in COSY and ROESY spectra also supports this assignment. Comparison with authentic 5-OH-Trp (Wako) spectra further rules out 5-OH-Trp. The NMR data for 4-OH-Trp and 7-OH-Trp as obtained by Van Wickern et al. (39) are also included in Table I even though these structures exhibit clearly different spectra. We have, thus, shown for the first time that myoglobin can stoichiometrically hydroxylate aromatic carbons.
Heme Dependence of Mb F43W/H64D/V68I Oxidation—
Heme was extracted from Mb WDI to determine whether apoMb could be modified by H$_2$O$_2$. No change in mass occurs with up to 20 eq of H$_2$O$_2$ or 5 eq of mCPBA. Heme is, thus, required for the formation of the +16-Da oxidized product, as depicted in Scheme 3. Heme was also extracted after formation of the +16-Da modified species. The resulting apo+16-Da Mb also did not show any mass increase when reacted with up to 20 eq of H$_2$O$_2$. These studies suggest that both the formation of the +16-Da intermediate and subsequent oxidation to form the final +30-Da product are catalyzed by the heme cofactor, most probably via the compound I intermediate.

Stopped Flow UV-Visible Studies of the Reaction of Mb WDI with Hydrogen Peroxide—The reaction of Mb WDI with H$_2$O$_2$ was monitored by stopped flow UV-visible spectroscopy. The spectra and absorbance change at 408 nm are shown in Fig. 6. The Soret band in the initial spectra is decreased in intensity and slightly blue-shifted from the resting state (not captured on this time scale), which is consistent with a partial accumulation of compound I. The spectrum rapidly reverted to the ferric resting state described above for the modified samples. No evidence of compound II formation was observed in the reaction of WT Mb with excess H$_2$O$_2$. The lack of compound II formation could potentially be due to high catalase activity. This is, however, not the case (see below). The shoulder at 380 nm is less prevalent. This feature is consistent with a conversion a hexacoordinated high spin heme state. A water or hydroxyl group of 6-OH-Trp could provide the sixth ligand and may result in a less reactive ferric state. A modification of the Trp-43 that hydrogen bonds to an axially bound water molecule as shown in Scheme 4 could result in such spectral features. Additional H-bonding may also be provided by Asp-64 (Scheme 4).

Catalase Activity Assay of Mb Variants—To investigate if the inability to detect compound II was due to high catalase activity, oxygen evolution by Mb WDI in the reaction with excess H$_2$O$_2$ was monitored. The results are shown in Table II. The initial rate was found to be only four times higher than that of WT Mb (Table II), which should not account for the lack of compound II detection. Interestingly, only about four turnovers...
have observed more than 90% incorporation of 18O in the products of sulfoxidation and epoxidation (4, 43, 44), we are not able to detect the possible involvement of oxygen exchange. This may indicate that the final product spectra might be of an inhibited form of the enzyme, possibly an unreactive ferric state with a strong hydrogen-bonding interaction with water as shown in Scheme 4.

**Determination of Oxygen Source with Labeled H218O2 and Possible Mechanism of Tryptophan Oxidation by Mb Trp-43 Variants**—To clarify the oxidation mechanism, labeled H218O2 was used to measure 18O incorporation with MALDI-TOF MS. In the stoichiometrically oxidized sample containing Trp-43, fragment +16 and +18 Da could be observed (data not shown). The extent of 18O incorporation into the modified Trp-43 was 48% when normalized to the source. The 18O-labeling study is consistent with the mechanism of Trp oxidation proposed by Hara et al. (12); i.e. compound I reacts with Trp-43 to generate the epoxy intermediate, which is subsequently attacked by a water molecule (H218O) either at C6 or C7 position to afford a 6,7-diol intermediate with 18O scrambling (Scheme 2–1). The diol intermediate readily aromatizes by eliminating a water molecule to yield 6-OH-Trp having ~50% incorporation of 18O derived from H218O2. Although direct opening of the epoxide to produce only 6-OH-Trp (Scheme 5) should be more likely than hydrolysis followed by elimination of a hydroxy group, this would result in 100% incorporation of labeled oxygen, contradicting the observed results. Monooxygenation by P450 enzymes has been proposed to occur via a number of mechanisms (6–8). In addition to the epoxidation mechanism, concerted direct insertion of oxygen or non-concerted stepwise insertion involving hydrogen abstraction and oxygen rebound could afford 6-OH-Trp (6–8). The direct hydroxylation mechanism is also expected to afford 6-OH-Trp with 100% incorporation of labeled oxygen. If we assume the oxygen exchange between compound I and water molecule, ~50% incorporation of labeled oxygen could be observed even though the oxidation proceeds without forming the diol intermediate (40–42). Although we have observed more than 90% incorporation of 18O in the products of sulfoxidation and epoxidation (4, 43, 44), we are not able to eliminate the possible involvement of oxygen exchange.

To our knowledge Mb WDI is the first enzyme to preferentially form 6-OH-Trp. P450 oxidation of indole (Trp side chain) has been shown to yield the pigments indigo and indirubin via 3-OH-indole (indoxyl) (45). In this reaction 6-OH-indole is detected as a side product. Although the mechanism of its formation was not discussed, the possibility that it is identical to that used by Mb WDI cannot be ruled out. Oxindole, which has an oxygen inserted at the C2 position, was also detected in that study. P450 BM-3 has also been engineered to form indigo and indirubin (46). It does so by forming a mixture of 2-OH-indole and 3-OH indole, which is then further oxidized. Naphthalene dioxygenase also preferentially oxidizes the C2 and C3 positions of indole to form 2,3-dihydroxyindolinol (47). Styrene monooxygenase also shows a preference for epoxidation of the double bond between the C2 and C3 positions of indole forming the 2,3-epoxyindole (48). In contrast to these enzymes, tryptophan hydroxylase hydroxylates Trp at the C5 position (49). The preference for oxidation at the C2 and C3 positions by the abovementioned enzymes and position C5 by tryptophan hydroxylase or C6 by Mb WDI cannot be due solely to the fact that the C2 position of Trp is substituted (β-carbon). Indole 2,3-dioxygenase, another heme enzyme which binds Trp as a substrate, specifically oxidizes the C2 and C3 positions to degrade Trp to N-formylkynurenine (50).

The specificity of these enzymes for yielding different products further highlights the importance of substrate binding orientation and position in the active site. Crystallographic studies of our P43W variants may yield further insight into the structural factors controlling the oxidation mechanism for selective hydroxylation at the C6 position.

Recently two Trp residues in cytochrome c oxidase were reported to be oxidatively modified, resulting in an increase of 16 Da (51). These two Trp residues are 44 and 62 Å from the CuB binuclear heme site. In this case the modification has been proposed to occur via the migration of radicals formed at the heme though the protein. A recent crystal structure of lignin peroxidase shows that the β-carbon of the catalytically active Trp at position 171 is hydroxylated (52). This is also proposed to occur via radical transfer from the heme. As discussed above, a radical mechanism would not likely account for ~50% incorporation of labeled 18O into 6-OH-Trp-43 and is, thus, not likely occurring in Mb WDI. The nature of the +16-Da products in cytochrome c oxidase may provide further insight into the difference between their respective mechanisms. Alternatively, a number of mechanisms may occur simultaneously to yield 46% incorporation of labeled 18O, but this warrants further investigation.

**Implications for Understanding and Engineering Monooxygenation Activity in Home Proteins**—Monooxygenation by cytochrome P450 enzymes has been well characterized (5–8, 53). One factor preventing monooxygenation of various substrates by WT Mb and many other heme proteins is likely the lack of an appropriate substrate binding site in these proteins. Even though Mb does oxidize a number of substrates including thioanisole and styrene in an enantioselective manner, evidence for a specific high affinity binding site for these substrates has not been provided. The covalently attached indole of Trp-43 provides a model system that can be compared with other proteins. Although class I peroxidases (cytochrome c peroxidase, ascorbate peroxidase, and bacterial catalase peroxidases) also contain Trp in the distal heme pocket, this Trp is never reported to be hydroxylated. The reason for this is likely based on orientation, since the distance from the heme iron to Trp is 4.1 Å, similar to that of camphor to the heme iron of P450cam (4.2 Å) (54) and the modeled distance of Trp-43 to the heme iron in Mb WL (4.9 Å) (12). In Mb WDI the Trp is thought to be perpendicu lar to the heme, and the C6-C7 bond may be almost directly over the heme iron such that the Trp π orbitals can interact with the lone pair electrons of the oxygen. Pouls and co-workers (55) have oxidized the distal Trp in cytochrome c peroxidase and cross-linked it to an engineered Tyr by replacing the distal His (55). A radical mechanism involving the
engineered Tyr is proposed in this case. Again, the engineered Tyr appears to be appropriately positioned for oxidation, forming a Tyr radical that then migrates to the adjacent Trp.

We have demonstrated that variants of Mb (and possibly many other heme proteins lacking thiolate ligation) can perform stoichiometric monooxygenation of aromatic carbons under certain conditions if the substrate can be appropriately positioned. Efforts are currently under way to engineer specific, high affinity binding sites that position exogenous substrate at a suitable distance and orientation for monooxygenation. Although our current computer model of Mb WL and native P450 structures provides suitable starting points, we hope to gain greater insight from the crystal structures (work in progress) of our Trp-43 variants, ideally in both the native and modified forms.

In summary, Mb WDI was stoichiometrically oxidized by $\text{H}_2\text{O}_2$. The addition of 1 eq of $\text{H}_2\text{O}_2$ results in a 16-Da mass increase. MALDI-TOF MS/MS shows the addition of 16 Da to be on Trp-43, and NMR data has been unambiguously assigned from the crystal structures (work in progress) of our Trp-43 variants, ideally in both the native and modified forms.

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