The Roles of His-64, Tyr-103, Tyr-146, and Tyr-151 in the Epoxidation of Styrene and \( \beta \)-Methylstyrene by Recombinant Sperm Whale Myoglobin*

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Previous studies demonstrated that at least two mechanisms are involved in the epoxidation of styrene and stilbene by myoglobin and \( \text{H}_2\text{O}_2 \) (Ortiz de Montellano, P. R., and Catalano, C. E. (1985) J. Biol. Chem. 260, 9265–9271). One mechanism, reaction of the olefin with the ferryl oxygen, preserves the olefin stereoechemistry and incorporates an oxygen from \( \text{H}_2\text{O}_2 \) into the epoxide. The second mechanism, proposed to be a protein-mediated co-oxidation process, results in loss of stereoechemistry and incorporation of an atom of molecular oxygen. To examine the role of individual residues in olefin epoxidation, we have examined the catalytic activities of the possible Tyr→Phe mutants, the His-64→Val mutant, and a protein combining all the tyrosine and histidine mutations. The latter protein is less stable than the other mutants and is the only one for which a protein radical is not detected in the reaction with \( \text{H}_2\text{O}_2 \). The \( k_\text{cat} \) and \( V_{\text{max}} \) for styrene epoxidation range, respectively, from 0.3–8 mM and 12–35 pmol/min/mmol of protein. Incubation with \( \text{H}_2\text{^{18}}\text{O} \) results in 20–30% incorporation of labeled oxygen into the epoxide with all the mutants except Y103F/Y151F, Y146F/Y151F, H64V, and H64V/Y103F/Y146F/Y151F, for which 52, 58, 89, and 96% of the epoxide oxygen, respectively, is labeled. Oxidation of cis-\( \beta \)-methylstyrene by wild-type myoglobin yielded a 54:46 ratio of cis- and trans-\( \beta \)-methylstyrene oxides. The cis-isomer accounts for 47–100% of the epoxide produced by the mutant hemoproteins, with the two H64V mutants yielding almost exclusively the cis-epoxide. The oxygen in the cis-epoxide derives primarily or exclusively from \( \text{H}_2\text{O}_2 \), and that in the trans-epoxide from an alternative source. These results indicate that tyrosine residues may participate in, but are not essential for, protein-mediated epoxidation. In contrast, His-64 appears to be essential for co-oxidative epoxidation because in its absence olefin epoxidation is mediated almost exclusively by ferryl oxygen transfer.

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Reaction of metmyoglobin with \( \text{H}_2\text{O}_2 \) results in net one-electron oxidation of the heme\(^1\) group to a long-lived ferryl (\( \text{Fe}^{IV}=\text{O} \)) species (1, 2). NMR (3), resonance Raman (4), and x-ray edge absorption (5) have clearly demonstrated that an oxygen atom is bound to the heme iron in the myoglobin ferryl complex. An EPR-detectable protein radical is also formed in the reaction, but it is unstable and rapidly disappears (1, 2, 6–8). The EPR signal observed by freeze-quench methods accounts for no more than 15% of the expected radical, but extrapolation of the rate of decay of the EPR signal to zero time suggests that it accounts for at least half of the second oxidation equivalent provided by the peroxide (2). The radical signal observed in the reaction of myoglobin with ethyl hydroperoxide by flow EPR methods directly accounts for one-half of the possible protein radical (8). The mechanism by which the protein radical is generated, its location in the protein, and the mechanism(s) by which it is dissipated are only partly understood despite the potential role of oxidized myoglobin as a mediator of myocardial reperfusion injury (9). If the second oxidation equivalent of the peroxide is used to oxidize the porphyrin to a radical cation, as in horseradish peroxidase (10), subsequent electron transfer from the protein would quench the radical cation and produce the protein radical. This mechanism would parallel that postulated for cytochrome \( c \) peroxidase (11). Alternatively, homolytic scission of the peroxide could generate a hydroxyl radical that directly abstracts an electron from the protein. A distinction between these mechanisms has not yet been made, but the evidence suggests that methemoglobin (12) and metmyoglobin\(^2\) cleave organic peroxides by both heterolytic and homolytic mechanisms. Hydroxyl radical mechanisms are likely to be less regiospecific than those involving a porphyrin radical due to the high reactivity of the hydroxyl radical.

The location of the protein radical depends, to some extent, on the mechanism by which it is formed. The saturation properties of the EPR signal of the myoglobin protein radical indicate that it is primarily located on aromatic residues (7). Furthermore, the chemical reactivity of the protein radical (see below) indicates that tyrosine residues are major loci of unpaired electron density. There are 3 tyrosines in sperm whale myoglobin (residues 103, 146, and 151) (13) and 2 in equine myoglobin (residues 103 and 146) (14), the 2 proteins in which protein radical formation has been primarily investigated. Analysis of the differences in the EPR spectra of horse, sperm whale, and chemically modified sperm whale myoglobin suggest that Tyr-103 is one of the residues that

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\( ^1 \)The abbreviations used are: heme, iron protoporphyrin IX regardless of the iron oxidation or ligation state; DETAPAC, diethyltriaminepentaacetic acid.

\( ^2 \)Analogous evidence has been obtained for metmyoglobin (41).
bears unpaired electron density (8). Site-specific mutagenesis studies have demonstrated, however, that none of the tyrosines of sperm whale myoglobin is essential for protein radical formation (15). This is most clearly demonstrated by the fact that an EPR-detectable protein radical is obtained even when all 3 tyrosines are replaced by phenylalanines. It appears that the unpaired electron density moves readily between multiple sites in the protein, including the three tyrosine residues.

Strong evidence that the protein radical is at least partially centered on the tyrosine residues is provided by evidence that these residues are primary participants in the mechanisms that dissipate the radical. Reaction of sperm whale myoglobin with \( \text{H}_2\text{O}_2 \) results in oligomerization of the protein due to the formation of tyrosine-tyrosine cross-links (16, 17). Digestion of the protein and sequencing of the major dityrosine-containing peptide indicates that it results from cross-linking of Tyr-103 to Tyr-151 (17). The studies with tyrosine site-specific mutants indicate that a cross-link between two Tyr-151 residues is also possible (15).

The H64V mutant was constructed by cassette mutagenesis as described for the other mutants. The cassette was inserted between the BglII and BstEII restriction sites using oligonucleotides that coded for a valine substitution. The oligonucleotides were made so that the BstEII site was lost in the mutant gene (pMbH64V). This allowed for rapid screening of the plasmid. The mutation was sequenced to confirm the codon changes. The H64V mutant protein was purified as described for the wild-type protein (15).

The H64V/K102Q/Y103F/Y146F/Y151F mutant gene was constructed by replacing the SphI/HindIII segment of the pMbH64V gene from the corresponding restriction site in the plasmid containing the K102Q/Y103F/Y146F/Y151F mutations. The ligation was carried out in a final volume of 10 \( \mu \)l containing 5 \( \times \) ligation buffer and 1 unit of T4 DNA ligase and allowed to proceed overnight at 16 °C. The ligation mix was diluted 5-fold, and 5 \( \mu \)l was used to transform Escherichia coli DH5α competent cells. The cells were plated on LB agar containing ampicillin. Ten colonies were picked and glycerol stocks were made from them. One of four colonies that was partially sequenced using the M13 universal primer was chosen for further work and was completely sequenced.

The purification scheme used for the H64V/K102Q/Y103F/Y146F/Y151F mutant differed from that used for all the other mutants. A different scheme was required by the fact that the heme was lost during purification and that the protein, unlike the other mutants, was not very soluble at pH 6.0. Fresh overnight cultures of cells were used to inoculate 6 liters of LB media containing 100 \( \mu \)g/ml ampicillin, and the cultures were incubated overnight. The cells harvested by centrifugation were resuspended in 50 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed in a bead beater and were sonicated before they were centrifuged at 140,000 g for 60 min at 4 °C. The pellet obtained between 50 and 95% ammonium sulfate saturation, resuspended in 50 mM sodium phosphate buffer (pH 7.0), was applied to an S-Sepharose fast flow (Pharmacia LKB Biotechnology Inc.) column. The column was washed initially with 50 mM sodium phosphate buffer (pH 7.0) and then with a linear gradient set up between 50 mM sodium phosphate buffer (pH 7.0) (buffer A) and 100 mM sodium phosphate buffer (pH 8.0) (buffer B). The myoglobin eluted in buffer B. Continuing elution with 25% buffer B causes elution of the mutant apoprotein. The fractions containing the desired protein were pooled.

The apoprotein was reconstituted by adding to it a freshly made solution of hemin in 50 mM sodium phosphate buffer (pH 7.0) buffer and stirring overnight at 4 °C. The resulting protein solution was concentrated on an Amicon concentrator (YM 10 membrane), and the protein was passed through a Sephadex G-25 column to remove excess heme.

**Analytical Methods**—Absorption spectra were recorded on a SLM-Aminco DW-2000 UV-Vis spectrophotometer. Gas chromatography was performed on a Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector and interfaced to a Hewlett-Packard 3365 Chemstation. GC/MS analyses were performed on a Hewlett-Packard Model 5890 gas chromatograph coupled to a VG-70 mass spectrometer.

**Oxidation**—Reaction mixtures (125 \( \mu \)l total volume) contained myoglobin (30 \( \mu \)M heme), the indicated amounts of styrene, and 450 \( \mu \)M \( \text{H}_2\text{O}_2 \) in 0.2 mM phosphate buffer (pH 7.4). The styrene was added as an acetonitrile solution to give a final acetonitrile concentration of 1%. The myoglobin/styrene solution was preincubated for 10 min at 0 °C before the reaction was initiated by adding the precooled peroxide solution. Incubations were carried out at 0 °C for 30 min. trans-\( \beta \)-Methylenestyrene was added at the end of the incubation period as an internal standard.

Methylene chloride (50 \( \mu \)l) was added and the reaction tubes were vortexed and centrifuged in an IEC Centra-8 centrifuge at 3000 rpm for 20 min. The methylene chloride layer was withdrawn and concentrated under a stream of argon for GLC analysis. Incubations containing DETAPAC (0.1, 0.5, 1, or 25 mM), desferoxamine (100 or 500 \( \mu \)M), or mannitol (20 or 50 mM) were carried out in a similar manner. Control incubations without myoglobin, or without \( \text{H}_2\text{O}_2 \), were also carried out. Mixtures containing known amounts of styrene oxide and benzaldehyde, but no styrene, were incubated and worked up in a similar manner to generate standard curves for product quantitation. Analysis of styrene oxidation was done by gas chromatography on a DB-1 column (0.25 mm x 30 m; J & W Scientific) programmed to hold at 80 °C for 3 min and then to rise at 4 °C/min to 150 °C.

Proteins—The preparation of the following single, double, and triple tyrosine mutants of sperm whale myoglobin has been described (15): Y103F, Y146F, K102Q/Y103F, K102Q/Y146F, Y103F/K151F, Y151F/Y146F, and K102Q/Y103F/Y146F/Y151F. The K102Q mutation is required to stabilize the protein with the Y103F mutation and is therefore present in all the Tyr-103 mutants (15).
concentrations of styrene were assayed, including concentrations both larger and smaller than the $K_n$, $K_0$, and $V_{max}$ values were obtained by fitting the data to the Michaelis-Menten equation $V = V_{max} [S]/(K_n + [S])$ using the KinetAsysta* program (Copyright 1989, James G. Robertson) on a Macintosh computer.

cis-β-Methylstyrene Oxidation—Reaction mixtures (125 μl final volume) contained myoglobin (30 μM), cis-β-methylstyrene (10 mM), and 450 μM $H_2O_2$ in 0.2 M phosphate buffer (pH 7.4). The olefin was added as an acetonitrile solution. Incubations and work-up were as described for the oxidation of styrene, except that no internal standard was added. GLC analysis was as described for styrene. The retention times for cis-P-methylstyrene oxide, trans-P-methylstyrene oxide, and phenylacetone were 21.5, 22.0, and 22.5 min, respectively.

RESULTS
Characterization of the H64V Mutant—The Soret maximum of the H64V mutant ($λ_{max} = 395$ nm) differs markedly from that of the wild-type or native proteins ($λ_{max} = 409$ nm). NMR studies of a slightly different H64V mutant support the conclusion that this spectroscopic difference is caused by the absence in the mutant of the water iron ligand that is present in the native protein (24, 25). Valine favors the pentacoordinate state because it eliminates a His-64 hydrogen bond that stabilizes the iron-coordinated water. The same conclusion was reached when the histidine was modified with cyanogen bromide (26). One consequence of the pentacoordinate state of the iron, and the smaller size of valine than histidine, is that ligands bind to the mutant protein at substantially (10<sup>2</sup>-10<sup>3</sup>) faster rates (27, 28). The mutant also autooxidizes more readily than native myoglobin (not shown). Addition of $H_2O_2$ to the H64V mutant results in a gradual concentration- and time-dependent decrease in the Soret absorbance without a detectable shift in the Soret maximum (Fig. 1). However, addition of 1 eq of $H_2O_2$ and freeze-quenching of the sample gives rise to an EPR signal at $g = 2.0014$ analogous to that obtained with wild-type myoglobin and the tyrosine mutants (Fig. 2) (15). In contrast to the results with the tyrosine mutants, however, no ferryl spectrum is observed when the EPR sample is thawed.

Characterization of the H64V/K102Q/Y103F/Y146F/Y151F Mutant—The quadruple Tyr/His mutant readily lost its heme group during purification and was generally less stable than the other mutants, presumably as a consequence of the accumulated structural perturbations caused by the five mutations. Its absorption maximum, however, unlike that of the simple H64V mutant, has a Soret maximum at 408 nm similar to that of the native protein (408 nm). This suggests that the iron may be hexacoordinate, as it is in native myoglobin. The spectra of the protein in the ferrous ($λ_{max} = 426$ nm), ferrous dioxy ($λ_{max} = 408$ nm), and ferrous carbon monoxy ($λ_{max} = 420$ nm) states are also similar to those of the corresponding states of the native protein (ferrous, 432 nm; ferrous dioxy, 416 nm; and ferrous carbon monoxy, 420 nm). As found for the H64V mutant (Fig. 1), addition of $H_2O_2$ results in a gradual decrease (Fig. 3) and concentration-dependent decrease in the Soret absorbance without a shift in the Soret maximum. No evidence is seen for formation of a

![Fig. 1. Absorption spectrum of the H64V mutant before (-----) and after addition of 1 (-----), 5 (-----), and 10 (-----) equivalents of $H_2O_2$. Similar changes are seen for the H64V/K102Q/Y103F/Y146F/Y151F mutant.](image)

![Fig. 2. EPR spectrum of the $H_2O_2$-treated H64V mutant. The EPR parameters are: gain, $4.0 \times 10^{3}$; microwave power, 2 milliwatts; modulation intensity, 8 G; time constant, 0.5 s; scan time, 4 min; magnetic field, 3269 G; range, 500 G. The signal is centered at $g = 2.0014$.](image)

![Fig. 3. Time dependence of the decrease in the Soret absorbance in incubations of the H64V/K102Q/Y103F/Y146F/Y151F mutant with $H_2O_2$ in the absence (■) and presence (□) of styrene. For comparison, the decrease in the Soret absorbance for the native enzyme is shown in the absence (■) and presence (□) of styrene. The precipitate decrease in the Soret absorption for the native enzyme is due to shift of the $λ_{max}$ from 408 nm to 422 nm immediately after addition of $H_2O_2$ (note that the resulting Soret band is relatively stable with time for the native protein).](image)
compound II-like species with a maximum at 422 nm analogous to that observed in the reaction of the native protein with H$_2$O$_2$ (1, 2). The rate of loss of the Soret absorbance is approximately the same in the absence or presence of styrene (Fig. 3) notwithstanding the fact that styrene is an acceptor for the putative ferryl oxygen (see below). In contrast to the H64V mutant, which did not give a detectable ferryl chromophore but did give an EPR-detectable protein radical, the quadruple Tyr/His mutant does not give a detectable protein radical (not shown). Parallel incubations of the quadruple Tyr/His mutant and native sperm whale myoglobin show that, under conditions that give a readily detectable protein signal for the native protein, no protein radical signal whatever is detectable for the mutant.

Substrate Oxidation by Recombinant Sperm Whale Myoglobin—The H$_2$O$_2$-dependent oxidation of styrene by recombinant sperm whale myoglobin, as previously reported for the native protein (19), yields styrene oxide and benzaldehyde (Table I). The identities of the two products have been established by chromatographic and mass spectrometric comparisons with authentic standards (not shown). A peak with the retention time of phenylacetaldehyde was also obtained but in such trace amounts that its identity could not be confirmed. The $K_m$ and $V_{max}$ for the formation of styrene oxide are 6 mM and 31 pmol/min/nmol, respectively, and the $V_{max}$ for benzaldehyde formation is 16 pmol/min/nmol. The corresponding values for formation of styrene oxide by native sperm whale myoglobin are $K_m = 1$ mM and $V_{max} = 31$ pmol/min/nmol. The values for the native and recombinant protein are very similar, in accord with the fact that the only difference between them is the presence of the terminal methionine in the recombinant protein. The data also agree with the fact that there is little difference between the crystal structures of native sperm whale myoglobin and a recombinant protein differing from the one described here only by the presence of an additional (N122D) mutation (29). Mass spectrometric analysis of the epoxide produced from styrene in incubations with metmyoglobin and [18O]H$_2$O$_2$ shows that only 27% of the epoxide oxygen derives from H$_2$O$_2$, whereas the remainder is from a source other than H$_2$O$_2$, presumably from O$_2$ (18, 19). Phenylacetonitrile is produced by a mechanism analogous to that which yields the cis-epoxide because the ketone oxygen also derives primarily from the peroxide (Table I).

To determine whether freely diffusible hydroxyl radicals are involved in the epoxidation reaction, styrene was incubated with myoglobin and H$_2$O$_2$ in the presence of chelating agents or a hydroxyl radical trap. The rates of formation of styrene oxide and benzaldehyde were not affected by concentrations of DETAPAC ranging from 0.1 to 1 mM, although they were decreased by 50% in the presence of a very high concentration (25 mM) of DETAPAC (not shown). Concentrations of mannitol up to 50 mM caused no change whatever in the rates of formation of styrene oxide or benzaldehyde. These results are in accord with previous results (19). In contrast, a 100 μM concentration of desferioxamine, a powerful chelating agent for iron, decreased the rates of formation of styrene oxide and benzaldehyde from 32 to 12 and 10 to 3 pmol/min/nmol of protein, respectively. Further decrease, to 9 and 3 pmol/min/nmol of protein, respectively. The cis:trans ratio of β-methylstylene oxides obtained from cis-β-methylstyrene was also altered by 100 μM desferioxamine from 74:26 to 90:10. Recent studies have shown, however, that desferioxamine directly reduces the ferryl species of myoglobin and therefore is expected to decrease epoxide formation (31). Furthermore, differential reduction of the ferryl species and protein radical by desferoxamine readily rationalizes the change in the cis:trans-isomer ratio. Desferoxamine therefore cannot be used to test for the involvement of chelatable iron in the myoglobin/H$_2$O$_2$ system.

Styrene Oxidation by Mutant Myoglobins—The oxidation of styrene by the three single, two double, and triple Tyr → Phe mutants of sperm whale metmyoglobin has been examined to determine the roles of the individual residues in the oxidation process. Oxidation of the olefin by the H64V mutant

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**Table I**

| Myoglobin | Styrene oxide | Benzaldehyde | $^{18}$O incorporation from H$_2$O$_2$ into styrene oxide |
|-----------|---------------|--------------|------------------------------------------------------|
|           | $K_m$ (mM)    | $V_{max}$ (pmol/min/nmol protein) | $V_{max}$ (pmol/min/nmol protein) | % |
| Native    | 1 ± 0.2       | 31 ± 2       | 14 ± 5      | 22 |
| Wild-type | 8 ± 2         | 31 ± 6       | 16 ± 2      | 27 |
| Y103F*    | 6 ± 2         | 35 ± 6       | 18 ± 1      | 19 |
| Y146F     | 7 ± 2         | 12 ± 2       | 15 ± 1      | 21 |
| Y151F     | 8 ± 1         | 14 ± 1       | 12 ± 2      | 23 |
| Y146F/Y151F | 8 ± 2      | 20 ± 2       | 25 ± 6      | 58 |
| Y103F/Y151F* | 2 ± 0.3  | 18 ± 1       | 10 ± 1      | 52 |
| Y103F/Y146F/Y151F* | 5 ± 1 | 24 ± 3       | 28 ± 6      | 28 |
| H64V      | 4 ± 0.3       | 29 ± 1       | 7 ± 1       | 89 |
| H64V/Y103F/Y146F/Y151F* | 0.3 ± 0.02 | 26 ± 0.5     | 8 ± 1       | 96 |

*All Tyr-103 → Phe mutants also carry the Lys-102 → Gln mutation required to stabilize the Tyr-103 alteration.*
has also been examined because His-64 is within hydrogen-bonding distance of the ferryl oxygen and is part of the protein structure which controls access of small substrates to the heme iron atom (27, 28, 32). Finally, the combined contributions of the tyrosines and His-64 have been evaluated with the quadruple Tyr/His mutant in which all the tyrosines are replaced by phenylalanines and His-64 by a valine. As shown in Table II, all the mutants oxidize styrene to styrene oxide and benzaldehyde in the presence of H₂O₂. The Kₐ values for the epoxidations differ little (4-8 mM) except for those of the K102Q/Y103F/Y151F (Kₐ = 2 mM) and H64V/K102Q/Y103F/Y146F/Y151F (Kₐ = 0.3 mM) mutants. The Vₘₐₚ values for the epoxidations range from 12 to 35 pmol/min/nmol, and those for the formation of benzaldehyde from 7 to 28 pmol/min/nmol (Table I).

Oxygen from H₂¹⁸O₂ is incorporated into the epoxide by all the mutants, but to different extents. The single tyrosine mutants give results similar to those obtained with the native enzyme, but the double tyrosine mutants Y146F/Y151F and K102Q/Y103F/Y151F yield epoxides with 58% and 52% incorporation of peroxide-derived oxygen. This is approximately double the incorporation seen with the wild-type enzyme. A similar process may be involved in the myoglobin-catalyzed reaction (Fig. 4). On the other hand, formation of the cis- and trans-epoxides from cis-β-methylstyrene is clearly differentiated in mechanistic terms by the degree of incorporation of oxygen from the peroxide (Table II). The cis-epoxide is obtained with very high (90-100%) incorporation of oxygen from the peroxide. In contrast, the oxygen in the trans-epoxide derives primarily (>80%) from a source other than the peroxide, presumably from molecular oxygen. The oxygen in phenylacetone, like that in the cis-epoxide, derives largely from the peroxide.

**DISCUSSION**

Incorporation of oxygen from the peroxide into the cis-epoxide in the oxidation of cis-β-methylstyrene is best rationalized by cytochrome P450-like transfer of the ferryl oxygen to the olefin. The mechanistic details of cytochrome P450-catalyzed epoxidations remain unclear, but studies of epoxidations catalyzed by simple metalloporphyrins have led Bruce and co-workers (33, 34) to propose that the first and rate-limiting step is the formation of a charge-transfer complex. A similar process may be involved in the myoglobin-catalyzed reaction (Fig. 4).

In the oxidation of the trans-epoxide with incorporation of oxygen from molecular oxygen presumably does not involve direct interaction of the olefin with the ferryl oxygen. The cis/trans-epoxide ratio therefore provides a measure of the relative importance of the two reaction pathways, although the ratios are approximate because there is some peroxide oxygen in the trans-epoxide and vice versa. The failure of DETAPAC and mannitol to alter the rates of styrene oxide and benzaldehyde formation.

![Fig. 4. Hypothetical mechanism for the formation of cis-β-methylstyrene oxide and phenylacetone from cis-β-methylstyrene in which the first and rate-limiting step is charge-transfer complex formation.](image-url)
confirms that freely diffusible hydroxyl radicals are not involved in the oxidation reactions.

The ratios obtained with the tyrosine mutants generally suggest little correlation between the proportion of the reaction that involves direct interaction of the olefin with the ferryl oxygen and the number of tyrosine residues (Table II). The two exceptions are the Y146F/Y151F and K102Q/Y103F/Y151F double tyrosine mutants, which produce a significantly higher yield of the cis- than trans-epoxide (Table II). The absence of a detectable relationship between the presence of the individual tyrosine residues and loss of the olefin stereochemistry provides no support for the postulated role of tyrosine residues in co-oxidative epoxidation (19, 20), although the expected decrease in the trans-epoxide is observed with the two double tyrosine mutants. It may be significant that the two double tyrosine mutants that show increased production of the trans-isomer are those with deletions of tyrosines 103 and 151. The crystal structure suggests that Tyr-103 and Tyr-151, in contrast to Tyr-146, are solvent-accessible. This inference is supported by the pKₐ values of the tyrosine residues: Tyr-151 (pKₐ = 10.3) has a pKₐ identical with that of tyrosine in water, Tyr-146 (pKₐ = 12.9) a value suggestive of a hydrophobic environment, and Tyr-103 (pKₐ = 11.8) a value suggestive of an intermediate environment (35, 36). Furthermore, Tyr-103 and Tyr-151 are involved in H₂O₂-dependent protein-protein and protein-heme cross-linking reactions. However, the yield of trans-product is not increased by individually replacing Tyr-103 or Tyr-151 with a phenylalanine, or by replacing all 3 tyrosines, results that are not readily reconciled with the hypothesis that one or more of the tyrosine residues is (are) essential for the epoxidation reaction (Table II).

The triple tyrosine mutant is also anomalous in that its Vₘₐₓ for the oxidation of styrene is similar to that for the wild-type enzyme, whereas all the other tyrosine mutants, except Y103F, have smaller Vₘₐₓ values. A smaller Vₘₐₓ value would be expected if tyrosine residues catalyze a fraction of the epoxidation reaction, assuming that there are no compensatory changes, such as an increase in the amount of the ferryl species, in the absence of the tyrosines. The cis-β-methylstyryle results show, however, that retention of the olefin stereochemistry is associated with incorporation of oxygen from the peroxide and therefore with oxygen transfer from the ferryl species. The fractions of the epoxide with oxygen from the peroxide, however, do not correlate with the Vₘₐₓ values, so that it is likely that the tyrosine alterations are causing structural perturbations that alter the Vₘₐₓ values.

The high cis/trans ratios of the β-methylstyrene epoxide produced by the H64V and H64V-containing quadruple mutants are consistent with the fact that His-64 blocks entry into the heme crevice. Its replacement by a smaller group would be expected to make the heme more accessible, and therefore to increase the proportion of the product formed by direct interaction with the ferryl oxygen. This is confirmed by the fact that 100% of the oxygen derives from the peroxide in the cis-β-methylstyrene epoxide (Table II) and a very high proportion in the styrene epoxide (Table I). The Vₘₐₓ values for the epoxidation of styrene, however, are similar to that for the wild-type enzyme, suggesting that epoxidation by the ferryl oxygen occurs as rapidly as the sum of the processes that epoxidize styrene in the wild-type enzyme.

Phenylacetone accounts for approximately 20% of the products formed in the oxidation of cis-β-methylstyrene by both the native and mutant hemoproteins. The phenylacetone appears to come from the oxidation pathway that produces the cis-epoxide, or a closely related pathway, because the oxygen of the ketone derives primarily (or exclusively) from the peroxide. The incorporation of oxygen from the peroxide into the ketone was comparable for the wild type enzyme and the mutants (Table II). This finding is consistent with the proposal that the first step of the interaction of the olefin with the ferryl oxygen is the formation of a charge-transfer complex that can decay by different mechanisms. One of these mechanisms leads, without loss of stereochemistry, to the epoxide. The other leads to a cationic species that undergoes a hydrogen shift to give the observed ketone (Fig. 4). The hydrogen shift is more favored for cis-β-methylstyrene than styrene because only a trace of phenylacetaldheyde, the corresponding styrene product, is detected. In both instances, the cation is formed adjacent to the phenyl ring, so that electronic differences caused by the methyl group are unlikely to be critical in determining the extent of the hydrogen shift. The higher yield of the ketone from cis-β-methylstyrene may be triggered by steric interactions between the phenyl and methyl groups that force them to twist out of the plane of the double bond. Conformational AM1 calculations suggest that the angle between the phenyl and methyl groups is 41° (37). The associated torsional distortion of the σ-bond may increase the fraction of the cis-β-methylstyrene-ferryl complex, relative to that of the styrene complex, that decays to the acyclic cationic intermediate in Fig. 4. It is to be noted that rotation about the carbon-carbon bond is required to bring the β-hydrogen into a position from which it can migrate to the sp²-hybridized α-carbon (38).

The present results confirm that there are at least two epoxidation mechanisms differing in their stereochemical specificity and source of epoxide oxygen. The results also show that tyrosine residues may be involved in protein-mediated olefin oxidation because their replacement by phenylalanines alters the Vₘₐₓ for styrene epoxidation, the cis/trans ratio of β-methylstyrene epoxide products, and the extent of incorporation of oxygen from the peroxide. However, the changes in the three parameters are not related in a straightforward manner to the Tyr → Phe mutations. This is evident in the fact that the mutant with no tyrosines is indistinguishable from the protein with 3 tyrosines in terms of the Vₘₐₓ for styrene epoxidation, cis/trans ratio of β-methylstyrene epoxide products, and degree of peroxide oxygen incorporation. The inescapable conclusion is that the tyrosine residues may participate in protein-mediated epoxidation but are not essential for that reaction. This is consistent with our finding that a protein-free radical is detected in the single, double, and triple tyrosine mutants (15).

In contrast, the evidence suggests that His-64 is essential for protein-mediated epoxidation. In the absence of His-64, the oxygen incorporated into the products derives almost exclusively from the peroxide, and cis-β-methylstyrene is oxidized with high specificity to the cis-epoxide. The increased accessibility of the heme iron made possible by the His → Val mutation may be one reason that the Vₘₐₓ for styrene epoxidation is the same as for the wild-type enzyme despite the fact that the second epoxidation mechanism is virtually suppressed. It has also been shown that modification of the imidazole by cyanogen bromide yields an enzyme with no water coordinated to the heme that consequently reacts with H₂O₂ at a rate (5.2 × 10⁷ dm³ mol⁻¹ s⁻¹) (26) comparable to that of the reaction with horseradish peroxidase (1.2 × 10⁷ dm³ mol⁻¹ s⁻¹) (39) rather than with intact metmyoglobin (1.0 × 10⁷ dm³ mol⁻¹ s⁻¹) (40). More rapid formation of the ferryl species could also contribute to the rate of styrene oxide formation if the rate-limiting step is not actual ferryl oxygen transfer to the olefin. The histidine, in addition, may either
act as a mediator of the transfer of unpaired electron density to the tyrosine residues or may participate directly in the epoxidation process. Support for the latter is provided by the finding that the His-64 mutant still reacts with H$_2$O$_2$ to give a protein radical, and is still readily cross-linked, implying that the tyrosine radicals, including Tyr-103, are readily formed. The histidine thus is clearly not required for formation of the tyrosine radicals. King et al. (7) reported that the tyrosine radical observed in the reaction of myoglobin with H$_2$O$_2$ is preceded by formation of another radical and suggested that His-64 was the site of the initial radical. Miki et al. (8) likewise reported that a transient radical precedes the accumulation of unpaired electron density on Tyr-103 (8). Evidence has been reported that His-64 is chemically modified in the reaction of myoglobin with H$_2$O$_2$ at acidic pH values, but the residue modified was not clearly identified (42). This observation is consistent with involvement of His-64 in styrene co-oxidation because the reaction would result in structural modification of the histidine residue. Although the role of His-64 remains to be defined, it is possible that histidine radicals such as those implicated in the copper-mediated oxidation of histidine are involved in styrene co-oxidation (43).

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