Conversion of Myoglobin into a Highly Stereospecific Peroxygenase by the L29H/H64L Mutation

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Myoglobin (Mb), normally a carrier for molecular oxygen, can catalyze hydrogen peroxide supported peroxygenation of a variety of substrates, including olefin epoxidation and thioether sulfoxidation.[1] However, the turnover numbers for sulfoxidation by Mb are lower than the values obtained by the incubation with peroxidases.[2] In contrast to the oxidation of alkene mediated by cytochrome *c* peroxidase (C*c*P), horseradish peroxidase (HRP) mutants, and chloroperoxidase (CPO) from the fungus *Caldariomyces fumago*, olefin epoxidation by Mb results in low yield incorporation of peroxide oxygen due to the competitive molecular oxygen incorporation.[2,3] Catalysis by Mb is presumably associated with an intermediate equivalent to compound I of peroxidase; however, the exact location of one of the two oxidation equivalents has not clearly been defined yet.[4] In order to identify active site residues controlling ferryl oxygen transfer reactions, we have performed site-directed mutagenesis studies of sperm whale myoglobin. Previous studies indicate that the replacement of His-64, 4.3 Å above the heme iron,[5] by unoxidizable amino acids such as a valine prevents protein-peroxy radical mediated epoxidation,[3b] but the formation of ferryl species with hydrogen peroxide appears to be slower than that for wild type Mb. The comparison of the X-ray structures of C*c*P[6] and Mb[5] suggests that the Leu-29 → His and His-64 → Leu double mutation of Mb would create a heme crevice similar to the active site structure of C*c*P, of which the distal histidine lies 5.6 Å above the heme iron, and we expect the L29H/H64L mutant would transfer the ferryl oxygen to the substrates efficiently (Figure 1). Thus, we have constructed L29H, H64L, and L29H/H64L mutants[7] and found that L29H/H64L Mb significantly increases the rate for the oxidation of both thioanisole and styrene and, more importantly, the enantioselectivity. In addition, the oxygen atom of epoxide formed by the incubation with the L29H/H64L mutant is mostly derived from peroxide.[8]

Mutating Leu-29 to a histidine residue improves the rate and enantioselectivity for the oxidation of thioanisole (Table 1).[9] The rate increases versus wild type Mb are 15-fold and 22-fold for L29H and L29H/H64L Mb, respectively. On the contrary, the elimination of His-64 in the distal pocket causes about 70% decrease in the oxidation rate with respect to the recombinant wild type. The L29H/H64L double mutation enhances the

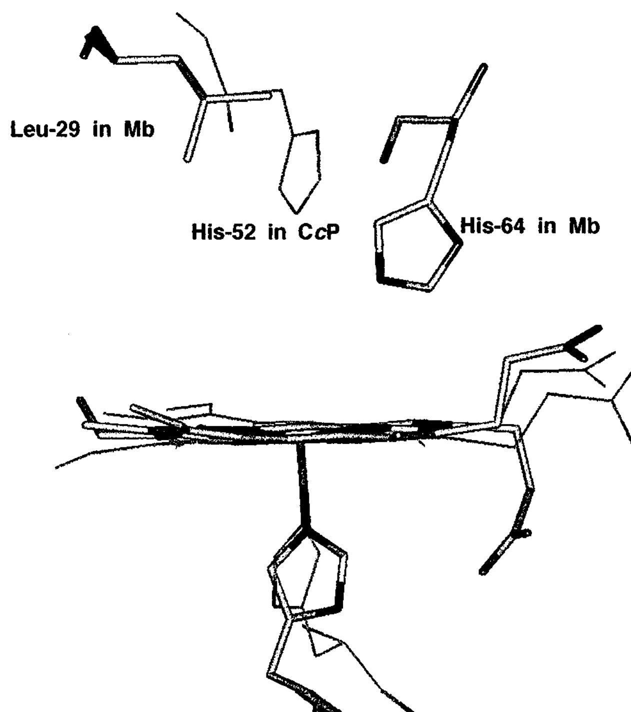

Figure 1. Superposition of the heme and some selected residues including proximal histidine (His-175 in C*c*P and His-93 in Mb) in the crystal structures of cytochrome *c* peroxidase (C*c*P) (thin line) and myoglobin (Mb) (thick line). The distance between Nϵ of distal histidine and iron is 5.6 and 4.3 Å in C*c*P and Mb, respectively.

Table 1. Enantioselective Oxidation of Methyl Phenyl Sulfide

	ee (%)[a]	rate[b]	^{18}O incorp from $H_2{}^{18}O_2$ (%)
wild type	25	0.25	92
H64L	27	0.072	89
L29H	91	3.9	100
L29H/H64L	97	5.5	97

[a] The absolute stereochemistry of the dominant isomer is *R*. [b] The unit for rate is turnover per minute.

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(7) Except for the H64L mutant, the purified ferric Mbs exhibit typical spectral features for the hexacoordinated state. The Soret of oxidized H64L metmyoglogin (λ_{max} = 393 nm) differs from that of the wild type L29H and L29H/H64L mutant (λ_{max} = 409 nm) due to the absence of water iron ligation in the mutant, as previously observed in the H64V mutant (Morikis, D.; Champion, P. M.; Springer, B. A.; Egeberg, K. D.; Sliger, S. G. *J. Biol. Chem.* **1990,** *265,* 12143). The spectra for deoxy and carbonmonoxy forms of the mutants are essentially identical to those of the wild type protein. The absorption spectrum for the hydroxy complex is identical to that in the previous report. However, hydroxy forms of the Leu-29 mutants are not clearly observed possibly due to the differences in the degree of interaction between His-29 and a water molecule bound to the heme iron.

(8) Although the peroxidase activity of Mb is not the focus of this paper, the rate of guaiacol oxidation was measured. In the presence of a large excess of guaiacol, the wild type oxidizes the substrate faster than the L29H/H64L mutant. Since the absorption spectrum during the turnover by the wild type shows the ferric resting state, the formation of a ferryl species is the rate-determining step for peroxidative cycle of Mb. The results imply that the enhancement of hydrogen peroxide supported peroxygenation activity by the L29H/H64L mutant is not due to the rate increase in the formation of a ferryl (Fe^{IV}=O) radical cation species, equivalent to compound I of peroxidase. Furthermore, the ferryl formation is not the rate-determining step in the peroxygenase cycle of Mb.

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Table 2. Oxidation of Thioether (R−S−R′)

R	R′	wild type ee (%)[a]	wild type rate[b]	L29H/H64L ee (%)[a]	L29H/H64L rate[b]
p-chlorophenyl	methyl	13	0.19	87	3.5
phenyl	methyl	25	0.25	97	5.5
p-methylphenyl	methyl	11	0.54	87	10
p-methoxyphenyl	methyl	2.8	1.1	45	1.7
benzyl	methyl	13	0.66	86	4.0
phenyl	ethyl	7.6	0.46	95	6.5

[a] The absolute stereochemistry of the dominant isomer is *R*. [b] The unit for rate is turnover per minute.

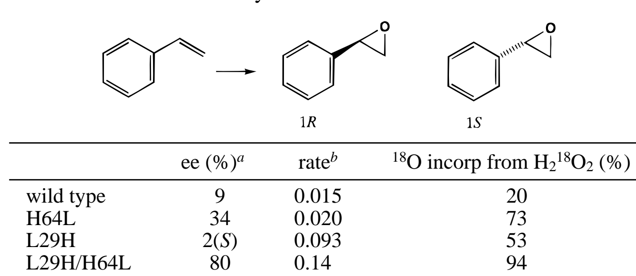
enantiomeric excess from 25 to 97%. The dominant formation of *R* by the mutation is in contrast with 97% ee for the *S* enantiomer given by F41L HRP.[2,10] The extremely high stereoselectivity with 97% incorporation of ^{18}O from $H_2{}^{18}O_2$ into the sulfoxide for L29H/H64L Mb[11] clearly indicates the ferryl oxygen transfer to thioanisole and rules out the involvement of molecular oxygen and hydroxyl radical.[12] Although the enantioselectivity is low for wild type and H64L Mb, $H_2{}^{18}O_2$-labeling experiments resulted in approximately 90% incorporation of the labeled oxygen into the sulfoxide.

Regardless of the size and electronic properties for *para* substituents of thioanisole, the L29H/H64L mutant oxidizes sulfides faster than the wild type with higher enantioselectivity (Table 2). However, for sulfoxidation by L29H/H64L Mb, the methoxy group at the *para* position drops the rate and enantiomeric specificity with respect to thioanisole. The L29H/H64L mutant oxidizes benzyl methyl sulfide and ethyl phenyl sulfide at rates comparable with that for thioanisole. The size of alkyl group and the distance between aromatic group and sulfur atom do not seem to be critical for enantioselective sulfoxidation by the L29H/H64L mutant.

Styrene epoxidation by H64L Mb was found to proceed at the similar rate for wild type Mb (Table 3).[13] In comparison with wild type Mb, the L29H mutant oxidizes six-times faster, and 9-fold enhancement with an improvement of enantioselectivity from 9 to 80% is seen for L29H/H64L Mb. Interestingly, preferred formation of the *R* methyl phenyl sulfoxide and *R* styrene oxide requires the opposite orientation of the phenyl group and the side chain with respect to ferryl oxygen; however, the structural information on the transition state cannot be deduced at this point.[14] The oxidation of styrene in the presence of $H_2{}^{18}O_2$ by wild type Mb produces epoxide with 20% ^{18}O-labeled oxygen.[11] The result is consistent with the competition of at least two mechanisms for epoxidation by wild type Mb: one that incorporates an oxygen atom from hydrogen peroxide employed and another that incorporates an atom of molecular oxygen.[1a,3b] Incubations of styrene and $H_2{}^{18}O_2$ with L29H/H64L, H64L, and L29H Mb resulted in incorporation of 94, 73, and 53% of ^{18}O in the epoxides, respectively. The value for L29H, bearing two histidines in the active site, is between the incorporation numbers for L29H/H64L and wild type Mb. The results indicate that the removal of His-64 decreases the incorporation of molecular oxygen and placement of a histidine residue at the 29 position enhances the ferryl oxygen transfer mechanism. Since wild type and L29H/H64L Mb can form phenyl−iron complex in the presence of phenylhydrazine, the wild type as well as the double mutant has an active site large enough for the substrate to access.[15] The kinetic constants for styrene oxidation by wild type and L29H/H64L Mb were found to be $K_m = 6 \pm 2$ mM, $V_{max} = 0.031 \pm 0.006$ turnover/min and $K_m = 9 \pm 2$mM, $V_{max} = 0.74 \pm 0.1$ turnover/min, respectively.[16] Since the K_m value for styrene epoxidation is not significantly different from that for the wild type, the double mutation does not appear to improve the substrate binding. Thus, the observed enhancement appears to be due to the increase in reactivity of ferryl species rather than the accessibility of ferryl oxygen for substrates.

In summary, the introduction of a histidine residue at the 29 position of H64L Mb enhances the reactivity of the ferryl species possibly due to the hydrogen bond between His-29 and the ferryl oxygen. The contribution this hydrogen bond network for the reactivity of the ferryl species of other hemoproteins was previously suggested.[17] On the other hand, the peroxygenase activity for L29H and wild type Mb is lower than that of the double mutant because the oxidation equivalents could be easily given to His-64, which lies closer to the heme iron than the residue at the 29 position (Figure 1), as well as to the substrate.[18] Details of the structure of L29H/H64L Mb active site and the substrate binding sites are under investigation.

Table 3. Oxidation of Styrene



	ee (%)[a]	rate[b]	^{18}O incorp from $H_2{}^{18}O_2$ (%)
wild type	9	0.015	20
H64L	34	0.020	73
L29H	2(*S*)	0.093	53
L29H/H64L	80	0.14	94

[a] The absolute stereochemistry of the dominant isomer is *R* except where indicated. [b] The unit for rate is turnover per minute.

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(9) H_2O_2 (1 mM) was added to a solution of either 5 μM Mb or the Mb mutants and 1 mM thioether in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) at 25 °C. Acetophenone was added as an internal standard, and the mixture was extracted with dichloromethane for HPLC analysis as reported.[2] Standard curves prepared with synthetic authentic sulfoxides were used for quantitative analysis, and the absolute stereochemistry was determined based on a retention time. In all cases, the dominant isomer was *R*. A linear relationship between time vs product formation was observed at least 15 min. The sulfoxide formed in control incubations without enzyme was subtracted when necessary. For the determination of K_m and V_{max}, the amounts of thioanisole varied.

(10) In order to compare the efficiency as sulfoxidation catalyst, we have determined the K_m and V_{max} values. The kinetic constants for thioanisole oxidation by wild type and L29H/H64L Mb were $K_m = 0.36 \pm 0.2$ mM, $V_{max} = 0.52 \pm 0.06$ turnover/min and $K_m = 0.17 \pm 0.01$ mM, $V_{max} = 9.5 \pm 3$ turnover/min, respectively. The V_{max} of L29H/H64L Mb was half of that for F41L HRP, but approximately 3-fold higher than the previously reported V_{max} for native HRP.[2]

(11) $H_2{}^{18}O_2$ was prepared by the procedure of Ortiz de Montellano (Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985,** *260,* 9265). The dichloromethane extracts of the incubation mixtures were analyzed by GC/MS equipped with Shimazu CBP1 capillary column.

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(13) The wild type or mutants (10 μM) in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) was incubated with 0.5 μL of neat styrene and 1 mM H_2O_2 at 25 °C. 2-Phenyl-2-propanol was added as an internal standard, and the dichloromethane extracts were analyzed by GC equipped with Chiraldex G-TA capillary column. The standard curve was prepared for quantitative analysis, and the absolute stereochemistry was determined on the basis of a retention time of the authentic 1*S* or 1*R* epoxide. The rates were determined from the linear portion of the product versus time plot. For the determination of K_m and V_{max}, the amounts of styrene varied.

(14) The oxidation of thioanisole by L29H/H64L Mb in the presence of styrene shows the noncompetitive kinetics.

(15) Reaction of wild type and L29H/H64L Mb with phenylhydrazine was carried out using the procedure of Kunze (Kunze, K. L.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1983,** *105,* 422). The UV spectra of the wild type and mutant were identical, and the spectrum of σ-bonded phenyliron complex for the wild type agreed with that previously reported.

(16) The V_{max} of styrene oxidation by the L29H/H64L mutant is 2 orders of magnitude greater than the previously reported values for F41L and F41T HRP.[2]

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