Communications to the Editor

Conversion of Myoglobin into a Peroxygenase: A Catalytic Intermediate of Sulfoxidation and Epoxidation by the F43H/H64L Mutant

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Received February 11, 1997

Myoglobin (Mb) has been one of the most intensively investigated hemoproteins as evident from the accumulated biochemical, biophysical, and spectroscopic data.[1] The heterologous overexpression system for recombinant sperm whale Mb in *Escherichia coli* has been developed,[2] and high-resolution X-ray crystal structures of the wild type as well as some Mb mutants are available.[3] Thus, superposition of the active site structures of Mb and other hemoproteins enables us to utilize Mb as heme enzyme models for the elucidation of structure−function relationships.[4] We have engineered sperm whale Mb based on the comparison of crystal structures of oxymyoglobin and an oxy form of cytochrome *c* peroxidase (C*c*P) (Figure 1).[5] Although the Leu-29 → His and His-64 → Leu double replacement of Mb seems to create a peroxidase-like active site, the imidazole is located too far from the heme center to interact with hydroperoxide bound to the iron. Our previous results suggest that L29H/H64L Mb cannot efficiently cleave O−O bond to generate a ferryl (Fe^{IV}=O) radical cation species, equivalent to compound I of peroxidase.[6] Thus, we have mutated Phe-43 to a histidine residue because the predicted distance between His-43 and the heme iron is approximately equal to that of C*c*P. The novel F43H/H64L Mb mutant oxidizes sulfide and styrene more efficiently than peroxidase. More intriguingly, we have identified a compound I-like species of the Mb mutant as the catalytic intermediate for the first time.

The replacement of Phe-43 in the wild type with a histidine residue increases the rate of thioanisole oxidation by 14-fold, and the mutation of His-64 → Leu in F43H Mb further enhances the sulfoxidation rate by 13-fold (Table 1). The enantiomeric excess is improved from 25% to 85% by the His-64 → Leu and Phe-43 → His double mutation of Mb, and the dominant enantiomer is *R*. More than 92% of ^{18}O incorporation in the sulfoxide from $H_2{}^{18}O_2$ in the oxidation by wild type, F43H, and F43H/H64L Mb indicates that the ferryl oxygen is transferred to thioether. In comparison with the wild type, the F43H/H64L mutant oxidizes styrene 300 times faster with an improvement of enantioselectivity from 9 to 68%. Incubations of styrene and $H_2{}^{18}O_2$ with wild type and F43H/H64L Mb resulted in incorporation of 20% and 94% of ^{18}O in epoxide, respectively. The low ^{18}O incorporation into the epoxide in the presence of the wild type and $H_2{}^{18}O_2$ could be rationalized by the competition of the ferryl oxygen transfer and cooxidation mechanism. The cooxidation mechanism requires protein radical formation followed by binding of molecular oxygen to generate a protein−peroxy radical, and His-64 was suggested as the initial radical site.[7] The replacement of His-64 with an unoxidizable leucine residue could prevent generation of the protein radical and decrease the cooxidation. In fact, the value of ^{18}O incorporation from ^{18}O-labeled hydrogen peroxide for the F43H mutant, bearing two histidines in the active site, is 54%, which is between the values for F43H/H64L and wild type Mb.[8]

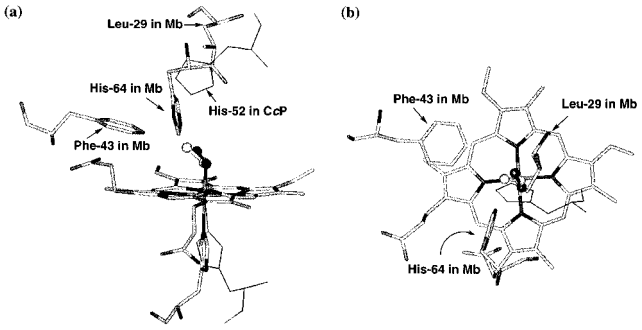

Figure 1. Superposition of the heme and some selected residues including distal histidine (His-64 in Mb and His-52 in C*c*P) in crystal structures of sperm whale myoglobin (Mb) and cytochrome *c* peroxidase (C*c*P). Light and dark balls indicate oxygen molecules bound to heme iron in Mb and C*c*P, respectively. The distances between N_ϵ of the distal histidine and iron are 4.3 and 5.6 Å in Mb and C*c*P, respectively. Only heme in Mb is shown in this figure. (a) Side view. (b) Top view.

We have attempted to identify the catalytic species of F43H/H64L Mb involved in a net two-electron oxidation of thioanisole and styrene. The horseradish peroxidase compound I-like spectrum is not observed by monitoring the changes in absorption spectra of the incubation mixture containing the mutant and hydrogen peroxide.[9] However, the mixing of F43H/H64L Mb and *m*-chloroperbenzoic acid (*m*CPBA) causes the decrease in absorbance at 406 nm, followed by the shift to longer wavelength by 12 nm (Figure 2a). The formation of the first intermediate proceeds at the rate of $k_{obs1} = 110\ s^{-1}$ (standard error 3.8), and the Soret shifts to 418 nm at the rate of $k_{obs2} =$

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(8) Peroxygenase activity of HRP was previously reported in: Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995,** *117,* 7056. The rate of sulfoxidation for F43H Mb is equal to that for native HRP, and the F43H/H64L mutant is found to oxidize thioanisole approximately 13 times faster than HRP. Styrene is oxidized by the Phe-43 → His mutants at least 100-fold faster than HRP and its mutants.
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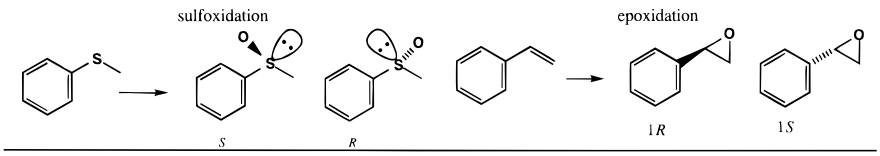
S0002-7863(97)00453-8 CCC: $14.00

Table 1. Peroxygenase Activity of Mb and Its Mutants[a]



	sulfoxidation			epoxidation		
	rate (min^{-1})	ee (%)[b]	^{18}O incorporation from $H_2{}^{18}O_2$	rate (min^{-1})	ee (%)[b]	^{18}O incorporation from $H_2{}^{18}O_2$
wild type Mb	0.25	25	92	0.015	9	20
F43H Mb	3.5	59	97	0.091	41	54
F43H/H64L Mb	47	85	96	4.5	68	94

[a] All the values are the average of at least two determinations. Incubations were carried out as described in ref 8. [b] The absolute stereochemistry of the dominant isomer is *R*.

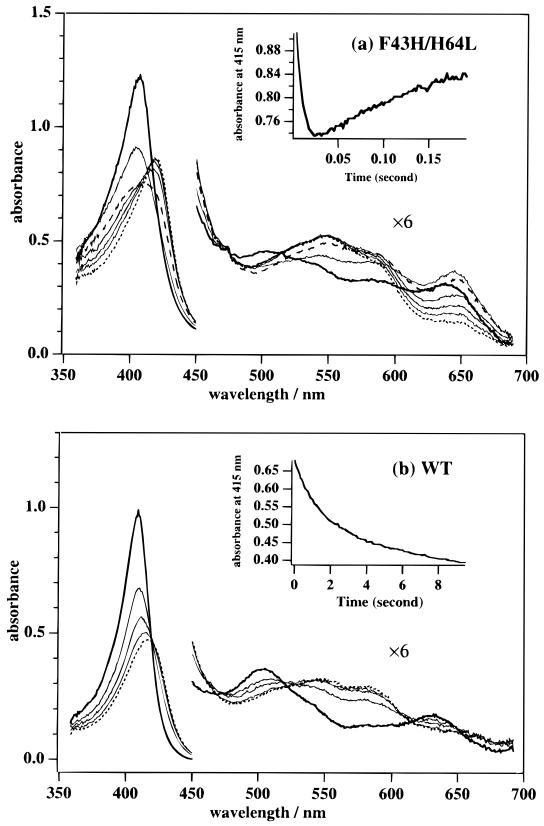


Figure 2. Absorption spectra of (a) ferric F43H/H64L (5 μM) or (b) wild type Mb (5 μM) with *m*CPBA (0.5 mM) in 50 mM sodium acetate buffer, pH 5.3. Spectra were recorded on a Hi-Tech SF-43 stopped-flow apparatus equipped with MG 6000 diode array spectrometer at 5 °C. Insets are changes in absorbance at 415 nm. The black line spectra represent ferric. Dashed and dotted line spectra are for compounds I and II, respectively.

9.2 s^{-1} (standard error 0.38). Decrease in the absorbance of the Soret and intense absorbance around 500−700 nm are characteristic for the conversion of ferric to ferryl porphyrin radical cation, and the following Soret shift is due to the decay of compound I ($Por^{+\bullet}Fe^{IV}{=}O$) to II ($PorFe^{IV}{=}O$) as observed for other heme containing peroxidases.[10] Upon the addition of styrene or thioanisole to the compound I-like intermediate, absorbance of the Soret band for the intermediate is recovered back to the level of ferric state. Thus, it is now convincing that the observed species bears two-electron oxidation equivalents.[11] Since the close examination of a crystal structure for sperm whale Mb suggests that the orientation of the distal histidine in F43H/H64L Mb could be somewhat similar to that of bovine liver catalase,[12] compound I of the mutant appears to be readily reduced to the ferric state in the presence of hydrogen peroxide.[13] On the contrary, the wild type reacts with *m*CPBA more slowly than the F43H/H64L mutant to generate compound II, and there is no accumulation of compound I (Figure 2b), presumably due to the rapid electron transfer from compound I to His-64, which lies closer to the heme iron.

In summary, we have engineered the distal pocket of Mb to mimic the active site of peroxidase, and the F43H/H64L mutant is found to be a much better peroxygenase than wild type Mb and even HRP. Furthermore, the utilization of *m*CPBA enables us to identify a compound I-like species of F43H/H64L Mb as a catalytic intermediate for peroxygenase activity. Our results clearly indicate that the alignment of the distal histidine is important for the reactivity as well as the direct observation of the transient catalytic species for Mb.

Acknowledgment. We thank Dr. John S. Olson (Rice University) for providing cDNA of sperm whale myoglobin and Dr. Akio Murakami (National Institute for Basic Biology) for his help in measuring catalase activity. This work was supported by Grant-in-Aid for Scientific Research (No. 07458147) and for Priority Areas, Molecular Biometallics, for Y.W.

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(11) The percentage of enantiomeric excess for thioanisole oxidation by F43H/H64L Mb with *m*CPBA is identical to the value with H_2O_2. The result implies that the same catalytic species is involved in the sulfoxidation.

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(13) The dismutation of hydrogen peroxide has been determined by monitoring oxygen evolution using an oxygen electrode, and the 50-fold increase in catalase activity versus the wild type (1.7 ± 0.2 turnovers/min) was observed for the F43H/H64L mutant (79 ± 2 turnovers/min). The catalase activity of F43H/H64L Mb is still less than 1% of that for bovine liver catalase.