Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 183–192

TETRAHEDRON: *ASYMMETRY*

Asymmetric oxidation catalyzed by myoglobin mutants

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Received 11 November 1998; accepted 7 December 1998

Abstract

The sperm whale myoglobin active site mutants (L29H/H64L and F43H/H64L Mb) have been shown to catalyze the asymmetric oxidation of sulfides and olefins. Thioanisole, ethyl phenyl sulfide, and *cis*-β-methylstyrene are oxidized by L29H/H64L Mb with more than 95% enantiomeric excess (% ee). On the other hand, the F43H/H64L mutant transforms *trans*-β-methylstyrene into the *trans*-epoxide with 96% ee. The dominant sulfoxide product in the incubation of alkyl phenyl thioethers is the *R* isomer; however, the mutants afford dominantly the *S* isomer of aromatic bicyclic sulfoxides. The results help us to rationalize the difference in the preferred stereochemistry of the Mb mutant-catalyzed reactions. Furthermore, the Mb mutants exhibit an improvement in the oxidation rate up to 300-fold with respect to wild type.

1. Introduction

Asymmetric biotransformations have been applied to organic synthesis as excellent alternatives to chemical procedures.[1] Chloroperoxidase from *Cadariomyces fumago* (CPO),[2] a heme enzyme, is one of the well studied enzymes for the enantioselective oxidation of sulfides and olefins.[3] Recently, a vanadium-containing non-heme bromoperoxidase from the alga *C. officinalis* (VBrPO)[4] has been shown to perform sulfoxidation of aromatic bicyclic sulfides in high enantiomeric excess (% ee).[5] In order to extend the biocatalytic methodology, we have examined the oxygenation by sperm whale myoglobin (Mb) mutants.

Mb has protoporphyrin IX as a prosthetic group, and its major physiological role is the storage and transfer of molecular oxygen.[6] However, Mb can support the peroxide-dependent one- and two-electron oxidation of a variety of substrates at a very slow rate.[7] Recently, we reported as communications that L29H/H64L and F43H/H64L Mb exhibit high catalytic turnover with high stereospecificity for the sulfoxidation of thioanisole and the epoxidation of styrene.[8] More importantly, a ferryl porphyrin radical

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0957-4166/99/$ - see front matter
PII: S0957-4166(98)00498-4

cation species ($O{=}Fe^{IV}$ $Por^{+\cdot}$), equivalent to compound I of peroxidase, has recently been confirmed as the catalytic species of a two-electron oxidation process by F43H/H64L, H64S, H64A, and H64L Mb.[8] The mutants were designed to increase the life time of catalytic intermediate as well as the accessibility of substrates. In this report, we have explored the scope of asymmetric oxygenation by the use of aromatic bicyclic sulfides and methyl styrene. The results help us understand the substrate binding orientation on the basis of the preferred stereochemistry for L29H/H64L and F43H/H64L Mb. Furthermore, we have confirmed that compound I of L29H/H64L Mb is also a catalytic species for the oxidation of sulfides and styrene.

2. Results and discussion

2.1. Asymmetric sulfoxidation of cyclic and acyclic sulfides

Among the Mbs studied here, L29H/H64L Mb is the best chiral catalyst (Table 1). The values of % ee for the L29H/H64L mutant are greater than those of wild type and F43H/H64L Mb for the oxidation of sulfides examined here. The largest improvement from 7.6% to 95% in enantioselectivity is observed for the sulfoxidation of ethyl phenyl sulfide (**2**) by the L29H/H64L mutant. On the other hand, the F43H/H64L mutant is the best catalyst in terms of the sulfoxidation rate. The Phe-43 → His and His-64 → Leu mutation increases the rate of thioanisole (**1**) oxidation by 190-fold with respect to the wild type, which is the largest enhancement achieved herein.

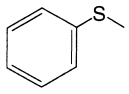
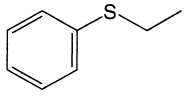
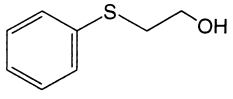
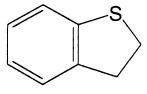
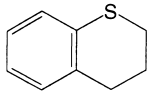
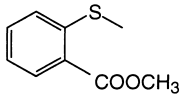
In order to elucidate the substrate recognition by the Mb mutants, we have determined the enantioselectivity for the oxidation of 2-hydroxyethyl phenyl sulfide (**3**). The absolute configuration for the sulfoxide isomers of **3** was determined by comparison of their CD spectra with that of (*R*)-methyl phenyl sulfoxide (Fig. 1). In the reaction with L29H/H64L and F43H/H64L Mb, the hydroxy group of **3** decreases the value of % ee by approximately 25% with respect to ethyl phenyl sulfide (**2**), but the *R* enantiomers are still the major sulfoxide products for **2** and **3** (Table 1). Thus, the orientation of substrate binding does not appear to be controlled by hydrophilic properties of alkyl groups in the case of sulfoxidation catalyzed by L29H/H64L and F43H/H64L Mb. Interestingly, cyclic sulfides give different stereoselectivity from acyclic thioethers for the L29H/H64L mutant. The values of % ee in the oxidation of 2,3-dihydrobenzothiophene (**4**) and 1-thiochroman (**5**) by L29H/H64L Mb are 67 and 66% ee, respectively, and the dominant isomers are (*S*)-sulfoxides (Table 1). On the contrary, *R* is the major enantiomer with 97% ee for thioanisole (**1**) oxidation by the L29H/H64L mutant. Since sulfide **6** bearing an *ortho* substituent is oxidized to the corresponding (*R*)-sulfoxide with 82% ee by L29H/H64L Mb, the cyclic moiety, not the *ortho* substituent of the benzene ring, appears to be important for the formation of the *S* isomer. The similar changes in dominant isomers are also observed for F43H/H64L Mb.

In this study, hydrogen peroxide was added in one portion at the beginning of the reaction to determine the initial rate, and the values can not be directly compared with the results from the continuous addition of oxidants to the reaction mixture with CPO[3g] or VBrPO[5] as previously reported. However, the sulfoxidation system with F43H/H64L appears to be comparable to that of CPO in terms of the rate.[9]

2.2. Catalytic species for the sulfoxidation reaction

In order to confirm that compound I is the catalytic species for the oxidation of sulfides by the L29H/H64L and F43H/H64L mutant, we have performed double mixing stopped-flow experiments. Compound I was generated in the first mixing, and sulfides were added to compound I in the second

Table 1
Enantiospecific sulfoxidation of cyclic and acyclic thioethers[a]

		wild type		L29H/H64L		F43H/H64L	
		rate[b]	%ee	rate[b]	%ee	rate[b]	%ee
1		0.25[c]	25[c] (*R*)	5.5[c]	97[c] (*R*)	47[c]	85[c] (*R*)
2		0.46[c]	7.6[c] (*R*)	6.5[c]	95[c] (*R*)	26	54 (*R*)
3		0.65	27 (*R*)	1.6	71 (*R*)	3.2	27 (*R*)[c]
4		2.2	0.2 (*R*)	24	67 (*S*)	95	17 (*S*)
5		0.8	5.4 (*R*)	3.2	66 (*S*)	50	34 (*S*)
6		0.4	4.3 (*S*)	2.4	82 (*R*)	7.5	8.5 (*R*)

(a) HPLC conditions: 20 % isopropanol : 80 % hexane for **1**, 10 % isopropanol : 90 % hexane for **2** and **6**, 15 % isopropanol : 85 % hexane for **3**, and, 5 % isopropanol : 95 % hexane for **4** and **5**. Retention times of the sulfoxide products: **1** 17 and 20 min, **2** 17 and 21 min, **3** 17 and 20 min, **4** 36 and 38 min, **5** 64 and 68 min, and **6** 19 and 21 min. Except for the sulfoxide of **6**, the *S* isomers always elute from the column first .
(b) The unit for rate is turnover/min.
(c) The results are taken from reference 8a and 8b.

mixing. UV–visible spectra began to be collected just after the second mixing to monitor the spectral changes.

The decrease in the absorbance of the Soret and the increase in the absorbance around 650 nm are characteristic for the formation of ferryl porphyrin radical cation from the ferric state (Fig. 2). Compound I ($O{=}Fe^{IV}$ $Por^{+\cdot}$) of L29H/H64L Mb is reduced back to the ferric state in the presence of an excess of thioanisole (**1**). Since compound II ($O{=}Fe^{IV}$ Por), bearing one oxidation equivalent with respect to the ferric state, is not observed, the sulfoxidation appears to proceed with a direct two-electron process. Essentially the same spectral changes are observed in the presence of 2,3-dihydrobenzothiophene (**4**) with L29H/H64L Mb. Therefore, the bicyclic sulfide does not change the oxidation mechanism. Thioanisole and dihydrobenzothiophene also reduce compound I of the F43H/H64L mutant directly to the ferric state.

The similar UV–visible spectral changes were observed in the reaction of compound I of both mutants

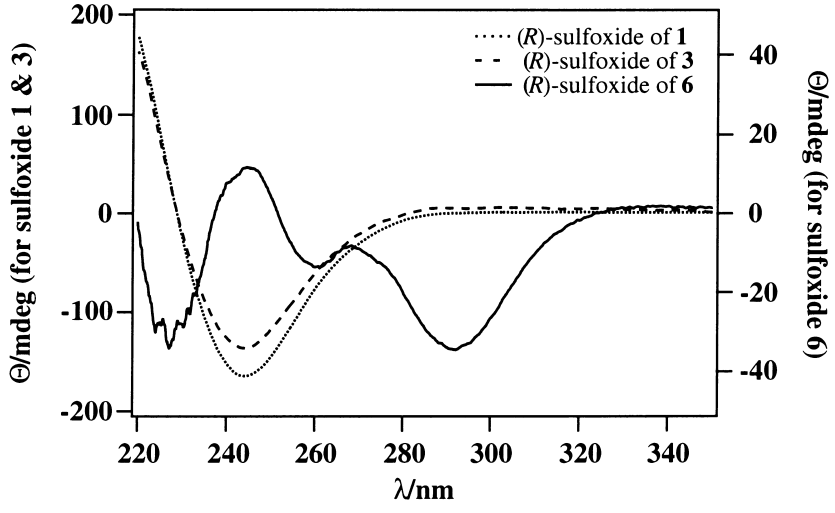


Figure 1. CD spectra of (*R*)-methyl phenyl sulfoxide, (*R*)-sulfoxide of **3** (eluted at 20 min), and **6** (eluted at 19 min)

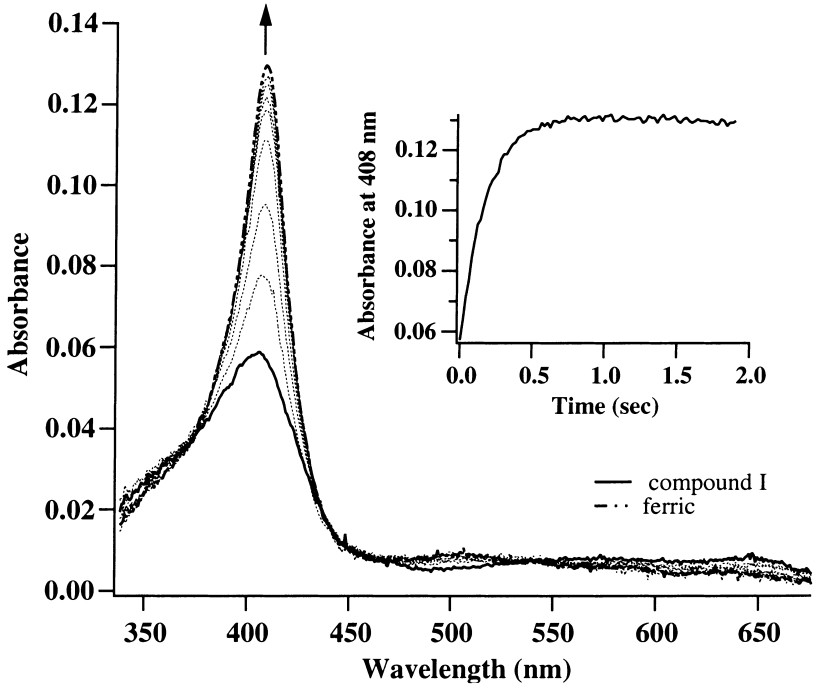


Figure 2. The UV–visible spectral changes from compound I of L29H/H64L Mb to the ferric state in the presence of thioanisole in 50 mM NaOAc buffer (pH 5.3) at 5°C

with styrene (**7**). Thus, the results indicate that the ferryl porphyrin radical cation ($O{=}Fe^{IV}$ $Por^{+\cdot}$) is the reactive species for the peroxide dependent monooxygenation by the mutants.

2.3. Asymmetric epoxidation of styrene and β-methylstyrene

In order to gain an insight into the highly stereoselective oxidation by the L29H/H64L and F43H/H64L mutant, we next studied epoxidation reactions. The epoxidation rate for styrene (**7**), *trans*- (**8**), and *cis*-

β-methylstyrene (**9**) catalyzed by the L29H/H64L mutant is increased by 9-fold, 4-fold, and 46-fold, respectively, compared with the rate of the wild type Mb (Table 2). The highest value of enantiomeric excess was 99% ee for *cis*-β-methylstyrene epoxidation catalyzed by L29H/H64L Mb. On the other hand, F43H/H64L Mb oxidized *trans*-β-methylstyrene to (1*R*,2*S*)-*trans*-epoxide with 96% ee. The F43H/H64L mutant is the best enzyme in terms of epoxidation rate, and styrene, *trans*- and *cis*-β-methylstyrene are found to be oxidized 300, 210, and 57 times faster by F43H/H64L than wild type Mb, respectively. Synthetic iron porphyrins, hemoprotein model compounds, oxidize the *cis*- much faster than the *trans*-isomer,[10] but *cis*-β-methylstyrene is less reactive to Mbs than the *trans* isomer (Table 2), presumably due to the limited accessibility of the *cis* isomer to the active site.

No more than a trace of *trans*-β-methylstyrene oxide is detected as a side product in the oxidation of *cis*-β-methylstyrene. Thus, the epoxidation proceeds with the retention of olefin stereochemistry. Since the *trans*-epoxide was generated in the oxidation of *cis*-β-methylstyrene by sterically unhindered Mn porphyrin, the active site of the mutants might prevent the rotation of the two vinyl carbons of *cis*-β-methylstyrene for steric reasons (Fig. 3).[10]

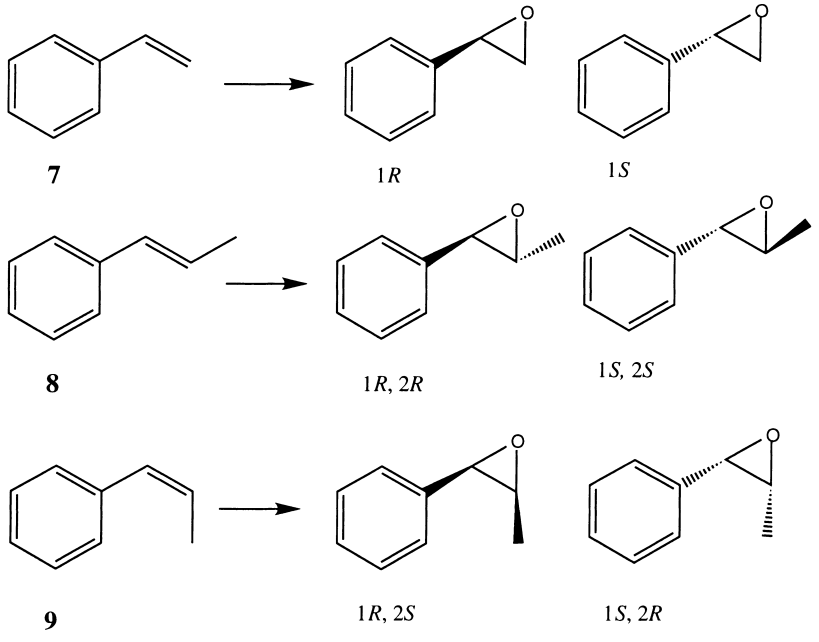
Only a trace of side product is observed in the oxidation of styrene and *trans*-β-methylstyrene; however, a significant amount of phenylacetone is generated in the incubation of *cis*-β-methylstyrene with F43H/H64L Mb. Compound I, an oxo-ferryl porphyrin π-cation radical species ($O{=}Fe^{IV}$ $Por^{+\cdot}$), is responsible for the epoxide production, but phenylacetone seems to be generated by the 1,2-hydrogen rearrangement of the cationic species as previously proposed for the cytochrome *c* peroxidase-catalyzed oxidation of olefin (Fig. 3).[11] Since β-methylstyrene oxide is not transformed into phenylacetone in the presence of F43H/H64L Mb and hydrogen peroxide, the carbocation at the benzylic position would be formed from the reaction of compound I with β-methylstyrene.

2.4. Substrate binding orientation

The dominant isomers observed in the oxidation of thioanisole, dihydrobenzothiophene, and styrene by L29H/H64L Mb are summarized in Fig. 4. We previously found that *para* substituents of thioanisole (**1**) did not change the Mbs' preference in enantioselectivity.[8a] In addition, a bulky substituent at the *ortho*-position of **1** did not change the enantioselectivity (Table 1). Thus, the origin of enantioselectivity could be rationalized by the conformational difference of sulfur or benzylic carbons in the putative reaction intermediates. If we assume the aromatic group is bound in a fixed position, dihydrobenzothiophene (**4**) and styrene seem to share a common binding orientation (Figs. 4 and 5). The vinyl group of styrene could be in the same plane of the benzene ring to form a flat molecule like dihydrobenzothiophene (Fig. 5). On the contrary, methyl phenyl sulfide approaches to the ferryl oxygen with the $S–CH_3$ bond approximately perpendicular to the plane of benzene ring. A similar substrate binding mode can be proposed for the F43H/H64L mutant (Fig. 5).

In order to elucidate the origin of enantioselectivity for L29H/H64L Mb, we have crystallized the mutant and performed the X-ray crystal structure analysis.[12] Energy minimization followed by docking either the substrates or products in the active site of L29H/H64L Mb did not immediately provide an obvious rationale for the difference in enantiospecificity.[13] Efforts to soak the substrate into the crystals are underway to elucidate the reason for high stereospecificity of the Mb mutants.

Table 2
Enantioselective epoxidation of olefins



		wild type Mb		L29H/H64L Mb		F43H/H64L Mb	
		rate[b]	ee(%)	rate[b]	ee(%)	rate[b]	ee(%)
7[c]	styrene	0.015	9 (1*R*)	0.14	80 (*R*)	4.5	68 (*R*)
8	*trans*-β-methylstyrene	0.076	39 (1*R*, 2*R*)	0.29	83 (1*R*, 2*R*)	16	96 (1*R*, 2*R*)
9	*cis*-β-methylstyrene	0.0026	3 (1*R*, 2*S*)	0.12	99 (1*R*, 2*S*)	0.15[d]	45 (1*R*, 2*S*)

(a) Retention times: styrene oxide 8.5 min (1*S*) and 10.6 min (1*R*), *trans*-methylstyrene oxide 13.5 min (1*S*, 2*S*) and 15.4 min (1*R*, 2*R*), *cis*-methylstyrene oxide 12.1 min (1*S*, 2*R*) and 17.1 min (1*R*, 2*S*).

(b) The unit for rate is turnover/min.

(c) The results are taken from reference 8a and 8b.

(d) Phenylacetone was also formed in the reaction. The ratio of *cis*-epoxide : phenylacetone was 1 : 3.

3. Experimental section

3.1. Materials

All substrates except for sulfide **4**, **5**, and **6** are commercially available. **4**, **5**, and $H_2{}^{18}O_2$ were chemically synthesized as previously reported.[3g,7a] Sulfoxides and epoxides were synthesized from the

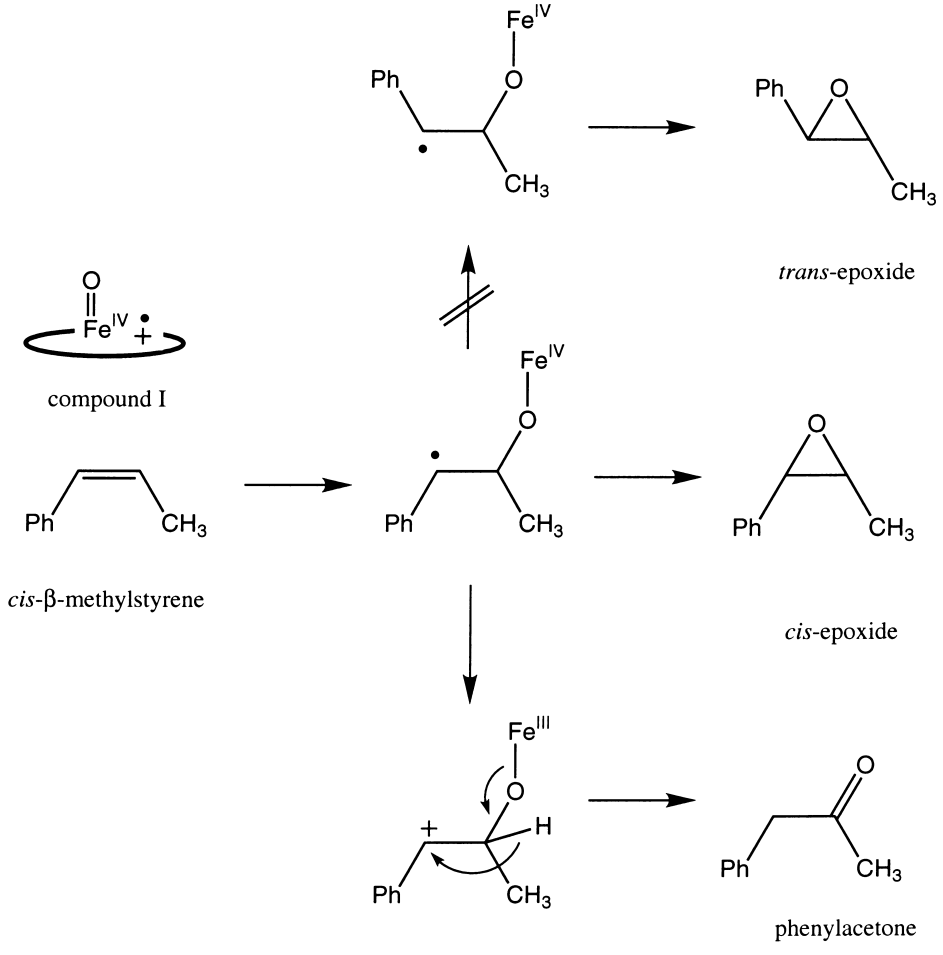


Figure 3. Proposed mechanism for the oxidation of *cis*-β-methylstyrene by Mbs[11]

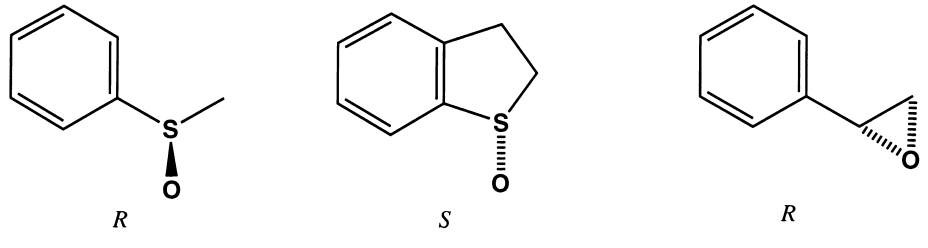


Figure 4. The dominant isomers observed for sulfoxidation and epoxidation

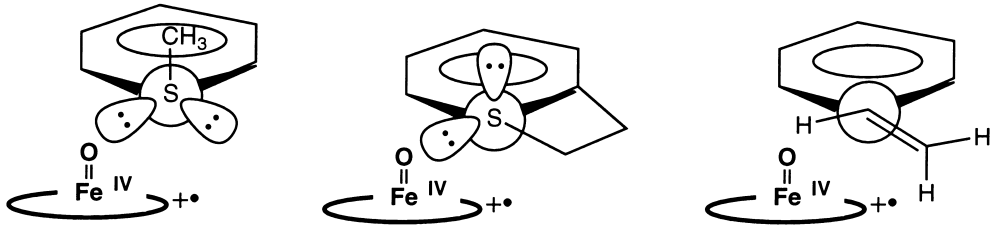


Figure 5. Proposed conformational rationalization for the asymmetric oxidation by the Mb mutants

corresponding starting substrates by the oxidation with *m*-chloroperbenzoic acid (mCPBA) or H_2O_2 and used to make standard curves.

The L29H/H64L and F43H/H64L Mb were constructed utilizing the polymerase chain reaction based method.[14] The Mbs were expressed and purified as previously reported.[15] The protein concentration was determined spectrometrically at 408 nm (ε=1.8×10^5 M^{-1} cm^{-1}).

GC–MS analysis of sulfide **6** and its sulfoxide was performed on a Shimadzu QP5000 equipped with a Shimadzu CBP1 capillary column. The column temperature was kept at 150°C for 2 min, then rose to 250°C at 20°C/min.

3.2. Synthesis of sulfide **6** and its sulfoxide

A mixture of thiosalicylic acid (4.0 g), methyl iodide (36 mL), and 28% ammonium hydroxide (24 mL) was stirred at 25°C for 48 h. The products (a mixture of **6** and its free acid) were extracted with dichloromethane, and purified by silica gel 60 (2.1 cm×8 cm). The column was washed with dichloromethane to elute **6** and then with 20% isopropanol containing hexane to recover the free acid. NMR: δ ($CDCl_3$) 2.49 (s, 3H), 3.95 (s, 3H), 7.18 (t, 1H, *J*=6.9 Hz), 7.31 (d, 1H, *J*=7.9 Hz), 7.50 (t, 1H, *J*=7.2 Hz) 8.03 (d, 1H, *J*=7.9 Hz). GC–MS: retention time 4.3 min, MS found 182.1 (calculated for $C_9H_{10}O_2S$ 182.23).

The sulfoxide of **6** was synthesized in a water–methanol mixture (2.0 mL) containing **6** (10 mg) and H_2O_2 (0.01 mol) at 25°C for 4.5 h. The sulfoxide product was purified by silica gel 60 (2.1 cm×8 cm) with the eluent system of isopropanol:hexane, 20:80. NMR: δ ($CDCl_3$) 2.85 (s, 3H), 3.96 (s, 3H), 7.57 (t, 1H, *J*=7.6 Hz), 7.83 (t, 1H, *J*=6.6 Hz), 8.09 (d, 1H, *J*=7.6 Hz), 8.32 (d, 1H, *J*=7.9 Hz). GC–MS: retention time 5.6 min, MS found 198.1 (calculated for $C_9H_{10}O_3S$ 198.24). The *S* and *R* isomers were separated and collected by isocratic HPLC on a Daicel chiral column OD (0.46 cm×25 cm) at a flow rate of 0.5 mL/min (isopropanol:hexane=20:80). The CD spectra were obtained on a JASCO J-40 spectropolarimeter, and the absolute configuration was assigned by comparison of the CD spectra of (*R*)-methyl phenyl sulfoxide and the synthetic sulfoxide of **6** (Fig. 1).

3.3. Enzymatic sulfoxidation

H_2O_2 (1 mM) was added to a solution of either Mb or the Mb mutants (5 μM) and 1 mM sulfide in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) at 25°C. A selected internal standard was added, the mixture was extracted with dichloromethane, and analyzed by isocratic HPLC on Daicel chiral column OD (0.46 cm×25 cm) at a flow rate of 0.5 mL/min.[16] The reaction time varies from 1 to 30 min, and the rates were determined from the linear portion of the product versus time plot. Since the enzymes were inactivated during the reaction, we normally observed up to 2500 turnovers. Standard curves prepared using synthetic sulfoxides were used for quantitative analysis, and the values of % enantiomeric excess were determined on a basis of peak area of HPLC traces. It was previously reported that the (*S*)-sulfoxides of **1** and **2** eluted from the column.[16] The absolute configurations for sulfoxides of **3** and **6** were confirmed by the comparison of the CD spectra of (*R*)-methyl phenyl sulfoxide and those of sulfoxide isomers for **3** and **6** collected from the chiral column. To determine the stereochemistry for sulfoxides of **4** and **5**, authentic (*R*)-sulfoxides were synthesized by CPO as described previously,[3g] and the retention times were determined as 21 (**4**) and 36 min (**5**), respectively. The sulfoxide formed in control incubations without enzyme was subtracted when necessary.

3.4. Reactions of compound I with sulfides or styrene

Rapid scan spectra were collected on a Hi-Tech SF-43 stopped-flow apparatus equipped with an MG 6000 diode array spectrometer. Single mixing experiments (i.e. mixing of ferric Mb and mCPBA) were performed to determine the rate of compound I formation. mCPBA was used as an oxidant because it was better than H_2O_2 at generating compound I on the time scale of stopped-flow experiments. The reaction of L29H/H64L Mb (10 μM) with mCPBA (250 μM) was performed in 50 mM sodium acetate buffer (pH 5.3) at 5°C. Since the F43H/H64L mutant (10 μM) does not require a large excess of mCPBA to generate compound I, the mCPBA concentration was reduced to 100 μM. Based on the results of single mixing experiments, the delay times, defined as the interval between the first and the second mixing, were set as 10 and 0.3 s for L29H/H64L and F43H/H64L Mb, respectively. Sulfides (100 μM) or styrene (100 μM) were added to compound I by the second mixing after the appropriate delay time to collect spectral changes.

The rates of compound I reduction for L29H/H64L Mb by thioanisole, 2,3-dihydrobenzothiophene, and styrene were 5.9 ± 0.1 s^{-1}, 4.5 ± 0.1 s^{-1}, and $0.92\pm0.02s^{-1}$, respectively. The values for the F43H/H64L mutant were 66 ± 3 s^{-1}, 44 ± 1 s^{-1}, and 44 ± 2 s^{-1}, respectively.

3.5. Enzymatic epoxidation

The wild type or mutants (10 μM) in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) were incubated with 0.5 μL of neat styrene, *cis*-, or *trans*-β-methylstyrene and 1 mM H_2O_2 at 25°C. A selected internal standard was added, and the dichloromethane extracts were analyzed by GC equipped with a Chiraldex G-TA capillary column at 80°C. The reaction time varies from 2 to 20 min, and the rates were determined from the linear portion of the product versus time plot. Since the enzymes were inactivated during the reaction, we normally observed up to 100 turnovers. The standard curve was prepared for quantitative analysis, and the absolute stereochemistry was determined based on a retention time of the authentic epoxide.

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