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Asymmetric Sulfoxidation and Amine Binding by H64D/V68A and H64D/V68S Mb: Mechanistic Insight into the Chiral Discrimination Step

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Myoglobin (Mb) is an oxygen transport hemoprotein that catalyzes a variety of oxidations, including sulfoxidation and epoxidation, in the presence of peroxides.[1−4] However, the reactivity and enantioselectivity for sulfoxidation by Mb are much lower than those by peroxidases.[5−8] We have recently shown that the distal histidine (His64) in sperm whale Mb is a critical residue in destabilizing a reactive intermediate, myoglobin compound I (Mb-I).[9,10] For example, the His64Asp mutant form (H64D Mb) reacts efficiently with H_2O_2 to form Mb-I with a rate constant of 2.5×10^4 M^{-1} s^{-1}.[10] The H_2O_2-dependent sulfoxidation and epoxidation activities of H64D Mb are 580- and 820-fold greater than those of wild-type Mb.[10] While these H_2O_2-dependent oxidation activities are higher than those of the rationally designed Mb mutants, F43H/H64L and L29H/H64L Mbs, which show up to 97% ee for sulfoxidation,[11] the H64D Mb gave almost racemic sulfoxide.[10] It has yet to be clarified how enantioselectivity of these Mb mutants is controlled in sulfoxidation.

We report herein that Mb mutants (H64D/V68A and H64D/V68S) are capable of selective (*S*)-α-methylbenzylamine binding over (*R*)-α-methylbenzylamine binding, as well as highly enantioselective sulfoxidation. Comparison between the enantioselective binding of α-methylbenzylamine and enantioselective sulfoxidation of thioanisole provides valuable mechanistic insight into the chiral discrimination step of the enzymes.

Structural studies of wild-type Mb indicated that access of small substrates such as O_2 and CO to the active site was limited by His-64 and Val-68 residues located immediate above the heme (Figure 1).[12−15] Thus, we prepared the double mutant forms H64D/V68A and H64D/V68S, which place a smaller residue in the distal pocket. The addition of H_2O_2 (1 mM) into a phosphate buffer solution (50 mM, pH 7.0) containing 1 mM thioanisole and Mb mutant (0.1−0.5 μM) at 25 °C resulted in formation of the corresponding sulfoxide. HPLC analysis (Daicel OD chiral-sensitive column) showed dramatic increased ee values for H64D/V68A and H64D/V68S in the H_2O_2-dependent thioanisole sulfoxidation with comparable catalytic activity to H64D Mb (Table 1). This indicates that the residue at position 68 plays an important role in the enantioselectivity of sulfoxidation.

Due to the structural similarity of α-methylbenzylamine and methylphenylsulfoxide, we have compared the enantioselective ligation of (*R*)- and (*S*)-α-methylbenzylamine to H64D/V68A and H64D/V68S Mbs to the sulfoxidation of thioanisole (Scheme 1). Ligation of the (*R*)- or (*S*)-amine to Mb was evidenced by UV−vis and EPR spectral changes due to the conversion from high-spin to low-spin Fe(III) caused by the ligation of the amine.[16] In contrast to the *R*-selective sulfoxidation by H64D/V68A and H64D/

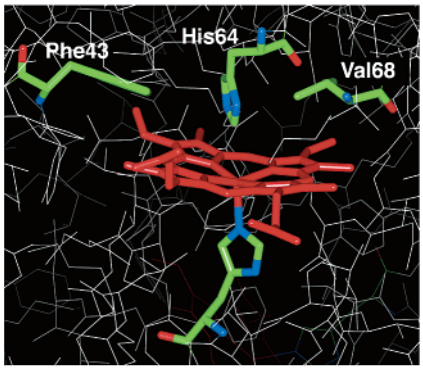

Figure 1. A distal site structure of Mb.

Table 1. Enantioselective Sulfoxidation of Thioanisole by Mbs with H_2O_2[a]

	rate[b]	ee % (*R*)
H64D/V68A	121	84
H64D/V68S	64	88
H64D[c]	145	6

[a] Sulfoxidation was carried out in phosphate buffer (50 mM, pH 7.0) at 25 °C in the presence of Mb mutant (0.1−0.5 μM), thioanisole (1 mM), and H_2O_2 (1 mM). [b] The unit for rate is turnover per minute. [c] Data refer to ref 10.

Scheme 1. Comparison of (*R*)-α-Methylbenzylamine Bound to Heme and an Expected Oxidation Intermediate for (*R*)-Phenylmethylsulfoxide Formation

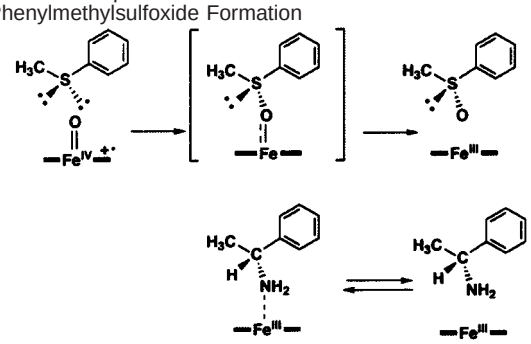

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Table 2. Kinetic Parameters for α-Methylbenzylamine Binding to Mb Mutants

	k_1 (M^{-1} s^{-1})		k_{-1} (s^{-1})		K (M^{-1})	
	R	*S*	*R*	*S*	*R*	*S*
H64D/V68A	$(1.3 \pm 0.1) \times 10^4$	$(1.3 \pm 0.1) \times 10^4$	16	0.59	8.1×10^2	2.2×10^4
H64D/V68S	$(2.2 \pm 0.3) \times 10^3$	$(2.7 \pm 0.1) \times 10^3$	22	0.24	1.0×10^2	1.1×10^4
H64D	30 ± 10	4 ± 2	0.096	0.057	3.1×10^2	70

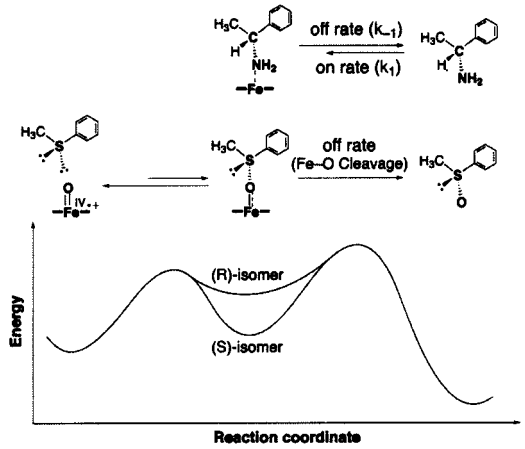

Figure 2. Proposed reaction coordinate for the thioanisole sulfoxidation and the amine binding in H64D/V68A and H64D/V68S Mb.

V68S (Table 1), the binding constant (K) values of (*S*)-α-methylbenzylamine with H64D/V68A and H64D/V68S are 27-fold and 112-fold larger than those of the corresponding (*R*)-amine, respectively (Table 2).[19] In the case of H64D Mb, the binding constant for the (*S*)-amine is significantly weaker than the others, whereas those for (*R*)-amine are about the same (Table 2).

To determine the chiral discrimination step in the amine binding, we have measured the on-rate (k_1) and the off-rate (k_{-1}) of amine binding to the Mb mutants by stopped-flow experiments.[19] The results are also summarized in Table 2. The on-rates (k_1) of (*R*)- and (*S*)-α-methylbenzylamine to H64D/V68A and H64D/V68S are almost identical, 1.3×10^4 and $2.2-2.7 \times 10^4$ M^{-1} s^{-1}, respectively. In contrast, a tremendous difference is seen for the off-rate. This indicates that the chiral discrimination of the (*S*)-amine ligation over the (*R*)-amine by H64D/V68A and H64D/V68S is exclusively caused by a very small off-rate of the (*S*)-amine relative to the (*R*)-amine, 1:27 for H64D/V68A and 1:92 for H64D/V68S. These selectivities would correspond to 93 and 98% ee for the amine binding, respectively.

Judging from a much smaller off-rate of the (*S*)-isomer in the amine binding than that of the (*R*)-isomer, the off-rate for the Fe−O bond cleavage in the (*S*)-sulfoxide formation may also be much smaller than that for the (*R*)-sulfoxide due to the higher activation energy of the former (Figure 2). Thus, enantioselectivity in the sulfoxidation of thioanisole by H64D/V68A and H64D/V68S Mb may be determined by the off-rate of sulfoxide.

In the case of H64D Mb, the on-rate of the (*S*)-amine to H64D Mb is 7.5-fold smaller than that of (*R*)-amine whereas the off-rate is only 1.7-fold smaller (Table 2). In this case, the (*R*)-sulfoxide formation is expected to be slightly faster than (*S*)-isomer formation. In fact, H64D Mb shows small enantiomeric excess for the (*R*)-sulfoxidation (Table 1).

In summary, we have engineered the distal pocket of Mb to convert the enantioselective cavity for the sulfoxidation of thioanisole. The H64D/V68A and H64D/V68S mutants are found to oxidize thioanisole with high enantioselectivity and reactivity. These mutants are also capable of enantioselective binding of α-methylbenzylamine, which mimics an expected sulfoxidation intermediate. The kinetic study of the amine binding suggests that the Fe−O bond cleavage in the intermediate is the chiral discrimination step of the sulfoxidation. We are undertaking the crystal structural analysis to elucidate the details of the substrate binding.

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(16) For example, H64D/V68A Mb has the Soret maximum at 408 nm, indicating that the ferric heme iron is in a hexacoordinated high-spin state.[17] Upon the addition of (*S*)-α-methylbenzylamine to H64D/V68A Mb, the Soret band shifts to 413 nm with a decrease in the absorbance. The absorption band at 632 nm of the ferric high-spin state disappears upon addition of the (*S*)-amine. The absorption spectrum of the H64D/V68A Mb-amine complex, having a β-band at 532 nm and an α-band shoulder around 560 nm, is typical for the hexacoordinated ferric low-spin state.[18]
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(19) The reactions of Mb mutants with the amine were performed in 50 mM Tris/HCl (pH 9.0) at 20 °C. The final concentration was 5.0 μM for Mbs and 0.5−5 mM for (*R*)- or (*S*)-α-methylbenzylamine. The kinetic traces at 408 nm were used for determining pseudo-first-order rates. The binding (k_1) and dissociation (k_{-1}) rates were given by the slope and intercept of a plot of the observed rates versus the (*R*)- and (*S*)-amine concentration. The binding constants (K) were given by k_1/k_{-1}.

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