Molecular Engineering of Horseradish Peroxidase. Highly Enantioselective Sulfoxidation of Aryl Alkyl Sulfides by the Phe-41 → Leu Mutant

Shin-Ichi Ozaki and Paul R. Ortiz de Montellano*

Department of Pharmaceutical Chemistry
School of Pharmacy, University of California
San Francisco, California 94143-0446

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Reaction of horseradish peroxidase (HRP) with H_2O_2 yields a ferryl (Fe^{IV}=O)/porphyrin radical cation state known as compound I that is reduced to the resting ferric state by two one-electron transfers.[1] The only two-electron reaction that HRP has clearly been shown to catalyze is thioether sulfoxidation, in which the ferryl oxygen is transferred to the sulfur with modest enantioselectivity.[2,3] The reason for the atypical oxidation of thioethers is unclear, but the observation of noncompetitive kinetics for the oxidation of guaiacol, a typical one-electron substrate, in the presence of thioanisole suggests that the two substrates bind at distinct sites.[3] In order to define structure–function relationships for HRP relevant to the preparation of novel catalysts, we have expressed the enzyme in a baculovirus/insect cell system[4] and initiated site-specific mutagenesis studies. In the absence of a crystal structure for HRP, we have chosen Phe-41 for initial mutagenesis. Sequence alignments of HRP with cytochrome *c* peroxidase (CcP) and lignin peroxidase (LiP),[5] two peroxidases for which crystal structures are available,[6] suggest that Phe-41 corresponds to Trp-51 in CcP and Phe-46 in LiP. Trp-51 precedes the catalytic histidine in the CcP sequence and lies 3.6 Å above, and roughly parallel to, the heme group.[6a] We report here that mutating Phe-41 to a leucine (F41L HRP) increases not only the rate but, most importantly, the enantioselectivity of thioether sulfoxidation.

The rates and enantioselectivities for the oxidation of alkyl aryl sulfides by F41L HRP are sensitive to the nature of the alkyl and aryl groups but are invariably higher than those for native HRP (Tables 1 and 2).[7,8] The largest enhancement is seen for cyclopropylmethyl phenyl sulfide, which is oxidized 10 times faster with an increase in enantioselectivity from 7% to 94% by F41L HRP. The smallest effect is seen for 4-acetamidothioanisole, for which a rate increase of 1.5-fold and a rise in enantioselectivity from 78% to 94% are observed. Neither native nor F41L HRP significantly oxidizes methyl benzyl sulfide even though its oxidation potential (1.71 V vs SCE) is only modestly different from that for thioanisole (1.44 V).[10]

Table 1. Enantioselective Sulfoxidation of Thioethers of Structure R_1SR_2

		native HRP		F41L HRP	
R_1	R_2	rate[a]	ee (%)	rate[a]	ee (%)
phenyl	ethyl	0.21	35	0.83	94
phenyl	cyclopropyl	0.047	7	0.49	94
p-chlorobenzyl	methyl	trace	nd[b]	trace	nd
benzyl	methyl	trace	nd	trace	nd
2-naphthalenyl	methyl	0.083	69	0.19	99

[a] (Nmol of sulfoxide) min^{-1} (nmol of enzyme)$^{-1}$. [b] Not determined.

Table 2. Electronic Effects of Para Substituents on the Rate and Enantioselectivity of Thioanisole Sulfoxidation

		native HRP		F41L HRP	
subst	σ^+	rate[a]	ee (%)	rate[a]	ee (%)
NO_2	0.79	0.30	66	0.33	72
CN	0.66	0.15	64	0.22	90
Cl	0.11	0.32	68	0.63	88
H	0.00	0.33	72	0.87	97
CH_3	–0.31	0.37	67	1.10	97
$NHCOCH_3$	–0.60	1.00	78	1.50	94
OCH_3	–0.78	0.61	75	1.70	97

[a] (Nmol of sulfoxide) min^{-1} (nmol of enzyme)$^{-1}$.

The catalytic mechanism does not appear to be altered by the F41L mutation. A correlation is observed between the substituent σ^+ values and the rates of oxidation of para-substituted thioanisoles for both native HRP ($\rho = -0.36$, $r^2 = 0.69$) and F41L HRP ($\rho = -0.54$, $r^2 = 0.93$) (Table 2).[11,12] Better correlation coefficients are obtained if two of the seven substituents (NO_2, $NHCOCH_3$) are excluded for native HRP ($\rho = -0.46$, $r^2 = 0.98$) and one (CN) for the F41L mutant ($\rho = -0.43$, $r^2 = 0.96$). Poorer correlations are obtained with σ than σ^+ values. The similar and negative ρ values indicate that a similar degree of positive charge develops on the substrate in the transition state of the reactions catalyzed by the native and mutant enzymes. The magnitude of the ρ values is considerably smaller than that for the two-electron sulfoxidations mediated by H_2O_2 ($\rho = -1.2$)[13a] and 4α-hydroperoxy-5-ethyl-3-methyllumiflavin ($\rho = -1.32$), a flavin monooxygenase model,[13b] but are somewhat larger than those for cytochrome P450 ($r = -0.16$)[13c] and a metalloporphyrin P450 model ($\rho = -0.26$).[11d] The sulfoxidation enantioselectivity appears also to be slightly sensitive to the electronic effects of the para substituents (Table 2).

Replacement of Phe-41 by a threonine gives a mutant (F41T HRP) that oxidizes thioanisole and *p*-chlorothioanisole more rapidly but with lower enantioselectivity than native HRP. The rates and enantiomeric excesses for the oxidation of thioanisole and *p*-chlorothioanisole by F41T HRP are, respectively, 0.93 nmol min^{-1} $nmol^{-1}$ (10% ee) and 1.1 nmol min^{-1} $nmol^{-1}$ (12% ee).

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(7) HRP and HRP mutants, expressed as described earlier,[4] were purified using the chromatography system of Chaga et al.: Chaga, G.; Andersson, L.; Ersson, B.; Berg, M. *Biomed. Chromatogr.* **1992**, *6*, 172. Incubations and product analyses were carried out as reported.[3] The yields of achiral sulfoxides formed in parallel incubations without the enzyme were subtracted from the enzymatic product yields in calculating the rates and enantiomeric excesses. In all cases, the *S* isomers were the major products. Standard curves prepared with synthetic standards were used for product quantitation.

(8) Although the peroxidase activity of HRP is not the focus of this report, the kinetic parameters for the peroxidative oxidation of guaiacol by F41L HRP (K_m = 1.1 mM, V_{max} = 0.37 nmol s^{-1} $pmol^{-1}$) differ little from those for native HRP (K_m = 1.4 mM, V_{max} = 0.19 nmol s^{-1} $pmol^{-1}$). The parameters for the oxidation of ABTS by F41L HRP (K_m = 0.42 mM, V_{max} = 14 nmol s^{-1} $pmol^{-1}$) and native HRP (K_m = 0.22 mM, V_{max} = 7.6 nmol s^{-1} $pmol^{-1}$) show, however, that the F41L mutation decreases substrate binding 2-fold but increases V_{max} 2-fold. An F41V mutation is reported to not alter the K_m but to decrease the V_{max} for ABTS 2-3-fold.[9]

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(11) The plots were of σ^+ vs log[(turnover number for para-substituted thioanisole)/(turnover number for thioanisole)]. The σ^+ values are from Hansch and Leo: Hansch, C.; Leo, A. *Correlation Analysis in Chemistry and Biology*; Wiley: New York, 1979; p 3.

(12) A ρ value of –0.31 (r = 0.57) vs σ^+ was reported earlier: Kobayashi, S.; Nakano, M.; Kimura, T.; Schaap, A. P. *Biochemistry* **1987**, *26*, 5019.

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The side chains of threonine and leucine are comparable in size but of different polarity. The rate increase observed when Phe-41 is replaced by the two smaller amino acids probably reflects increased access of the thioanisoles to the ferryl oxygen within an active site known to restrict such access.[1b,14] The opposite changes in enantioselectivity caused by the leucine and threonine mutations suggest, if the aromatic ring is bound in a common site in both enzymes, that the thioether alkyl group binds in either of two lipophilic pockets, one of which is enlarged by the mutations. The pocket remains lipophilic in the leucine mutant and thus favors increased binding of the alkyl group and formation of the *S* enantiomer. In contrast, the increased polarity of the enlarged pocket in the threonine mutant shifts binding of the alkyl group to the smaller pocket and decreases formation of the *S* enantiomer.

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The results clearly identify Phe-41 as a major determinant of peroxygenase substrate binding in the HRP active site and suggest a model of the features of the active site that determine stereospecificity. Furthermore, conversion of HRP into a nearly stereospecific sulfoxidation catalyst by the F41L mutation is the first step in the conversion of HRP into a versatile oxygenative catalyst.

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Supplementary Material Available: Linear free energy plots for native HRP and F41L HRP (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.