Rescue of His-42 → Ala Horseradish Peroxidase by a Phe-41 → His Mutation

ENGINEERING OF A SURROGATE CATALYTIC HISTIDINE

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The reaction of HRP with H2O2 is catalyzed by an active site histidine that is postulated to (a) facilitate formation of the ferric peroxyde (Fe-OOH) complex by deprotonating the peroxide, and (b) promote cleavage of the oxygen-oxygen bond by transferring the proton to the distal oxygen of the Fe-OOH complex (Fig. 1) (3). The catalytic role of the histidine, first proposed on the basis of the crystal structure of CcP (3), is confirmed by a decrease of 10^5 in the rate of formation of Compound I when the catalytic histidine (His-52) of CcP is replaced by a leucine (4). A high resolution crystal structure is not yet available for HRP, but sequence alignment of the peroxidases suggests that His-42 is the catalytic histidine in HRP (5, 6).† This is confirmed by our demonstration that mutation of His-42 of HRP to an alanine causes a 10^6-fold decrease in the rate of Compound I formation and a 10^3-fold decrease in the rate of guaiacol oxidation (8). Similar results have been reported independently for the His-42 → Leu mutant (9).

An aromatic residue is adjacent to the catalytic histidine in all the known crystal structures of plant and fungal peroxidases (Fig. 2) (10–13). In HRP, this aromatic residue is Phe-41. As found previously for Trp-51 of CcP (14, 15), mutation of Phe-41 to an alanine (8), valine (17, 18), or threonine (17, 18) has only minor effects on HRP Compound I formation or guaiacol peroxidation. However, these mutations greatly improve the ability of HRP to catalyze peroxygenase reactions such as styrene epoxidation and thioanisole sulfonation, in which the ferryl oxygen is transferred to the substrate (17, 18). The increases in the rates of peroxygenase reactions without significant changes in the rates of Compound I formation or peroxygenase reactions are consistent with the proposal that, in the native enzyme, the ferryl species is partially shielded from direct interaction with substrates. This

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*The abbreviations used are: HRP, horseradish peroxidase isozyme c; hHRP, polyhistidine-tagged recombinant HRP; CcP, cytochrome c peroxidase; heme, iron protoporphyrin IX regardless of oxidation and ligation state; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

The reaction of HRP with H2O2 produces a two-electron oxidized species known as Compound I (1, 2) in which the ferric iron is oxidized to a ferryl (FeIV=O) species and the porphyrin to a porphyrin radical cation. Stepwise reduction of Compound I by two substrate-derived electrons produces Compound II, in which the porphyrin radical cation has been quenched, and subsequently the resting ferric state (see Reactions 1–3).

\[
\begin{align*}
&k_1 \\
&\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} \\
&\text{REACTION 1} \\
&k_2 \\
&\text{Compound I} \rightarrow \text{Compound II} \\
&\text{REACTION 2} \\
&k_3 \\
&\text{Compound II} \rightarrow \text{HRP} \\
&\text{REACTION 3}
\end{align*}
\]

The crystal structure of peanut peroxidase, which is closely related to HRP, confirms the identity and location of the histidine in HRP (7).
The cells (2000 × 10^6, 10 min) was concentrated and ultrafiltered at −25 °C with an Amicon spiral wound cartridge concentrator (C12PRS (SY10, M, 10,000 cut-off spiral membrane) to a final volume of 150–200 ml. The buffer was 20 mM NaHPO₄ (pH 8.0) containing 500 mM NaCl. After centrifugation (12,000 × g, 30 min) of the ultrafiltrate, the supernatant was stirred with 7.5–10 ml of Ni(II)NTA (Invitrogen) at 4 °C for 1.5–2 h. The resin was then collected in a 1.5-cm diameter column support, and the resulting column was washed with 1 ml/min of the same buffer until the eluent was clear. The resin was then washed with 20 mM NaHPO₄ (pH 6.0) buffer containing 500 mM NaCl, followed successively by washes with the same buffer containing 0.1 and finally 1.0 M imidazole. The final wash was dialyzed against 20 mM NaHPO₄ buffer (pH 8.0) before it was run through a 1.5 × 10-cm Pharmacia Q-Sepharose Fast Flow column (gravity flow). The protein was quantitated after each step by the Bradford assay (Bio-Rad) with bovine serum albumin as the standard.

Spectroscopic Characterization of Compound I and Compound II Formation—Compound I was generated by adding 1 eq of H₂O₂ to the ferric enzyme. In the case of the His-42 mutants, excess H₂O₂ was required to obtain Compound I. Efforts to detect the F41H/H42A hHRP Compound II intermediate included the addition of 1–10 eq of K₄Fe(CN)₆ to Compound I and allowing the Compound I obtained with 1 eq of H₂O₂ to decay spontaneously.

**Determination of the Rate of Compound I Formation—**An Applied Photophysics model SF.17MV stopped flow spectrophotometer with a slit width of 0.25 mm was used to determine the rate of Compound I formation at 25 °C. The decay of the absorption at 414, 416, 411, or 415 nm, the isosbestic points between Compound II and Compound I for native, wild-type, H42A, and F41H/H42A HRP, respectively, was monitored. To follow Compound I formation under pseudo-first order conditions, the mutant enzyme (0.25 mM) in 20 mM NaHPO₄ (pH 7.0) buffer was treated with excess (2.5, 5.0, 8.0, 11.0, 14.0, and 30.0 μM) H₂O₂. The following equations, based on the kinetic scheme in the Introduction, were used to derive the rate constant for Compound I formation.

**ABTS Oxidation—**Steady-state kinetic constants were obtained by measuring the initial rates of ABTS oxidation at 25 °C and 10 mM H₂O₂ as the ABTS concentration was varied (0.02–5.0 mM). The 1-ml final assay volume contained the enzyme (0.2 nM HRP or hHRP, 20 nM F41H/H42A hHRP), or 143 mM H42A hHRP) in 50 mM sodium acetate buffer, pH 4.6. ABTS oxidation was monitored at 414 nm (ε₄1₄ = 3.6 × 10³ M⁻¹ cm⁻¹). A Hanes plot of [S] versus [v] was used to estimate the Kₘ and kₐ values.

**Guaiacol Oxidation—**Steady-state kinetic constants for guaiacol oxidation were obtained in a manner similar to that used to obtain the ABTS constants. The 1-ml reaction mixtures contained 1.0 mM to 5.0 mM guaiacol, 1.0 mM H₂O₂, and enzyme (2.0 nM hHRP or hHRP, 204 mM F41H/H42A hHRP, or 1.78 μM H42A hHRP) in 50 mM sodium acetate buffer, pH 6.0. In some experiments, 1 μM 1,2-dimethylimidazole was included in the assay mixture. Guaiacol oxidation was followed at 470 nm using the absorbance change ε₄₇₀ = 2.6 × 10⁴ M⁻¹ cm⁻¹ (25). For routine guaiacol peroxidation assays, the reaction mixture contained 10.0 mM guaiacol and 1.0 mM H₂O₂ in 50 mM sodium acetate buffer, pH 6.0, and initial rates were measured.

**Thioanisole Oxidation—**1 ml at 25 °C of a solution of native or mutant HRP and thioanisole in 50 mM sodium phosphate buffer, pH 7.0, was added H₂O₂ (0.5 mM). The thioanisole concentration range used for Kₘ and kₐ determinations was 0.05–0.5 mM. The enzyme concentration is given in the appropriate table. After a 2-min incubation, the solution was extracted with 1 ml of CH₂Cl₂, benzophenone (20 mmol) was added as an internal standard, the extract was concentrated nearly to dryness, and the residue was taken up in 50 μl of high performance liquid chromatography solvent (8.2 hexane:isopropanol). The sample was analyzed by isocratic high performance liquid chromatography on a Chiralcel OD chiral column (Daicel Chemical Industries) on a Hewlett Packard model 1040A system with a Varian 1090 solvent pump system and a diode array detector set at 242 nm. The column was eluted with 8.2 hexane/isopropanol at a flow rate of 0.5 ml/min. The retention times
of the (S)- and (R)-methylphenyl sulfoxide enantiomers were 13.6 and 15.9 min, respectively. A standard curve was constructed by injecting known amounts of methylphenyl sulfoxide. To test the linearity with time of product formation, 0.5-ml incubations containing 0.5 mM H$_2$O$_2$, known amounts of methylphenyl sulfoxide, and the indicated concentration of native or mutant HRP in 50 mM sodium phosphate buffer, pH 7.0, were carried out. The reactions were stopped at various time points and were analyzed as above. The reaction was found to be linear for at least 3–5 min.

**Styrene Oxidation**—To 0.5 ml at 23 °C of a solution of the indicated amount of native or mutant HRP and styrene in 50 mM sodium phosphate buffer, pH 7.0, was added H$_2$O$_2$ (15 mM). The styrene concentration range used for $k_0$ and $k_{cat}$ determinations was 0.1–5.0 mM. The enzyme concentration is given in the appropriate table. After 30–60 min, the incubations were extracted with 1 ml of CH$_2$Cl$_2$, the extracts remained for 1 min before recycling. The injector and detector temperatures were 200 and 250 °C, respectively. The retention times were: styrene, 5.2 min; benzaldehyde, 6.6 min; phenylacetaldehyde, 9.7 min; and styrene oxide, 11.4 min. Product standard curves were constructed by injecting known amounts of material. To calculate the percentage of the observed products, the sum of the substrate and the products, corrected for differences in detector response to each compound, was found (Fig. 3), essentially identical to that obtained with native HRP.

The Soret maximum at 405 nm in Figure 4 was determined by monitoring the decay of the absorption at 406 nm without significantly shifting the position of the Soret band, in accord with the formation of Compound I (Fig. 4). However, addition of 1 eq of K$_4$Fe(CN)$_6$ does not produce the 10–12 nm shift of the Soret band expected for the reduction of Compound I to Compound II (17). The only change observed on addition of 1 eq of ferrocyanide is a partial recovery of the Soret band intensity with a slight shift in its position to 407 nm (Fig. 4). The addition of up to 10 eq of ferrocyanide only slightly increases the absorbance with a slight shift of the absorption maximum to 409 nm (Fig. 4).

The Soret maximum at 409 nm with a somewhat lower intensity than that of the original ferric protein. The reaction was linear for at least 60 min.

**RESULTS**

**Expression and Purification of F41H/H42A hHRP**—The F41H/H42A hHRP double mutant constructed by cassette mutagenesis has been expressed in *Escherichia coli* as reported previously for the F41A and H42A mutants (8). All three recombinant proteins have a polyhistidine tag at the amino terminus that facilitates their purification (8). The F41H/H42A double mutant can thus be obtained in highly purified form by a simple purification protocol based on Ni(II)NTA affinity chromatography. The protein is typically obtained in a yield of 15 mg/liter of pure protein and is pure as judged by SDS-polyacrylamide gel electrophoresis analysis (not shown).

**Spectroscopic Properties**—The Soret maximum at 405 nm in the absorption spectrum of the ferric F41H/H42A double mutant (Fig. 3) was red-shifted by 3 nm from the maximum at 402 nm of the native and recombinant wild-type proteins (8). Addition of cyanide produces the ferric cyano complex with an absorption maximum at 421 nm and a broad absorption centered at 540 nm (Fig. 3), as found for the native and wild-type proteins (17). Dithionite-reduction of the ferric protein to the ferrous state in the presence of CO produces a ferrous-CO complex with $\lambda_{max} = 422$ nm and $\alpha$, $\beta$ bands at 570 and 540 nm (Fig. 3) essentially identical to that obtained with native HRP.

**Compound I Formation**—The rate of formation of Compound I was determined by monitoring the decay of the absorption at 415 nm, an isosbestic point between Compound II and the ferric state. The rate $k_1 = 3.0 \pm 0.3 \times 10^{4} M^{-1} s^{-1}$ at pH 7.0 and 25 °C (Table I). This rate of Compound I formation is approximately 10$^3$ times faster than that for the H42A mutant but 10$^5$ times slower than that for native, wild-type, or F41A HRP (Table I) (8).

**Steady-state Kinetics of the Oxidation of Guaiacol and ABTS**—The F41H/H42A double mutant is active with respect to the oxidation of peroxidase substrates such as guaiacol and ABTS. The pH profile for guaiacol oxidation shows that the pH maximum is only slightly shifted with respect to that of the native enzyme (Fig. 5). The pH profile is broader, however, and there is less loss of activity at pH values above the optimum than is observed with the native enzyme.

**Kinetic studies of the oxidation of guaiacol by F41H/H42A hHRP, H42A hHRP, HHRP, and HRP** have been carried out to evaluate the effect of a histidine at position 41 on the catalytic properties of an enzyme without the catalytic histidine at position 42 (Table II). The $K_m$ for the oxidation of guaiacol by F41H/H42A hHRP is 43 $\mu M$, a value to be compared with the values of 5800, 3000, and 3.8 $\mu M$ for the native, wild-type, and H42A proteins, respectively (Table II). The $k_{cat}$ for the oxidation of guaiacol by the F41H/H42A double mutant is 1.8 s$^{-1}$, whereas the $k_{cat}$ values for the native, wild-type, and H42A mutant are 420, 300, and 0.015 s$^{-1}$, respectively. The activity...
of the double mutant, as judged by the $k_{cat}$ values, is 120-fold higher than that of the H42A single mutant but 164-fold lower than that of the wild-type enzyme. The $k_{cat}/K_m$ values for the various proteins are much closer, however, because the H42A substitution decreases not only the $k_{cat}$ but also the $K_m$.

The catalytic role of the missing histidine in the H42A mutant can be partially satisfied by exogenous imidazole (8). The effect of exogenous 1,2-dimethylimidazolodiazole on the oxidation of guaiacol by the F41H/H42A mutant was therefore compared with its effect on the oxidation catalyzed by the H42A mutant (Table II). Exogenous 1,2-dimethylimidazolodiazole raises the $K_m$ value for guaiacol 2.6-fold for the H42A mutant and 4.3-fold for the F41H/H42A mutant, as expected if the imidazole competes with guaiacol for occupancy of the active site. In catalytic terms, the $k_{cat}$ value for the H42A mutant is increased 7.8-fold but that for the F41H/H42A mutant only 1.4-fold (Table II). Thus, exogenous imidazole does not significantly promote catalytic turnover of the double mutant but, as shown earlier (8), facilitates catalysis in the case of the H42A Imidazole.

Analogous studies of the oxidation of ABTS by F41H/H42A hHRP show that its $K_m$ value is 60 μM (Table II). This value is essentially the same as the $K_m$ for H42A hHRP (62 μM) but is considerably smaller than that for oxidation of ABTS by the native (800 μM) or wild-type (610 μM) enzymes. The $k_{cat}$ for the oxidation of ABTS by the F41H/H42A double mutant is 100 s⁻¹, whereas the $k_{cat}$ values for the native, wild-type, and H42A mutant are 4100, 4900, and 0.41 s⁻¹, respectively. The catalytic activity ($k_{cat}$) of the double mutant with respect to ABTS is thus 260-fold higher than that of the H42A mutant but 39-fold lower than that of native HRP. Peroxygenase Reactions—The kinetic parameters for thioanisole sulfoxidation by the F41H/H42A mutant are $K_m = 8.4$ mM and $k_{cat} = 5.33$ s⁻¹ (Table III). Thioanisole thus binds 14 and 28 times more weakly to the F42A/H42A mutant than it does to native HRP or H42A hHRP, respectively. On the other hand, the catalytic rate is 110-fold greater than that of native HRP and 180-fold greater than that of the H42A mutant. An additional characteristic of the F41H/H42A mutant is that it produces almost exclusively the (R)-enantiomer of methyphenyl sulfoxide, whereas native HRP and the H42A mutant yield approximately a 1:3 (S)/(R) enantiomer ratio (Table III).

Styrene oxidation by native HRP and the H42A and F41H/H42A mutants produces three metabolites: styrene oxide, phenylacetaldehyde, and benzaldehyde. The H42A and F41H/H42A mutants produce less benzaldehyde but more styrene oxide than the native enzyme. Native HRP, with a $K_m$ of 0.3 mM and a $k_{cat}$ of $9.9 \times 10^{-5}$ s⁻¹ (Table IV), is a very poor catalyst for styrene oxidation. Native HRP was previously reported to have little if any activity as a styrene epoxidation catalyst (8, 17). Comparison of the catalytic constants shows that the H42A mutation decreases the affinity of the enzyme for styrene 10-fold. As reported previously, the $k_{cat}$ for the H42A mutant is 70 times higher than that for native HRP (Table IV). The presence of a surrogate catalytic histidine in the F41H/H42A mutant further improves $k_{cat}$, making the double mutant 240 times better than native HRP and 3.5 times better than the H42A mutant as a catalyst.

**DISCUSSION**

Replacement of His-42 by an alanine, as expected from its catalytic role, decreases the rate of Compound I formation by a factor of $4.6 \times 10^5$ (Table I) (8). Nevertheless, the peroxygenase activity of the H42A mutant, as judged by thioanisole sulfoxidation and styrene epoxidation, is higher than that of wild-type HRP (8). The increase in peroxygenase activity has been proposed to reflect better substrate access to the Compound I ferryl oxygen. Further improvement of the peroxygenase activity should be possible if the decrease in the rate of Compound I formation caused by the H42A mutation could be prevented without sacrificing the improved access to the ferryl oxygen. One possible approach is to introduce a surrogate histidine at a position that allows it to promote Compound I formation without blocking access to the ferryl oxygen. To explore this approach, we have replaced Phe-41 by a histidine in the H42A mutant. Phe-41 was chosen as the site of the compensatory mutation because the corresponding residues in the available peroxidase structures are close to the iron atom but do not directly block the substrate access channel (Fig. 2) (10–13). Evidence that Phe-41 of HRP occupies a position very similar to that of the corresponding residues in peroxidase crystal structures is provided by studies of the reactions of the F41A, F41V, H42A, and H42L mutants with phenylidrazine (8, 26).

F41H/H42A hHRP is readily expressed and purified and has spectroscopic properties (Fig. 3) similar to those of both native and recombinant HRP. Reaction with 1 eq of H₂O₂ produces a species with a Compound I spectrum very similar to that of the wild-type Compound I intermediate (Fig. 4). However, 1 eq of K₃Fe(CN)₆ does not reduce Compound I to an intermediate with the spectroscopic properties of Compound II. Instead of the 10–12-nm Soret band shift with the major increase in absorbance expected for Compound II formation, a shift of only 2 nm with a modest increase in absorbance is observed (Fig. 4). Similar spectroscopic changes are observed if 10 eq of ferrocyanide are added to Compound I, or if Compound I is allowed to decay in the absence of exogenous reducing agents (Fig. 4). Thus, Compound I decays directly to a ferric-like state without the observable formation of a Compound II intermediate. In this regard, F41H/H42A hHRP resembles the H42A mutant, which gives a Compound I species that decays directly to the ferric enzyme without the detectable formation of a Compound II intermediate (8). The reason for the abnormal lower stability of Compound II than Compound I is unclear, but resonance
Raman studies have shown that in native HRP the Compound II ferryl oxygen is hydrogen-bonded, presumably to His-42 (27, 28). This hydrogen bond would be lost in the H42A mutant. If loss of the hydrogen bond is responsible for the instability of Compound II in H42A hHRP, it appears that the surrogate histidine in the F41H/H42A mutant does not provide a comparable hydrogen bond. Incomplete recovery of the Soret absorbance when preformed Compound I is reduced with ferrocyanide indicates that, in the absence of protection by reducing agents, Compound I formation results in partial degradation of the heme group.

His-41 in the F41H/H42A mutant functions as a partial surrogate for the normal histidine in catalyzing Compound I formation. The rate of Compound I formation for the F41H/H42A mutant is approximately 1500 times faster than for the H42A mutant (Table I). Not surprisingly, the surrogate histidine is not as effective as His-42 in the native enzyme, as shown by the fact that the formation of Compound I is still 300 times faster in the native protein than in F41H/H42A hHRP. Nevertheless, it appears that His-41 facilitates the formation of Compound I, presumably by partially satisfying one or both of the catalytic roles played by the normal His-42: (a) deprotonation of the peroxide in formation of the Fe-OOH complex, and (b) transfer of the proton to the terminal oxygen of the Fe-OOH complex to promote oxygen-oxygen bond scission.

The higher rate of Compound I formation in the F41H/H42A than H42A mutant increases the guaiacol and ABTS peroxidase activities (Table II). Peroxidation of guaiacol by the F41H/H42A mutant is 120 times faster than peroxidation by H42A hHRP but 160 times slower than peroxidation by the wild-type enzyme. Likewise, the $k_{cat}$ (104 s$^{-1}$) for peroxidation of ABTS by F41H/H42A hHRP is 260-fold higher than that for the H42A mutant but 39-fold lower than that for the wild-type enzyme (Table II). These $k_{cat}$ changes qualitatively parallel the changes in the rates of Compound I formation. The absence of a quantitative correlation is consistent with the fact that the ratelimiting step of the reaction differs in the wild-type and mutant proteins. In the wild-type protein the reduction of Compound II is rate-limiting, whereas Compound II is not detected with either of the two mutants. The finding that the $K_m$ values for guaiacol and ABTS are orders of magnitude lower for the H42A and F41H/H42A mutants than for the wild-type enzyme (Table II) suggests that the mutations may also alter the interactions of Compound I with the two substrates. As a result of this tighter substrate binding, the $k_{cat}$/$K_m$ values for the oxidation of guaiacol and ABTS by the mutant and wild-type enzymes differ by less than the $k_{cat}$ values. The increase in the binding affinity of guaiacol and ABTS in the two mutant proteins may be related to the increase in the size of the active site cavity caused by the H42A mutation (8). The increase in the $K_m$ values for styrene and thioanisole increase (Tables III and IV) complicates this question, although we have provided evidence that these peroxygenase substrates bind in a different location than the peroxidase substrates (29).

We demonstrated earlier that imidazoles improve the catalytic activity of H42A hHRP by binding in its active site as surrogate acid-base catalysts (8). However, exogenous 1,2-dimethylimidazole only slightly increases the $k_{cat}$ for guaiacol peroxidation by the F41H/H42A mutant (Table II). The guaiacol oxidation pH profile for the F41H/H42A mutant is broader and slightly shifted toward a more basic optimum than that for the wild-type enzyme (Fig. 5), but the profile otherwise exhibits the same concave shape as the wild-type. In contrast, the rate for H42A hHRP increases linearly with pH, in agreement with a quantitative correlation is consistent with the fact that the ratelimiting step of the reaction differs in the wild-type and mutant proteins. In the wild-type protein the reduction of Compound II is rate-limiting, whereas Compound II is not detected with either of the two mutants. The finding that the $K_m$ values for guaiacol and ABTS are orders of magnitude lower for the H42A and F41H/H42A mutants than for the wild-type enzyme (Table II) suggests that the mutations may also alter the interactions of Compound I with the two substrates. As a result of this tighter substrate binding, the $k_{cat}$/$K_m$ values for the oxidation of guaiacol and ABTS by the mutant and wild-type enzymes differ by less than the $k_{cat}$ values. The increase in the binding affinity of guaiacol and ABTS in the two mutant proteins may be related to the increase in the size of the active site cavity caused by the H42A mutation (8). The increase in the $K_m$ values for styrene and thioanisole increase (Tables III and IV) complicates this question, although we have provided evidence that these peroxygenase substrates bind in a different location than the peroxidase substrates (29).

The improvement in the thioanisole sulfoxidation and styrene epoxidation activities of the F41H/H42A mutant with respect to hHRP and H42A hHRP supports the view that the peroxygenase activity of wild-type HRP is limited by restricted access to the ferryl oxygen, and of the H42A mutant by the rate of formation of Compound I. Compound I formation may be only partially rate-limiting for the H42A mutant, however, because the rate of Compound I formation of F41H/H42A hHRP is increased 1500-fold higher but that of thioanisole oxidation only 180-fold and of styrene epoxidation 3.4-fold. The relatively small increase in styrene epoxidation when the F41H mutation is combined with the H42A mutation may reflect continued steric limitations within the cavity created by the H42A mutation. Support for this is provided by the fact that the sulfoxidation rate is enhanced more than the epoxidation

### Table II

**Steady state kinetic parameters for the oxidation of guaiacol and ABTS**

| Substrate | Enzyme     | Enzyme concentration | $K_m^{\mu M}$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m^{\mu M}$ |
|-----------|------------|-----------------------|---------------|-----------|---------------|------------------------|
| Guaiacol  | Native     | 2                     | 5800 ± 700    | 420 ± 40  | 7.2 ± 1.1 × 10^{-2} | 7.2 ± 1.1 × 10^{-2} |
|           | WT         | 2                     | 3000 ± 100    | 300 ± 10  | 10 ± 2 × 10^{-2}  | 10 ± 2 × 10^{-2} |
|           | H42A       | 1780                  | 3.8 ± 0.5     | 0.015 ± 0.001 | 0.4 ± 0.06 × 10^{-2} | 0.4 ± 0.06 × 10^{-2} |
|           | H42A       | 1420                  | 9.9 ± 1       | 0.12 ± 0.01 | 1.2 ± 1.0 × 10^{-2} | 1.2 ± 1.0 × 10^{-2} |
| F41H/H42A | F41H/H42A  | 204                   | 43 ± 1        | 1.8 ± 0.1  | 4.2 ± 0.8 × 10^{-2} | 4.2 ± 0.8 × 10^{-2} |
|           | F41H/H42A  | 204                   | 190 ± 2       | 2.5 ± 0.1  | 1.3 ± 0.1 × 10^{-2} | 1.3 ± 0.1 × 10^{-2} |
| ABTS      | Native     | 0.2                   | 800 ± 2       | 4100 ± 100 | 5.1 ± 0.2       | 5.1 ± 0.2 |
|           | WT         | 0.2                   | 610 ± 2       | 4900 ± 100 | 8.0 ± 0.3       | 8.0 ± 0.3 |
|           | H42A       | 143                   | 6 ± 1         | 0.41 ± 0.02 | 6.6 ± 1.0 × 10^{-2} | 6.6 ± 1.0 × 10^{-2} |
|           | F41H/H42A  | 20                    | 60 ± 7        | 100 ± 10   | 1.7 ± 0.1       | 1.7 ± 0.1 |

* pH 6.0, 1 mM H$_2$O$_2$.
* pH 4.6, 10 mM H$_2$O$_2$.

### Table III

**Kinetic parameters for thioanisole sulfoxidation**

| Enzyme     | Enzyme concentration | Thioperoxide | $K_m^{\mu M}$ | $k_{cat}$ | Enantiomer ratio |
|------------|-----------------------|--------------|---------------|-----------|------------------|
| Native HRP | 25                    | 0.6          | 0.05          | 21.79     |                  |
| H42A       | 10                    | 0.3          | 0.03          | 25.75     |                  |
| F41H/H42A  | 0.5                   | 8.4          | 5.3           | <1.99     |                  |

### Table IV

**Kinetic parameters for styrene epoxidation**

| Enzyme     | Enzyme concentration | $K_m^{\mu M}$ | $k_{cat}$ | Ph$_4$CHO:Ph$_3$CHO:Ph$_2$CHO:SO$_2$ |
|------------|-----------------------|---------------|-----------|-----------------------------------|
| Native HRP | 98                    | 0.3           | 1 × 10^{-6} | 22.36:42                         |
| H42A       | 26                    | 2.9           | 7 × 10^{-3}  | 08.30:62                         |
| F41H/H42A  | 17                    | 2.5           | 2.4 × 10^{-3} | 03.34:63                         |

* SO = styrene oxide.
rate in going from the H42A to the F41H/H42A mutant. Thioanisole sulfoxidation is a less sterically demanding reaction than olefin epoxidation because it requires addition of the ferryl oxygen to one rather than two atoms of the substrate.

In summary, the F41H mutation introduces a histidine into the active site of the H42A mutant of HRP that partially satisfies the catalytic role normally played by His-42. This leads to substantial rescue of the rates of Compound I formation and peroxidase catalysis caused by the H42A mutation. Furthermore, the F41H mutation does not appear to significantly decrease the active site access provided by the H42A mutation, and F41H/H42A hHRP has elevated peroxygenase activity.

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