Rescue of the Catalytic Activity of an H42A Mutant of Horseradish Peroxidase by Exogenous Imidazoles*

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His-42 plays a critical role in the H$_2$O$_2$-dependent catalytic turnover of horseradish peroxidase (HRP). This is clearly illustrated by the finding that an H42A mutation decreases the rate of Compound I formation by a factor of $\sim 10^4$. As shown here, the addition of 2-substituted imidazoles partially rescues both the rate of formation of Compound I and the peroxidase activity of the H42A mutant. 2-Substituted imidazoles are the most effective because they do not coordinate to the iron. In contrast to native HRP, which exhibits a parabolic pH profile, and the H42A mutant, for which the activity increases linearly with increasing pH, the activity of the H42A mutant in the presence of 1,2-dimethylimidazole ($pK_a = 8.0$) exhibits a sigmoidal pH dependence with a midpoint at pH 8.0 $\pm$ 0.2. Similar results are obtained with 2-methylimidazole. These results establish that the free base forms of these imidazoles facilitate HRP turnover. The spectroscopic binding constants for 1,2-dimethylimidazole and 2-methylimidazole are $K_a = 2.9 \pm 1.3$ and $2.5 \pm 0.2$, respectively. When cyanide is bound to the heme, the $K_a$ for 1,2-dimethylimidazole is 0.17 M. This >10-fold decrease in $K_a$ may reflect hydrogen bonding of the protonated imidazole to the iron-coordinated cyanide. The log of the rate of Compound I formation exhibits a linear dependence on the molecular volume of the imidazoles used to rescue the activity. If the rates are corrected for differences in the size of the imidazoles, the log of the rates is linearly related to the $pK_a$ of the imidazoles. This Bronsted analysis predicts that $\sim 60\%$ of a positive charge develops on the imidazole in the transition state of Compound I formation. The results confirm the acid-base role of the distal histidine, demonstrate that exogenous histidines can function as surrogates for the missing histidine in the H42A mutant, and provide a transition state model of relevance to the formation of Compound I in the native protein.

The distal histidine (His-42) of HRP$^1$ plays an important role in peroxidase catalysis. His-42 is thought to promote the formation of Compound I by acting as a general acid-base catalyst that (a) deprotonates H$_2$O$_2$, facilitating the formation of a ferric peroxide (Fe-OOH) complex and (b) subsequently transfers the proton to the distal oxygen of the ferric peroxide complex in the dioxygen bond cleavage step (1–3). This catalytic sequence produces the HRP ferryl porphyrin radical cation species (P$^+\cdot$Fe$^{V=O}$) known as Compound I. A network of hydrogen bonds is thought to assist the distal histidine in its catalytic role. Alignment of the sequence of HRP with those of cytochrome c peroxidase, lignin peroxidase, and Arthromyces rhamosus peroxidase, for all of which crystal structures are available (4–6), suggests that in HRP Asn-70 is hydrogen bonded to the N$_6$ of His-42 (7). In accord with this view, mutation of Asn-70 to a valine decreases the rate of HRP Compound I formation from 1.6 $\times$ 10$^5$ to 6 $\times$ 10$^4$ s$^{-1}$ and the rate of Compound I reduction by guaiacol from 7.8 $\times$ 10$^6$ to 1.2 $\times$ 10$^5$ m$^{-1}$ s$^{-1}$ (7).

Compound I formation is followed in the catalytic cycle of HRP by two one-electron reduction steps:

$$k_1$$

HRP + H$_2$O$_2$ $\rightarrow$ Compound I

$$k_2$$

Compound I $\rightarrow$ Compound II

$$k_3$$

Compound II $\rightarrow$ HRP

The first of these one-electron transfers reduces the porphyrin radical cation of Compound I to an intermediate known as Compound II that retains the ferryl species. In addition to its role in Compound I formation, His-42 is known to take up a proton when Compound I is reduced to Compound II (8). Resonance Raman shows that the ferryl oxygen of Compound II is hydrogen bonded to the distal histidine, presumably via the proton taken up during the reduction of Compound I to Compound II (9, 10). When Compound II is reduced to the ferric state, the proton is completely transferred to the ferryl oxygen, which is then eliminated as a molecule of water.

In accord with the proposed catalytic role of His-42, mutation of His-42 to an alanine decreases the rate constant for Compound I formation from 8.9 $\times$ 10$^6$ to 19.4 M$^{-1}$ s$^{-1}$ and the rate of guaiacol oxidation from 7.5 $\times$ 10$^6$ to 10.5 nmol s$^{-1}$ $\mu$mol$^{-1}$ (11). Furthermore, Compound II is not observed as an intermediate when Compound I of the H42A mutant is reduced by one-electron donors. The H42A mutation converts HRP the ability to epoxidize styrene (11). Analysis of the architecture of the active site by reaction with phenyldiazene indicates that the H42A mutation opens up the active site without altering its gross structure, making the ferryl species more accessible to styrene and thus facilitating the oxygen transfer reaction. The data specifically suggests that replace-

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1 The abbreviations used are: HRP, horseradish peroxidase isozyme c; HRP$_{Gfp}$, glyoxisome-targeted recombinant horseradish peroxidase isozyme c; heme, iron protoporphyrin IX regardless of oxidation and ligation state; ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); I m, imidazole; 2-MeIm, 2-methylimidazole; 1-MeIm, 1-methylimidazole; 2,6-diMeIm, 1,2-dimethylimidazole; 2-Bi3-MeIm, 2-bromo-1-methylimidazole.
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EXPERIMENTAL PROCEDURES

Materials and Methods—Recombinant, polyhistidine-tagged hHRP and H42A hHRP were heterologously expressed in E. coli and were purified as reported (11). These proteins are designated as hHRP to indicate that they bear a polyhistidine extension that facilitates their purification. NMR spectra were determined on a General Electric QE 300 instrument. Kinetic assays and spectra were obtained on either a Cary 1E or Hewlett Packard 8452A diode array spectrophotometer.

Results

Effect of Imidazoles on hHRP Activity

We report here that exogenous imidazoles partially rescue the catalytic activity of the enzyme and provide evidence that the imidazoles bind within the active site and function as catalytic surrogates for His-42.

pH Profiles for the Oxidation of Guaiacol by H42A hHRP in the Presence and Absence of Substituted Imidazoles—Guaiacol oxidation was monitored spectrophotometrically at 470 nm ($\epsilon_{470} = 2.75 \times 10^3 M^{-1} cm^{-1}$) with a 10 $\mu M$ concentration of the imidazole. In order to assay a wide pH range, 25 $\mu M$ acetaldehyde (pH 4.5–5.5), phosphate (pH 6–8), and borate (pH 8.5–10) were used as the reaction buffers. The pH was adjusted to 0.1 with NaCl. H42A hHRP was assayed both alone and in the presence of 1 $\mu M$, 2$\pm$1$\mu M$, and 2$\pm$2$\mu M$ imidazole.

The pH profiles of 2-MeIm and 1,2-diMeIm—Solvents containing a 400 $\mu M$ volume of 10 $\mu M$ H42A hHRP and varying amounts (0.24 to 2.3) of 1$\pm$1$\mu M$ or 2$\pm$2$\mu M$ in 100 $\mu M$ Na$_2$HPO$_4$ buffer (pH 7.0) were prepared. A concentrated aqueous solution of the imidazole (4 $\mu M$, pH 7.0) was used to adjust the imidazole concentration. The reference cuvette contained the same amount of the imidazole in 100 $\mu M$ Na$_2$HPO$_4$ buffer (pH 7.0) buffer because the imidazoles display residual absorption in the visible region. Both solutions were allowed to equilibrate for 1 h with periodic mixing prior to recording the 300–550 nm difference absorption spectrum. The $K_a$ values for the imidazoles were calculated from the difference obtained when the spectrum of imidazole-free H42A hHRP was subtracted from that of imidazole-bound H42A hHRP after correction for the imidazole absorption.

Rescue of H42A hHRP-catalyzed Guaiacol and ABTS Oxidation by Imidazoles—The oxidations of guaiacol and ABTS by H42A hHRP were assayed at 25 $^\circ$C in the presence of 1 $\mu M$, 1$\pm$1$\mu M$, 2$\pm$2$\mu M$, or 1,2-diMeIm. In the guaiacol assay, H$_2$O$_2$ was added (0.5 $\mu M$ final concentration) to a solution containing 500 $\mu M$ H42A hHRP, varying concentrations of imidazoles, and 5 $\mu M$ guaiacol in 100 $\mu M$ K$_2$HPO$_4$ (pH 7.0). Guaiacol consumption was monitored as described in the ABTS assay. H$_2$O$_2$ (0.5 $\mu M$ final concentration) was added to 50 $\mu M$ H42A hHRP, varying concentrations of imidazoles, and 5 $\mu M$ ABTS in 100 $\mu M$ Na$_2$HPO$_4$ (pH 7.0). The formation of the ABTS radical cation was monitored at 414 nm ($\epsilon_{414} = 3.6 \times 10^4 M^{-1} cm^{-1}$). The imidazole levels were adjusted with 5 $\mu M$ stock solutions of the imidazoles adjusted to pH 7.0, giving 5–50 $\mu M$ concentrations of the unprotonated form of the imidazole. NaCl (500 $\mu M$) was added to maintain constant ionic strength.

Rescue of Compound I Formation by 1,2-DiSubstituted Imidazoles—1,2-DiMeIm (Aldrich) was purified by distillation under reduced pressure. 2-Ethyl- and 2-propylimidazole were N-methylated with iodomethane as reported for the N-methylation of ethyl imidazol-4- carboxylate (12). 2-Br-1-MeIm was synthesized from 1-MeIm by reaction with n-butylithium followed by 1,2-dibromoethane (13). 1-Methyl-2-butilimidazole was similarly generated by allowing the imidazolium ion to react with 1-bromobutane. Reaction of 1-MeIm with N,N,N-trimethylmethylenimine (14). The structures and purities of the synthetic products were verified by $^1$H NMR and mass spectrometry.

Compound I formation was assayed at 25 $^\circ$C by following the decrease in the Soret absorption at 402 nm in 1 ml reaction mixtures containing 2 $\mu M$ H42A hHRP and various concentrations of 1,2-disubstituted imidazoles in 100 $\mu M$ potassium phosphate buffer (pH 7.0). The reactions were initiated by adding various amounts of H$_2$O$_2$. The raw data was converted to rates using the absorption coefficient $\epsilon_{402} = 5.24 \times 10^4 M^{-1} cm^{-1}$ determined from the decrease in the absorption at 402 nm when partial equivalents of H$_2$O$_2$ were added to wild-type hHRP.

RESULTS

pH Profile of the 2$\pm$2$\mu M$ and 1,2-DiMeIm—Figure 1 shows the pH profile for the oxidation of guaiacol by H42A hHRP in the presence of varying concentrations of 2-MeIm and 1,2-diMeIm. The pH values in the figure are those measured after imidazole addition because 1 $\mu M$ imidazole concentrations slightly alter the pH (pH units). As shown in the inset, the catalytic activity of H42A hHRP increases linearly with increasing pH in the absence of an added imidazole. This profile differs significantly from that of wild-type HRP, which displays a broad, bell-shaped pH profile with an optimum at approximately pH 6.5 (7). When 2$\pm$2$\mu M$ is added to the assay mixture, the pH profile for the H42A mutant shows that deprotonation of a group with $K_a = 7.8 \pm 0.4$ improves activity (Fig. 1). This $K_a$ value corresponds well with that of 2$\pm$2$\mu M$ (pK$_a = 7.85$) (15). 1,2-DiMeIm (pK$_a = 8.0$) (15) gave a similar pH profile with an inflection point at pH 8.0 $\pm$ 0.2 (Fig. 1).

Imidazole Rescue of the Oxidation of Guaiacol and ABTS by H42A hHRP—Im, 1$\pm$1$\mu M$, 2$\pm$2$\mu M$, and 1,2-diMeIm enhance the guaiacol and ABTS peroxidase activities of H42A hHRP. For 1$\pm$1$\mu M$ and 2$\pm$2$\mu M$ the improvement in activity increases and then plateaus off with increasing concentration of the imidazole (Fig. 2). This leveling off is consistent with the ability of Im and 1-MeIm to coordinate to the heme iron atom, as demonstrated spectrophotometrically by a shift of the Soret band from 402 to 412 nm with increasing Im concentrations. Formation of the Im-imiron complex is detectable at Im concentrations above 18 $\mu M$ (not shown). A similar pattern is observed for rescue of the catalytic activity of H42A hHRP by 1$\mu M$. In contrast, the red shift of the Soret band indicative of nitrogen coordination to the iron is not observed with the 2-methylimidazoles and the peroxidase activity increases in a linear fashion as the 1,2-diMeIm free-base concentration is increased from 5 to 50 $\mu M$ (14). 2-MeIm also causes a concentration-dependent increase in the activity of the H42A mutant, but the magnitude of the increase is greater than that observed with 1,2-diMeIm and the concentration dependence is curved rather than linear (Fig. 2).

Determination of the $K_a$ for 2$\pm$2$\mu M$ and 1,2-DiMeIm—The difference spectra for the binding of 2$\pm$2$\mu M$ (Fig. 3A) and 1,2-diMeIm (Fig. 3B) to H42A hHRP have minima at 383 and 402 nm and maxima at 416 and 419 nm, respectively. The absorbance differences between the minima and maxima
the corresponding imidazole concentrations can be fit to the hyperbolic function:

\[
\frac{\Delta \text{Abs}}{\Delta \text{Abs}_{\text{max}} [S]} = \frac{K_d + [S]}{K_d}
\]

Kd values of 2.5 ± 0.2 and 2.9 ± 1.3 M are thus obtained for 2-MeIm and 1,2-diMeIm, respectively. To confirm that these high concentrations of 1,2-diMeIm do not result in coordination to the heme, the same experiment was run in the presence of 1 mM cyanide. The resulting difference spectra displayed a maximum at 426 nm and a minimum at 399 nm. The Kd value determined as above was 174 mM.

Rescue of Compound I Formation by a Series of 1,2-Disubstituted Imidazoles—In view of the postulated role of His-42 in the activation of H2O2, the effect of exogenous imidazoles on the catalytic rate of H42A hHRP is likely to be reflected in the rate of Compound I formation. Indeed, in the presence of 100 mM 2-MeIm and 1,2-diMeIm, partial formation of Compound I is observed immediately on addition of 1 equivalent of H2O2, and virtually complete formation of Compound I is observed immediately on addition of 10 equivalents of H2O2 (Fig. 4). In contrast, 500 equivalents of H2O2 are required to observe the relatively slow (~10 min) formation of Compound I in the absence of exogenous imidazole. Decay of Compound I to the resting state without the detectable formation of a Compound II intermediate is observed within a few minutes in the presence or absence of imidazoles. In the absence of an imidazole, the initial resting state spectrum is only partially regained when the oxidized protein is fully reduced with K4Fe(CN)6, presumably because heme degradation occurs. The heme destruction observed for the H42A mutant is suppressed in the presence of an imidazole, as the initial Soret band intensity is fully recovered upon addition of K4Fe(CN)6 to the oxidized protein (Fig. 4).

To clarify the steric and electronic requirements for the rescue of activity by imidazoles, the effect of a series of 1-methyl-2-substituted imidazoles on the rate of Compound I formation has been evaluated (Table I). This series of compounds was...
chosen because the 1-methyl substituent suppresses hydrogen bonding effects involving the exchangeable imidazole proton and placement of the variable substituent at position 2 prevents coordination to the heme iron atom. In monitoring the rescue of Compound I formation by imidazoles, relatively low (1–25 mM) imidazole concentrations and a high buffer concentration were used to minimize the effect of fluctuating imidazole levels on the ionic strength and pH. In view of its high K_d value, the 1,2-diMeIm concentrations used in this experiment only partially saturate H42A hHRP. H_2O_2 concentrations were also kept relatively low (14–110 μM) to avoid the formation of Compound III (the ferrous dioxy complex), as this would shift the Soret maximum and decrease the absorbance at 402 nm. Compound I formation was monitored by the exponential decay of the 402-nm absorbance over a period of 3–5 min. Only partial conversion of the resting state to Compound I is indicated by the decrease in the Soret absorption, which is less than the 50% of the decrease expected for complete Compound I formation. The maximum decrease in the 402 nm absorption increased with increasing imidazole and peroxide concentrations. The rate of Compound I formation was determined by performing a linear least squares fit to the initial decay in absorbance (10% of total absorbance change). A plot of the rate of Compound I formation versus the H_2O_2 concentration gives a series of lines that intersect at the origin according to the equation:

\[ \log k_{\text{imid}} = \log k_{\text{imid}}(\text{corr}) + \log k_{\text{imid}}^* \]

(Eq. 1)

To obtain a peroxide-independent rate, the slopes of the lines can be plotted versus the imidazole concentration:

\[ \text{Slope}[\text{H}_4\text{A hHRP}] = k_{\text{imid}}^* [\text{imid}] + k_1 \]  

(Eq. 2)

The slope of this secondary plot provides the imidazole-dependent rate constant for Compound I formation. The y-intercept of the plot indicates that an imidazole-independent rate of Compound I formation contributes to the measured velocities. The imidazole-independent rate calculated from the y-intercept, 16 M^{-1} s^{-1}, is similar to that deduced from a steady state kinetic analysis (19 M^{-1} s^{-1}) (11). The rate of Compound I formation with 1-methyl-2-ethylimidazole was examined not only at pH 7.0 (Table I) but also at pH 7.3, where k_{\text{imid}}^* = 19 × 10^{-3} M^{-1} s^{-1} relative to (Im + 1H^+). At this pH, twice as much of the deprotonated form is present as at pH 7.0, and the resulting imidazole-dependent rate constant is approximately 1.9-times that obtained at pH 7.0 (k_{\text{imid}}^* = 10 × 10^{-3} M^{-2} s^{-1}). This confirms that the deprotonated form is the active form. The measured imidazole-dependent rate constants were therefore corrected using the measured pK_a values to reflect the actual concentration of the unprotonated imidazole that is present.

A plot of the rate constants for Compound I formation for the 2-methyl-, 2-ethyl-, 2-propyl-, and 2-butyl-substituted derivatives of 1-MeIm versus the molecular volumes of the imidazoles shows that the two parameters are linearly related (Fig. 5). The correlation is described by the equation \[ \log k_{\text{imid}}^* = 0.54 - \frac{0.12}{\text{molecular volume}}, \]

with r^2 = 0.92. The abilities of the imidazoles to promote Compound I formation thus depend inversely on their molecular volume. The value of the slope of the line (B) was used to obtain rate values that are independent of the size of the substituent using the equation \[ \log k_{\text{imid}}^*(\text{corr}) = \log k_{\text{imid}}^* - (B \times \text{molecular volume}). \]

A plot of \[ \log k_{\text{imid}}^*(\text{corr}) \]

versus the pK_a values of the 2-substituted imidazoles (Fig. 6) yields a linear Brönsted relationship described by the equation \[ \log k_{\text{imid}}^*(\text{corr}) = -4.33 + (0.6 + pK_a) \]

with a correlation coefficient r^2 = 0.95. The slope of this plot (B = 0.6) indicates that the imidazole is approximately 60% protonated in the transition state leading to the formation of Compound I in the H42A mutant.

**DISCUSSION**

Replacement of His-42 by an alanine drastically diminishes the peroxidative activity of hHRP with respect to guaiacol and ABTS (Table II) (11). This decrease in activity is presumably due to elimination from the active site of a critical acid-base catalyst. As shown here, the acid-base role of His-42 can be partially satisfied by exogenous imidazoles. A comparison of the abilities of Im, 1-MeIm, 2-MeIm, and 2-diMeIm to rescue the catalytic activity of H42A hHRP shows that 2-MeIm is the most and 1-MeIm the least effective of the four imidazoles (Fig. 2). A comparison of the effectiveness of the four imidazoles in stimulating catalysis identifies two factors that modulate the observed effect: (a) coordination of the imidazole to the heme iron atom, and (b) the ability of the imidazole to participate in hydrogen bonding interactions.

Spectroscopic studies show that Im and 1-MeIm at high concentrations coordinate to the heme iron atom of the H42A mutant to give hexacoordinated complexes. In contrast, 2-MeIm and 1,2-diMeIm bind to the enzyme with K_d values of 2.5 ± 0.2 and 2.9 ± 1.3 μM, respectively, without detectably coordinating to the iron atom (Fig. 3). A 2-methyl substituent normally suppresses such coordination (16). In agreement with these observations, the enhancements of catalytic activity by Im and 1-MeIm level off at higher concentrations of the imidazoles (Fig. 2), whereas the catalytic enhancements produced by 2-MeIm and 1,2-diMeIm do not level off.

The nonlinear dependence of the catalytic enhancement on the 2-MeIm concentration, indicative of a higher than first order dependence of the rate on the imidazole concentration, is due to hydrogen bonding interactions because the concentration dependence of the rate enhancement by 1,2-diMeIm is

**Table I**

| 2-Substituent | pK_a | Volume | k_{\text{imid}}^* (M^{-1} s^{-1}) | k_{\text{imid}} (M^{-2} s^{-1}) |
|---------------|------|--------|-------------------------------|-------------------------------|
| Methyl        | 8.18 | 109.4  | 9.9 × 10^{-3}                 | 1.6 × 10^{-1}                 |
| Ethyl         | 8.13 | 106.9  | 10.0 × 10^{-3}                | 1.5 × 10^{-1}                |
| Propyl        | 8.13 | 145.8  | 3.9 × 10^{-3}                 | 5.7 × 10^{-2}                |
| Butyl         | 8.09 | 164.7  | 3.1 × 10^{-3}                 | 4.2 × 10^{-2}                |
| Hydroxymethyl | 6.86 | 116.5  | 4.6 × 10^{-3}                 | 7.9 × 10^{-3}                |
| Bromo         | 4.08 | 113.9  | 7.5 × 10^{-4}                 | 7.5 × 10^{-4}                |
The rate of Compound I formation (7). In principle, exogenous
noted, mutation of Asn-70 of HRP causes a 25-fold decrease in
His-42, by analogy to similar interactions in the peroxidases for
Asn-70 has been postulated to facilitate the acid-base role of
cyanide. Cyanide coordinates to the heme iron atom of native
i.e.
linear ($k_1$) 

\[
\begin{align*}
&k_1 = 1.4 (\pm 0.1) 	imes 10^3 \\
&k_1 = 4.6 (\pm 0.7) \\n&k_1 = 1.2 \text{ DiMeIm} \\
&k_1 = 4.6 (\pm 0.7) \\
\end{align*}
\]

**TABLE II**

| Enzyme          | Imidazole | Guaiacol | ABTS |
|-----------------|-----------|----------|------|
| Native HRP      | None      | 1.4 ($\pm$ 0.1) x $10^3$ | 1.2 ($\pm$ 0.1) x $10^2$ |
| Wild-type hHRP  | None      | 1.4 ($\pm$ 0.1) x $10^3$ | 1.2 ($\pm$ 0.1) x $10^2$ |
| H42A HHRP       | None      | 5.4 ($\pm$ 0.1) x $10^3$ | 6.9 ($\pm$ 2.3) x $10^2$ |
| H42A HHRP$^a$   | 1,2-DiMeIm| 4.6 ($\pm$ 0.7) x $10^3$ | 3.9 ($\pm$ 0.9) x $10^2$ |

$^a$ Actual units for this entry are s$^{-1}$ m$^{-1}$, with the error based on the mean of the slope (from Fig. 2).

The rates of guaiacol and ABTS oxidation were measured under conditions used to obtain the data in Fig. 2. For guaiacol oxidation, 5 nM, 50 nM, and 50 μM concentrations of native HRP, wild-type hHRP, and H42A were assayed, whereas for ABTS 5 nM, 50 nM, and 0.5 μM concentrations of native HRP, wild-type hHRP, and H42A were assayed. The values for the 1,2-diMeIm-rescued activity were determined from linear fits of the data in Fig. 2.

The correlation between the size of the 2-substituent and the ability of the unprotonated imidazoles to serve as His-42 surrogates in promoting Compound I formation (Fig. 5) is consistent with a requirement for the binding of the unprotonated histidines in the active site. Although HRP can oxidize large substrates, evidence that the active site is sterically constrained in the vicinity of the ferryl oxygen is provided by the low peroxygenase activity of the enzyme (2, 11), chemical modification experiments (25, 26), and the crystal structures of related peroxidases (4–6, 17, 18). The inverse effect of the size of the 2-substituent on the reaction may also include a direct steric effect of the substituted on deprotonation of H$_2$O$_2$ by the imidazole.

The linear Brønsted plot obtained when the logs of the rates

![Fig. 6. Relationship between the size-corrected rate of Compound I formation and the pK$_a$ values of the 2-substituted 1-MeIm employed as catalysts.](image)
of Compound I formation, corrected for differences due to the volume of the 2-substituent, are plotted against the \( K_a \) values of the imidazoles (Fig. 6) clearly indicates that the imidazoles function in the reaction as base catalysts. This provides direct evidence for the role of the imidazole in deprotonating \( \text{H}_2\text{O}_2 \) and thus in facilitating formation of the \( \text{Fe}^{\text{III}}\text{OOH} \) complex. The inference from the Brønsted plot that the imidazoles acquire approximately 60% of a positive charge in the transition state of the reaction suggests that hydrogen transfer from the peroxide to the imidazole occurs at the same time as formation of the iron-oxygen bond, as shown schematically in Fig. 7. It is likely that the protonated imidazole subsequently transfers the proton to the distal oxygen of the \( \text{Fe}^{\text{III}}\text{OOH} \) complex, but this study provides no specific evidence for or against this role of the imidazole because the rate-limiting effect on formation of the \( \text{Fe}^{\text{III}}\text{OOH} \) complex masks subsequent effects on decomposition of the peroxide complex to Compound I. In agreement with this conclusion, the increases in the rates of Compound I formation with pH and with increasing imidazole \( K_a \) values indicate that the rate accelerations are promoted by the neutral rather than protonated imidazoles.

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