Stimulation of the Activity of Horseradish Peroxidase by Nitrogenous Compounds*

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(Received for publication, November 5, 1987)

THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC.

The abbreviations used are: HRP, horseradish peroxidase.

A variety of nitrogenous compounds broaden the activity versus pH profile for the peroxidation of dianisidine catalyzed by horseradish peroxidase (HRP), but not by myelo- or chloroperoxidase, Escherichia coli hydroperoxidase I, methemoglobin, or microperoxidases. The peroxidation of dianisidine catalyzed by cytochrome c peroxidase was affected by the nitrogenous compounds, but to a lesser extent than was the action of HRP. The peroxidations of a variety of phenols by HRP exhibited broad activity versus pH profiles and were unaffected by the nitrogenous compounds. The energy of activation for the peroxidation of dianisidine by HRP was unaffected by changes of pH in the range 6.5–8.5 and was unchanged by the presence of the nitrogenous compounds. The nitrogenous compounds markedly increased Vₜ for the peroxidation of dianisidine by HRP, but did not change the slope of Lineweaver-Burk plots of kinetic data.

These results are accommodated by a mechanism in which nitrogenous compounds hydrogen-bond to the distal histidine of HRP and in so doing raise its pKₐ. Since the acid form of the distal histidine is thought to facilitate peroxidations catalyzed by HRP by hydrogen bonding to the ferryl oxygen of compound II, raising its pKₐ broadens the activity versus pH profile for the peroxidation of anilino substrates, such as dianisidine. We propose that phenolic substrates hydrogen-bond directly to the ferryl oxygen, thus displacing the distal histidine and eliminating the possibility of being influenced by nitrogenous compounds.

The mechanism of action of horseradish peroxidase (HRP) appears to involve divalent oxidation of the ferriheme prosthetic group by H₂O₂, followed by successive univalent reductions, by the electron donor substrate, as follows (1, 2).

\[ \text{HRP-Fe(III) + H}_2\text{O}_2 + 2\text{H}^+ \xrightarrow{k_1} \text{HRP-Fe(V) + 2H}_2\text{O} \] (compound I)

\[ \text{HRP-Fe(V) + DH} \xrightarrow{k_1} \text{HRP-Fe(IV) + DH}^+ + \text{H}^+ \] (compound II)

\[ \text{HRP-Fe(IV) + DH} \xrightarrow{k_1} \text{HRP-Fe(III) + DH}^+ + \text{H}^+ \] (3)

\[ 2\text{DH} \rightarrow \text{HD-DH or D + DH}_2 \] (4)

*This work was supported by research grants from the United States Army Research Office, the Council for Tobacco Research U. S. A., Inc., the National Science Foundation, and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The rate constant k₁ is much greater than k₇, and the overall process may be made dependent upon k₇ when [H₂O₂] is rate-limiting or upon k₁ when the concentration of the electron donor substrate (DH₃) is rate-limiting.

We have previously reported (3) that the peroxidation of anilino substrates, such as dianisidine or p-phenylenediamine, by HRP could be markedly stimulated (boosted) by a variety of nitrogenous compounds such as ammonia, imidazole, pyridine, and alkylamines; whereas the peroxidation of phenolic substrates, such as guaiacol, was unaffected. A recent report of the boosting effect (2) pointed to the importance of the pKₐ of some activity-limiting ionizable group being raised. Boosting by nitrogenous compounds required an unshared pair of electrons on nitrogen since ammonia and the mono- and, trimethylamines were all active, but tetramethylammonium was not. More recently, the boosting effect of variously substituted imidazoles has similarly been explored (4, 5), but the mechanism of this effect remains a mystery. We now describe further studies of this phenomenon and propose a mechanism.

MATERIALS AND METHODS

HRP (RZ = 1.88, 241 units/mg) was purchased from Cooper Biomedical, Inc. and was either used as received or purified to RZ 3–3.6 by chromatography over Bio-Gel A–0.5m. Purification did not affect responsiveness to nitrogenous boosters. Acidic isozymes of HRP (types VII and VIII) and a basic isozyme (type IX) were from Sigma. HRP isozyme C (type I–C, RZ = 3.13) was a gift from Toyobo Biochemicals. Microperoxidases 8, 9, and 11 and chloroperoxidase were from Sigma. The catalase/peroxidase (hydroperoxidase I) of Escherichia coli was isolated as previously described (6, 7). Myeloperoxidase was purified from HL-60 cells as described by Anderson et al. (8). The HL-60 cells were generously provided by Dr. W. Lynn. Human hemoglobin (Pentex Biochemicals) was oxidized to the met state with ferricyanide and then dialyzed. Cytochrome c peroxidase was prepared from baker’s yeast (9, 10). It exhibited A40nm/Amnm = 0.85. A stock solution of A40nm = 3.3 was diluted a total of 10⁵-fold when ferrocyanochrome c was used as the electron donor substrate, but only 250-fold when dianisidine was used. o-Dianisidine from Eastman was twice recrystallized from 95% ethanol and was stored in the dark. Imidazole was from Aldrich, and H₂O₂ was from Mallinckrodt Chemical Works. The concentration of solutions of H₂O₂ was based upon E₂₄₀nm = 43.6 m⁻¹ cm⁻¹ (11). Other reagents were commercially available and of analytical grade.

The peroxidation of dianisidine was followed at 28°C at 400 nm in reaction mixtures containing 50 mM buffer, 0.52 mM H₂O₂, 0.36 mM dianisidine ± boosters. The concentration of peroxidase was varied to compensate for the effect of boosting compounds so that a convenient rate was measured in all cases. Buffering was achieved with sodium acetate (pH 2.3–5.5), sodium phosphate (pH 5.8–8.0), sodium pyrophosphate or borate (pH 8.5–10.0), and sodium carbonate (pH 10 and above).
RESULTS

Do Boosters Influence Binding of Substrate?—Since boosting was seen only when \( k_i \) was rate-limiting, i.e. when \( \text{H}_2\text{O}_2 \) was present in saturating amounts and [dianisidine] was rate-limiting, it appeared possible that boosters increased the affinity of HRP compound II for dianisidine. The effect of varying [dianisidine] in the absence and presence of imidazole was therefore examined. Fig. 1 which presents the results on reciprocal coordinates, demonstrates that imidazole, while sharply increasing the activity of the enzyme, did not increase its apparent affinity for dianisidine. The parallel pattern of lines in Fig. 1 suggests that imidazole could bind to HRP only in the presence of the dianisidine and that it caused parallel increases in the \( K_a \) value for dianisidine and \( V_m \).

Is Boosting a General Property of Dianisidine Peroxidases?—The effect of imidazole on the peroxidation of dianisidine was examined when HRP was replaced by a variety of catalysts of this reaction. Since, in the case of HRP, the boosters have the effect of shifting the alkaline limb of the activity versus pH profile (3) and since the optimum pH was likely to differ from one catalyst to the next, we examined each catalyst over a range of pH. Myeloperoxidase catalyzed the peroxidation of dianisidine over a wide range of pH values, with optima at pH 5.5 and 9.0; and imidazole exerted only marginally significant effects on its activity at any pH, as shown in Fig. 2. HRP was compared with \( E. \) coli hydroperoxidase I; and as shown in Fig. 3, HRP was much more susceptible to boosting by imidazole than was hydroperoxidase I. Thus, lines 1 and 4 in Fig. 3 are the activity versus pH profiles for HRP without and with imidazole, respectively; whereas lines 2 and 3 are the corresponding profiles for hydroperoxidase I. Methemoglobin can catalyze the peroxidation of dianisidine, albeit not as efficiently as does HRP, and the data in Fig. 4 demonstrate that imidazole slightly suppressed the activity of methemoglobin over most of the pH ranges examined. The effects of imidazole on the activities of chloroperoxidase and microperoxidases 8, 9, and 11 were examined at pH 8.0 and 10.0; and as shown by Fig. 5, in no case was boosting seen. The peroxidation of ferrocychrome c by the yeast cytochrome c peroxidase was also explored. A sharp optimum in activity was noted at pH 5.0, but imidazole at 10.0 mM exerted only small effects on the alkaline limb of the activity versus pH profile. These results are shown in Fig. 6. Cytochrome c peroxidase is also able to catalyze the peroxidation of dianisidine, albeit not as well as it does that of cytochrome c. This was examined at pH 8.0 and 9.0 without and with 10 mM imidazole. As shown in Fig. 7, imidazole boosted approximately 3-fold at pH 8.0, whereas there was very little activity at pH 9.0 with and without imidazole. We may note by way of comparison that this level of imidazole stimulates HRP approximately 50-fold at pH 9.0. HRP exists as a family of isozymes (12). It should also be noted that acidic (Sigma type VIII) and basic (Sigma type IX) isozymes of HRP exhibited

![Fig. 1. Effect of imidazole on kinetics of peroxidation of dianisidine by HRP. Reaction mixtures contained 50 mM potassium phosphate, 1.0 mM H\(_2\)O\(_2\), 0.3 mM HRP, the indicated concentrations of dianisidine, and the following concentrations of imidazole (all at pH 8.0 and 25°C): line 1, none; line 2, 50 mM; line 3, 250 mM. Initial rates, measured at 460 nm, are presented here as a function of [dianisidine] on reciprocal coordinates.](image1)

![Fig. 2. Effect of imidazole on peroxidation of dianisidine by myeloperoxidase. Reaction mixtures contained myeloperoxidase, 50 mM buffer, 0.4 mM H\(_2\)O\(_2\), and 0.36 mM dianisidine with (C) or without (●) 12 mM imidazole at the indicated pH and at 25°C. The buffers used were as described under "Materials and Methods."](image2)

![Fig. 3. Effects of imidazole on peroxidation of dianisidine by HRP and \( E. \) coli hydroperoxidase I. Reaction conditions were as described for Fig. 2 except that the catalysts were HRP without imidazole (line 1), HRP + 12 mM imidazole (line 4), hydroperoxidase I without imidazole (line 2), and hydroperoxidase I + 12 mM imidazole (line 3).](image3)

![Fig. 4. Effect of imidazole on peroxidation of dianisidine by methemoglobin. Reaction mixtures were as described for Fig. 2 except that H\(_2\)O\(_2\) was at 0.8 mM, and the catalyst was methemoglobin at 46 μg/ml. Line 1, no imidazole; line 2, 10 mM imidazole.](image4)
Activation of Horseradish Peroxidase

**Fig. 5.** Effect of imidazole on peroxidation of dianisidine by chloroperoxidase and microperoxidases. *Upper*, reaction mixtures contained 50 mM potassium phosphate, 0.52 mM H$_2$O$_2$, 0.36 mM dianisidine, and enzyme ±15 mM imidazole at pH 8.0 and 25°C. *Line a*, HRP; *line a’,* HRP + imidazole; *line b*, microperoxidase 8; *line b’,* microperoxidase 8 + imidazole; *line c*, chloroperoxidase; *line c’,* chloroperoxidase + imidazole. *Lower*, reaction conditions were same as described (upper), except that 50 mM pyrophosphate (pH 10.0) was used. *Lines a-c* represent the tracing of activity of microperoxidases 8, 9, and 11 in the absence of imidazole, respectively; *lines a’-c’* represent the corresponding tracings in the presence of 15 mM imidazole.

**Fig. 6.** Effect of imidazole on peroxidation of ferrocytochrome c by cytochrome c peroxidase. Reaction mixtures contained 50 mM buffer, 0.2 mM H$_2$O$_2$, 10 μM ferrocytochrome c, and cytochrome c peroxidase ±10 mM imidazole at the indicated pH and at 25°C. *●*, no imidazole; *○*, plus imidazole.

**Fig. 7.** Effect of imidazole on peroxidation of dianisidine by cytochrome c peroxidase. Reaction mixtures contained 50 mM buffer, 0.02 mM H$_2$O$_2$, 0.36 mM dianisidine, and enzyme ±10 mM imidazole at pH 8.0 or 9.0 as follows: *line a*, pH 8.0, no imidazole; *line a’,* pH 8.0 + imidazole; *line b*, pH 9.0, no imidazole; *line b’,* pH 9.0 + imidazole.

**Fig. 8.** Effect of imidazole on activation energy for peroxidation of dianisidine by HRP. Reaction mixtures contained 50 mM potassium phosphate, 0.52 mM H$_2$O$_2$, 0.35 mM dianisidine, and 0.8 nM HRP ±15 mM imidazole at pH 7.1 and over a range of temperatures. The log of the initial rate of peroxidation of dianisidine is presented here as a function of reciprocal absolute temperature. *●*, no imidazole; *○*, plus imidazole.

It is apparent, from the above, that HRP is almost unique in its response to nitrogenous compounds. Of the several catalysts of the peroxidation of dianisidine which were examined, HRP showed, by far, the greatest boosting by imidazole with cytochrome c peroxidase a poor second. It follows that boosting by nitrogenous compounds reflects some feature of the active site of HRP, rather than an interaction with an intermediate common to the peroxidation mechanism, however catalyzed.

**Does Boosting Involve a Change of Mechanism?**—One way to approach this question would be to measure energy of activation in the absence and presence of imidazole. The data in Fig. 8, plotted according to the Arrhenius equation, in which case the slope is 2.3 times the energy of activation, demonstrate that the boosting effect of imidazole is not accompanied by a change in energy of activation and therefore in enthalpy of activation. It follows that boosting compounds do not make available a new reaction pathway, with a lower energy of activation, but rather allow the same mechanism,
which occurs optimally at pH 5.5, to occur over a wider range of pH. Arrhenius plots of data collected at pH 6.5, 7.1, and 8.5 demonstrated that the energy of activation for the peroxidation of dianisidine by HRP was invariant with pH in this range (data not shown).

Is the Peroxidation of Phenols Unresponsive to Boosters?—We had previously noted that peroxidation of a phenol, such as guaiacol, by HRP was unresponsive toward boosters (3). This was re-examined using phenols chosen to vary considerably in acidity and in standard redox potential. The peroxidation of these phenols was examined over a wide range of pH with and without imidazole. The data in Fig. 9 demonstrate that HRP-catalyzed peroxidation of phenols was entirely unresponsive toward the boosting effect of imidazole. The data also show that the activity versus pH profiles for the oxidation of phenols extended much further into the alkaline range than was the case for the peroxidation of dianisidine. Indeed, the profiles for the peroxidation of phenols resembled that seen with dianisidine in the presence of imidazole (see Fig. 9).

Nucleophilicity and Steric Factors in Boosting—A variety of compounds were examined for their ability to boost the peroxidation of dianisidine by HRP. Thiocyanate and iodide, tested at 10 mM, were totally ineffective; and acetate was only marginally active (data not shown). Several nitrogenous compounds were tested at 12 mM and were found to differ markedly in their activities. Thus, as shown in Table I, imidazole was most effective, followed by pyridine and dipyridyl; whereas bipiperidine and quinuclidine were inactive.

Because an unshared pair of electrons on nitrogen appears essential for boosting (3), only the free base form of nitrogenous boosters should be active. Compounds with different basicities would therefore appear to differ in effectiveness merely on this basis. There is also a possible distinction between maximum boosting, seen at an effectively infinite concentration of the boosting agent, and its apparent affinity for HRP, as reflected in the concentration needed to achieve half of the maximum boosting. When the increment in activity was plotted as a function of the concentration of the boosting compound on reciprocal coordinates, straight lines were obtained whose extrapolated ordinate intercept gave the maximum boost and whose slope divided by the ordinate intercept gave the concentration of booster needed for half-maximal boosting (Kb) so obtained reflects both the free base and the conjugate acid forms of the booster, and this could be corrected to the value for the free base form by applying the Henderson-Hasselbalch equation.

Data collected in this way are presented in Table II. When maximum boosting is considered, the compounds which were very active were all comparable. These active compounds were imidazole, pyridine, ammonia, cyclohexylamine, and trimethylamine; whereas piperidine was substantially less active. When apparent affinity of the free base form of the

**Table I**

Effect of nitrogenous compounds on peroxidation of dianisidine by HRP.

| Addition | Dianisidine peroxidation rate |
|----------|-------------------------------|
| None     | 0.07                          |
| Imidazole| 1.97                          |
| Pyridine | 0.34                          |
| 4,4'-Dipyridyl hydrate | 0.32                      |
| Quinuclidine HCl | 0.07                     |
| 4,4'-Bipiperidine 2-HCl | 0.09                     |

**Table II**

Kinetic constants for boosting compounds.

| Compound            | Vmax * | pKb  | [AH+] | [A]  |
|---------------------|--------|------|-------|------|
| None                | 0.7    |      |       |      |
| Imidazole           | 80     | 7.1  | 8.3   | 8.3  |
| Pyridine            | 37     | 5.2  | 267   | 267  |
| Ammonia             | 51     | 9.2  | 17    | 14   |
| Cyclohexylamine     | 46     | 10.6 | 348   | 64   |
| Trimethylamine      | 98     | 9.7  | 3200  | 2060 |
| Triethylamine       | 0.7    |      |       |      |
| Piperidine          | 11     | 11.2 | 150   | 9    |
| Phosphite           | 0.7    |      |       |      |
| Hypophosphite       | 0.7    |      |       |      |

*Values are expressed as (moles of dianisidine/mole of HRP/minute) × 1000. Reaction mixtures contained 50 mM sodium carbonate, 0.36 mM dianisidine, 0.5 mM H2O2, 0.8 mM HRP, and a range of concentrations of the compounds tested, all at pH 10.0 and 25°C. Initial rates were followed at 460 nm. Increments in rate were plotted as a function of the concentration of the boosting compound on reciprocal coordinates, and Vmax and Kb were calculated from the resultant straight lines.

**Fig. 9. Effects of pH and imidazole on peroxidation of phenols by HRP.** Reaction mixtures contained 50 mM buffer (pH 8.2), 8 × 10^-4 M H2O2, 3.6 × 10^-4 M dianisidine, and 0.8 mM HRP. Nitrogenous compounds were added to a final concentration of 12 mM.
boosting agent for HRP was considered, large differences were apparent. It thus appears that apparent affinity varies from booster to booster, much more than do their maximal effects; and there was no obvious relationship between $pK_a$ and $K_m$.

**Role of Calcium—**HRP has been reported to contain tightly bound Ca$^{2+}$ which, in the case of isozyme C, could be removed by treatment with guanidinium chloride plus EDTA, with loss of approximately half of the enzymic activity, and which could subsequently be replaced, with restoration of activity (13–16). Since Ca$^{2+}$ removal was reported to decrease $k_4$ when measured at pH 7.0, but not at pH 4.4 (15), it appeared possible that ligations of boosting compounds to this Ca$^{2+}$ on the enzyme might be involved in their stimulatory activity. Treatment of HRP isozyme C with guanidinium chloride ± EDTA, followed by dialysis, as described (13–16), did cause loss of activity; but a proportional loss of heme was also evident, in terms of a decrease in absorbance at 403 nm. Moreover, the lost activity could not be restored by treatment with Ca$^{2+}$, and the residual activity of the Ca$^{2+}$-depleted enzyme was as susceptible to boosting by imidazole as was the native enzyme. Atomic absorption spectrophotometry was used to verify that the treatment with guanidinium chloride did result in calcium removal from the HRP. These data are summarized in Table III. It is not clear why we failed to achieve selective removal of Ca$^{2+}$ by the published procedure, but the parallel loss of heme is not surprising in view of the harsh treatment (6.0 M guanidinium chloride) used in this procedure. The activity which survived this treatment was as responsive to boosting by imidazole as was the native enzyme.

**Do Boosting Compounds Shift the $pK_a$ of an Activity-limiting Ionization?**—We previously suggested (3) that the action of nitrogenous boosters was to raise the $pK_a$ of an activity-limiting ionization. At that time, the only booster which was examined over a wide range of pH was imidazole. It appeared possible that nitrogenous bases with very different $pK_a$ values might shift the activity-limiting ionization to different degrees. Pyridine and ammonia differ markedly in $pK_a$ and in the concentration of the free base form required for half-maximal boosting. Accordingly, the boosting actions of ammonia and pyridine were examined from pH 6 to 11. At each pH, the concentration of booster was varied so that the boosting effect could be extrapolated to provide values of the maximum rate possible, as described for Table II. The data presented in Fig. 10 indicate that ammonia and pyridine shifted the apparent activity-limiting ionization in the same way and to the same limit.

![Graph](image)

**FIG. 10. Effect of pH on boosting by ammonia or pyridine.** Reaction mixtures contained 0.5 mM H$_2$O$_2$, 0.36 mM dianisidine, 50 mM buffer, and HRP as described under “Materials and Methods” at the indicated pH and at 25 °C. The boosting compounds were tested over a range of concentrations, and initial rates were plotted as a function of the concentration of the boosting compound on reciprocal coordinates. The lines so generated were extrapolated to $\pm$ boosting compound. The resultant maximum velocities are presented here as a function of pH. $\bullet$, no boosting compound; $\Delta$, plus ammonia; $\Delta$, plus pyridine.

### DISCUSSION

Compound I of HRP is thought to contain an Fe(IV)=O in a heme $\pi$-cation radical (17–21), and donation of an electron to compound I reduces the $\pi$-cation radical, leaving an Fe(IV)=O heme, as compound II (20, 22–27). The rate of reduction of compound II by electron donor substrates (i.e. $k_4$) decreases with increasing pH (28–32), and this effect of pH seems due to titration of a catalytically important residue, rather than to changes in the net charge on the enzyme (33). One histidine residue is thought to be ligated to the heme iron and is referred to as the proximal histidine. Another histidine residue appears to be hydrogen-bonded to the ferryl oxygen atom of compound II and is called the distal histidine (34–36). Hydrogen bonding from the imidazolium ring of the distal histidine facilitates rapid exchange of the ferryl oxygen with water, and the rate of this exchange correlates with the rate of the catalytic process (34). Absence of hydrogen bonding to the ferryl oxygen, as shown by lack of an effect of pH on the Fe(IV)=O stretching frequency, is seen with metmyoglobin and correlates with a lack of significant peroxidase activity (35).

It thus appears that hydrogen bonding from the distal imidazolium to the ferryl oxygen of compound II is important for the reduction of compound II by the electron donor substrate, and the hydrogen from the distal imidazolium probably leaves with the ferryl oxygen as OH$^{-}$ when electron transfer from the donor substrate has occurred. The decrease in $k_4$ with increasing pH may thus be attributed to ionization of the distal imidazolium. Since the effect of nitrogenous boosters was seen only on the alkaline limb of the activity versus pH curve, these compounds must elevate the $pK_a$ of the distal imidazolium. They might exert this effect by hydrogen bonding to it, as shown in Fig. 11A. In this figure, pyridine is shown hydrogen-bonded to the distal imidazolium of compound II, and the electron donor substrate (aniline) is shown donating an electron via the heme edge. The electron from the aniline donor substrate would be conducted to the ferryl oxygen through the heme, and the hydrogen-bonded hydrogen from the distal imidazolium would then leave with that oxygen as OH$^{-}$.

This scheme can account for the boosting of the peroxidation of dianisidine and other anilino substrates by nitrogenous

| Treatment* | Ca$^{2+}$/HRP | $A_{440}$ nm * | Relative activity* | Boosting* |
|------------|--------------|----------------|-------------------|-----------|
| None       | 2.1          | 0.23           | 100               | 12        |
| Guanidinium chloride | 0.4          | 0.04           | 46                | 12        |
| Guanidinium chloride plus EDTA | 0.6          | 0.05           | 16                | 11        |
| Reconstitution with Ca$^{2+}$ | 0.5          | 0.03           | 20                | 14        |

*Removal of Ca$^{2+}$ and reconstitution with Ca$^{2+}$ were performed as described by Haschke and Friedhoff (13).

*$A_{440}$ nm was recorded as a measure of heme content.

*Peroxidase activity was assayed in reaction mixtures containing 50 mM potassium phosphate, 0.52 mM H$_2$O$_2$, 0.36 mM dianisidine, ± 10 mM imidazole at pH 8.0 and 25 °C.

*Boosting refers to the ratio of the peroxidase activities seen in the presence of imidazole to that seen in its absence.
compounds, but not for the lack of boosting seen with phenolic substrates. Paul and Ohlsson (37) have suggested that substrates for HRP fall into two groups, i.e. phenolic and anilino, with the former class of substrates exhibiting greater affinity for the enzyme. They interpreted this as being due to hydrogen bonding between the phenol and the enzyme. Roles for hydrophobic interactions and for hydrogen bonding in the binding of phenols to HRP were also proposed by Schejter et al. (38). In studies of the reaction of phenols with HRP compound II, Dunford and Adeniran (39) noted that p-amino-phenol did not fit the Hammett plot for phenols and suggested that its preferred binding orientation favored electron donation from the amino, rather than from the phenolic, group.

We apply these findings to our data by suggesting that phenolic substrates displace the distal histidine from the ferryl oxygen and, in turn, hydrogen-bond directly to that oxygen, as depicted in Fig. 11B. This displacement of the distal histidine has the consequence that ionization of its imidazolium is no longer rate-limiting. Hence, the enzyme should be active over a wider range of pH with phenolic substrates than with anilino substrates; and the reduction of HRP compound II by phenols should not be subject to boosting by nitrogenous compounds which can hydrogen-bond to the distal imidazolium since its pKₐ is no longer relevant.

Fig. 11 appears to accommodate the available data, yet one would expect that nitrogenous boosters of different basicity should shift the pKₐ of the distal histidine to different degrees. The data in Fig. 10, however, show that ammonia and pyridine appeared to shift the activity-limiting ionization to the same degree. This apparent discrepancy between the expectations from the proposed mechanism and the data can be accommodated. Thus, suppose that both of these boosters shift the pKₐ of the distal histidine to such a high value that ionization of one or more other groups then becomes rate-limiting before titration of the distal histidine occurs.

Fig. 11 explains the kinetic data shown in Fig. 1. Thus, the compound II form of HRP would be produced only in the presence of H₂O₂ plus dianisidine. Since it is only this form whose catalytic activity should be increased by the booster, it would appear that the booster (imidazole) could bind only in the presence of the donor substrate. Moreover, since a rate-limiting step of the catalytic cycle other than substrate binding was increased by the boosting compound, one would expect a parallel increase in Km for dianisidine and Vmax. Fig. 11 also explains the constancy of energy of activation ± booster, which was shown in Fig. 8. Thus, if the booster raises the effective pKₐ of the distal imidazole, the reaction pathway and the activation energy would be unchanged by its presence. In effect, at any given pH in the alkaline range, the booster increases the fraction of HRP compound II whose distal histidine is protonated and so increases rate, but does not change mechanism. The inactivity of some nitrogenous bases, such as quinuclidine and bipiperidine (Table I), and the very wide range of apparent affinities of boosters for HRP compound II (Table II) could be explained in terms of ease of access of these compounds to the active site.

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