Effects of Imidazoles and pH on the Peroxidase Activity of the Hemin−Hydrogen Peroxide System

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The reaction of hydrogen peroxide with protohemin has been examined in aqueous solution (pH 5−10) with potassium ferrocyanide as a substrate. Kinetic studies were carried out in the presence and absence of imidazole bases (B: imidazole, 1-methylimidazole, 2-methylimidazole, 4-methylimidazole, and 1,2-dimethylimidazole). The apparent second-order rate constant (k_{app}) for the formation of ferricyanide was used as the index of the peroxidase activity. In the absence of bases, hemin showed weak and constant activity at alkaline pH but has lost it at acidic pH. A plot of log k_{app} vs pH has revealed that the association of one H^+ equivalent with apparent $pK_a = 6.9$ has inhibited the reaction. Along with the pH-dependency of the absorption spectra of ferric hemin, it was suggested that the hydroxide coordination to the ferric hemin is essential to the generation of the peroxidase activity. On the other hand, addition of bases resulted in an enhancement of the reaction rate at alkaline pH. This effect was well accounted for by the mono-ligation of a base to the iron atom in the high-valent porphyrin intermediate(s). However, the reaction was found to be inhibited in the presence of large excess of unhindered bases, and the inhibition was strongly correlated to the formation of bis-coordinated ferric hemin. Therefore, bis-ligating bases have prevented the access of H_2O_2 to the ferric hemin and the subsequent formation of the intermediates. At acidic pH, the bis-coordination was promoted and the unhindered bases were less effective activators. Addition of sterically hindered 2-methylimidazole, by contrast, resulted in the higher activity at acidic pH than that in the base-free system. This may suggest that oxo-ligand in the intermediate was activated by the hydrogen bonding of the protonated 2-methylimidazole. A reaction scheme that satisfies the above criteria is proposed, and the correlation with the reactions of heme enzymes is discussed.

Introduction

Reaction mechanisms of metalloporphyrins with various oxidants have received considerable attention since they are relevant to the catalytic mechanisms of heme enzymes, such as peroxidases,[1] catalases,[2] and cytochrome P-450s,[3] all of which contain iron(III) protoporphyrin IX (hemin) at their active centers. Intensive studies on the peracid,[4−6] iodosobenzene,[6−9] and hypochlorite[10] oxidations of iron(III) porphyrin complexes have been done, and it is generally agreed that these oxidants transfer an oxygen atom to the iron(III) porphyrin to form an iron(IV)−oxo porphyrin π-cation radical species, which is relevant to the compound I intermediate of horseradish peroxidase (HRP[11]) and catalase formed upon reaction with H_2O_2.[1,2]

On the other hand, oxidation mechanism of iron(III) porphyrin by hydroperoxides has been a subject of much debate. Considerable evidence has been offered[7b,12] in support of the one-electron oxidation of iron(III) porphyrin (homolytic O−O bond cleavage) to provide iron(IV)−oxo porphyrin species, which is relevant to the compound II intermediate of HRP and is ineffective in epoxidizing alkenes.[13] Other kinetic and product studies on the epoxidation of alkenes by hydroperoxides and iron(III) porphyrin have offered evidence in support of heterolytic mechanism.[5,8,14]

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(11) Abbreviations used: HRP, horseradish peroxidase; DMF, dimethylformamide; PP, protoporphyrin IX; OEP, octaethylporphyrin; TPP, tetraphenylporphyrin; ImH, imidazole; 4-MeImH, 4-methylimidazole; 1-MeIm, 1-methylimidazole; 2-MeImH, 2-methylimidazole; 1,2-Me_2-Im, 1,2-dimethylimidazole; 3,4-Me_2py, 3,4-dimethylpyridine, 4-Me_2-Npy, 4-(dimethylamino)pyridine.
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The rate enhancement brought about by the heme enzyme, compared with iron porphyrin models, has been explained in part by general acid[15] and general base catalysis.[5a] This is supported by the X-ray structure analysis of cytochrome *c* peroxidase,[16] and hence water molecule should play a key role in the oxidation reaction of the enzyme. However, mechanistic studies of ferric porphyrin models in water[15,17] have been complicated by their low solubility in this solvent and their tendency to aggregate[18] and to form a μ-oxo dimer species.[19] These problems were circumvented by employing methanol as solvent,[5,8b] but it seems hard to determine precisely the proton activity and acid dissociation constants of starting species and intermediates in such an organic solvent.

Recently, mechanistic studies in aqueous solution have been done[20] on the oxidations of water-soluble and non-μ-oxo dimer-forming TPP derivatives with 2,6-dichloro-3-sulfonato and 2,6-dimethyl-3-sulfonato substituents on the phenyl periphery. These derivatives were comprised of at least three of four possible atropisomers,[20j] and all the atropisomers possess indistinguishable pK_a values; thereby the electronic nature of the isomers would be the same.[20k] However, it was shown[7c] that the nature and location of the phenyl ring substituents affect markedly the yields and product distributions in the oxidation of hydrocarbons catalyzed by iron TPP derivatives. In addition, the electron-withdrawing power of the substituents was shown to affect the electronic structures of oxo−iron(IV) porphyrin π-cation radicals.[21]

To best understand the reaction mechanism of the heme enzymes, therefore, detailed mechanistic studies are necessary in water with naturally occurring iron porphyrin, i.e., hemin. We have employed a solvent system containing a minimal amount (0.1%) of DMF in water to prevent the aggregation of the hemin. We could successfully indicate the effects of pH and imidazole derivatives on the peroxidase activity of the hemin in this solvent system. A reaction scheme that satisfies the kinetic results, as well as the spectrophotometric results of the ferric hemin, is proposed, and the correlation with the reaction mechanisms of the heme enzymes will be discussed.

Experimental Section

Materials. Deionized, glass-distilled water was further purified by reverse osmosis (EASYpure, Barnstead) and used for all experiments. Hemin chloride (equine, from Sigma) and potassium ferrocyanide (Wako Chemicals) were used without purification. The concentration of the hemin was determined by using a millimolar extinction coefficient (ϵ_{mM}) of 34.4 at 557 nm for its pyridine hemochrome.[22] The concentration of hydrogen peroxide (Mitsubishi Kasei) was determined spectrophotometrically (ϵ_M = 39.1 at 240 nm).[23] The ferrocyanide and hydrogen peroxide solutions were prepared just prior to use. ImH, 2-MeImH, and 4-Me_2Npy (Wako Chemicals) were recrystallized from benzene. 4-MeImH, 1,2-Me_2Im, 3,4-Me_2py (Tokyo Kasei), and 1-MeIm (Aldrich) were used as received. Other chemicals used were of reagent grade.

Kinetics. Hemin, potassium ferrocyanide, and bases, if necessary, were successively added into the cuvette containing 0.1% DMF and 50 mM buffer solution. The following buffers were used: sodium citrate (pH 5−6), sodium phosphate (pH 6−8), and sodium borate or sodium pyrophosphate (pH 8−10). Hydrogen peroxide was then added by syringe to the solution to initiate the reaction. The cell compartments were thermostated at 25.0 ± 0.1 °C throughout the measurements. The initial rates (V_0) were determined by the appearance of the 420-nm absorbance of the ferricyanide (ϵ_{mM} = 1.04)[24] after addition of hydrogen peroxide with Shimadzu UV-2100 spectrophotometer.

Titrations. Ferric hemin (ca. 0.1 mM) was titrated with imidazole derivatives (ImH, 1-MeIm, 2-MeImH, 4-MeImH) at desired pH in the buffer systems described above, which contained 10% DMF. The pH values were not corrected for DMF. The pH of the reaction mixtures was checked before and after each titration experiment, and was identical within 0.1 pH units. The absorbance changes at about 540 nm were monitored, and the base concentration ($[B]_{mid}$) that was necessary to half-produce the final hemin complex and the number of base molecules (n) of concern in the binding reaction were obtained by following the procedure given elsewhere.[25] The pH dependency of the ferric hemin was also examined by the addition of small aliquots of sodium hydroxide solution to the mixture containing ferric hemin, 10% DMF, and 50 mM sodium phosphate buffer. The pK_a value of the ferric hemin (pK_{a1}) was determined spectrophotometrically at 596 nm.

Results

Initial Rates. In Figure 1 is shown the dependency of V_0 on the hemin (catalyst), H_2O_2 (oxidant), and ferrocyanide (substrate) concentrations. As seen in each panel, V_0 values were greatly enhanced by the addition of ImH at pH 10.0, although at pH 6.4, the V_0 values were low and the effect of ImH was less apparent. It is clear that V_0 is linearly correlated to the hemin and H_2O_2 concentrations irrespective of pH or the existence of ImH. In the presence of ImH at pH 10.0, however, the V_0 values began to deviate from the linear correlation to the hemin concentration above 2 μM (Figure 1, top), which may be attributed to the dimerization of the ferric hemin.[15a] In the absence of either hemin or H_2O_2, the production of ferricyanide was not detected (Figure 1, top and middle). Therefore,

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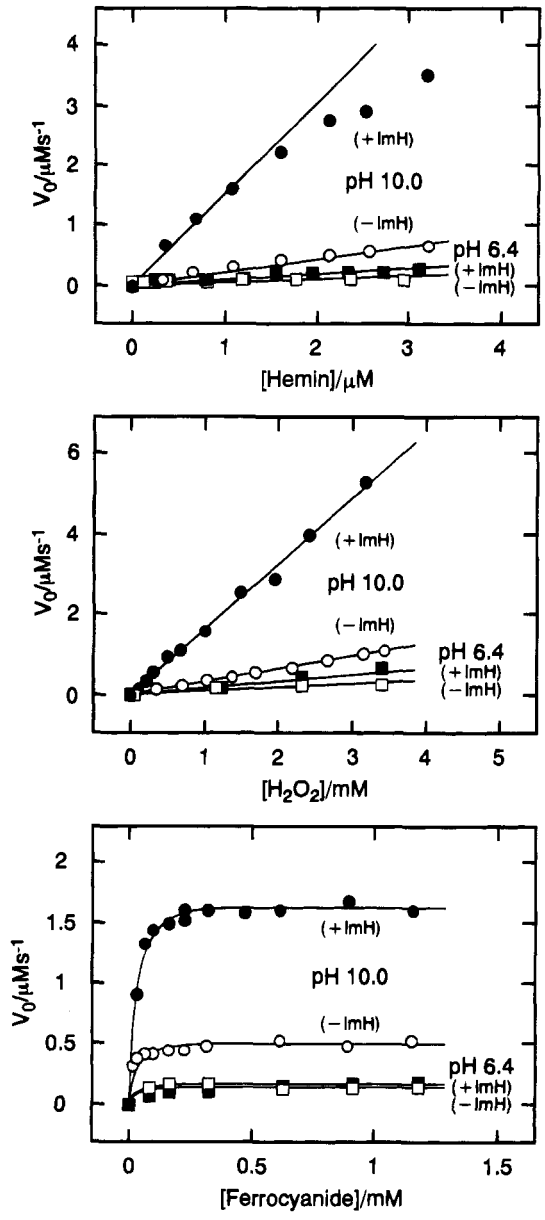

Figure 1. Dependencies of initial rates (V_0) on the concentrations of hemin (top), hydrogen peroxide (middle), and ferrocyanide (bottom). The V_0 values were determined at 25 °C in the absence (open circles) or presence (closed circles) of 10 mM ImH in 50 mM sodium borate buffer (pH 10.0), and in the absence (open squares) or presence (closed squares) of 3.0 mM ImH in 50 mM sodium phosphate buffer (pH 6.4). Standard reaction mixture contained 1.0 μM hemin, 1.0 mM hydrogen peroxide, and 300 μM ferrocyanide, and the concentrations were varied as shown in respective panels. All the reaction mixtures contained 0.1% DMF.

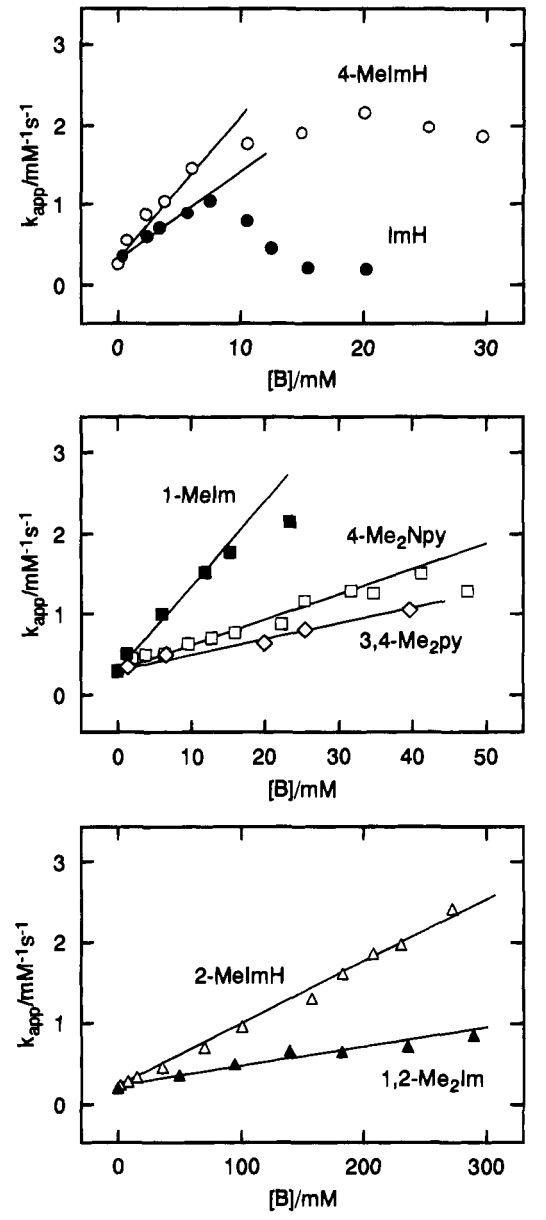

Figure 2. Dependencies of k_{app} on the base concentrations. The added bases were 4-MeImH (open circles), ImH (closed circles), 1-MeIm (closed squares), 4-Me_2Npy (open squares), 3,4-Me_2py (open diamonds), 2-MeImH (open triangles), and 1,2-Me_2Im (closed triangles). Standard reaction mixture was used as in Figure 1.

autooxidation of ferrocyanide did not occur, and the hemin and H_2O_2 are both indispensable to the generation of the peroxidase activity.

On the other hand, V_0 value was found to be saturated on increasing the ferrocyanide concentration irrespective of pH or the existence of ImH (Figure 1, bottom). This indicates that kinetically active intermediate(s) is present during the catalytic cycle. The saturation occurred where ferrocyanide concentration was greater than 0.2 mM in every case, although the V_0 value at the respective plateau region depended on the reaction conditions. On the basis of these results, we typically employed the following reaction conditions: [hemin] = 1 μM, [H_2O_2] = 1 mM, and [ferrocyanide] = 0.3 mM, where V_0 values depend linearly on both [hemin] and [H_2O_2], and are independent of [ferrocyanide]. The apparent second-order rate constant (k_{app}) was obtained by dividing the V_0 value by the initial concentrations of hemin ($[E]_0$) and hydrogen peroxide ($[S]_0$)

$$k_{app} = V_0/([S]_0[E]_0) \quad (1)$$

and was used as the index of the peroxidase activity.

Effect of Bases on k_{app}. Since the V_0 values were greatly enhanced by the addition of ImH at pH 10.0 but not at pH 6.4 (Figure 1), we have studied intensively the effect of bases on the k_{app} at pH 10.0. As seen in Figure 2 (bottom), k_{app} increased linearly to the concentrations of 2-MeImH and 1,2-Me_2Im, both of which possess a 2-methyl substituent on the imidazole ring. The linearity was kept even when the concentrations of the two bases were raised up to 300 mM. On the other hand, addition of ImH first increased the k_{app} value linearly to [ImH] (promotion process) and then it decreased (inhibition process) (Figure 2, top). A similar tendency was also observed in the case of

Table 1. Effects of Bases on the Peroxidase Activity of Hemin

bases	pK_a	pH	k_{app}/[B]/(mM^{-2} s^{-1})[a]	[B]$_{bp}$/mM[b]
4-Me$_2$Npy	9.70	10.0	0.0316	ND[c]
1,2-Me$_2$Im	7.85	10.0	0.00240	ND
2-MeImH	7.56	10.0	0.00764	ND
1-MeIm	7.33	10.0	0.105	97.4
4-MeImH	7.22	10.0	0.180	43.8
ImH	6.65	10.0	0.111	9.26
		8.5	ND	2.04
		6.7	ND	5.67
3,4-Me$_2$py	6.46	10.0	0.0195	ND

[a] The values were obtained from the slope of the linear lines in Figure 2. [b] [B]$_{bp}$ denotes the base concentration at the break point in Figure 3 where the log k_{app} value begins to drop. [c] ND, not determined.

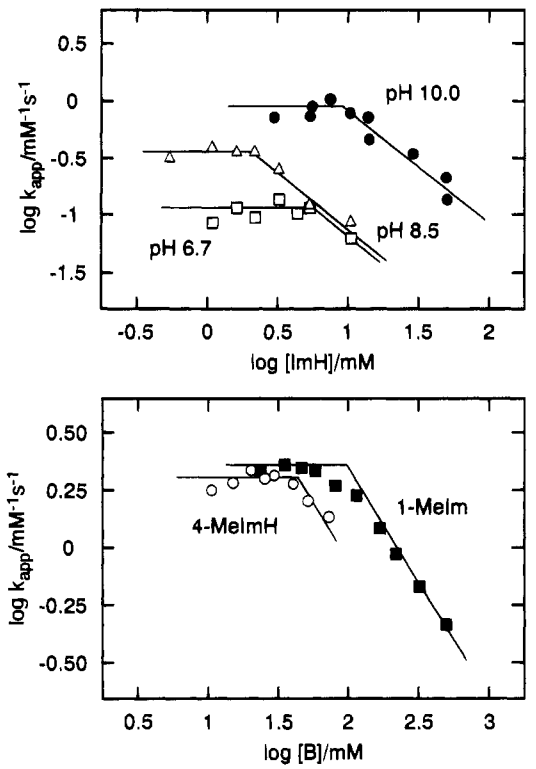

Figure 3. Dependencies of log k_{app} on the log [B] in the inhibition process. The added bases and the pH were 4-MeImH at pH 10.0 (open circles), 1-MeIm at pH 10.0 (closed squares), and ImH at pH 10.0 (closed circles), at pH 8.5 (open triangles), and at pH 6.7 (open squares). The buffers employed were sodium borate (pH 10.0), sodium pyrophosphate (pH 8.5), and sodium phosphate (pH 6.7). Standard reaction mixture was used as in Figure 1.

other unhindered bases. ImH and 4-MeImH (with NH proton) showed maxima of the k_{app} at relatively low concentrations, while 1-MeIm and 4-Me$_2$Npy (without NH proton) had maxima at higher concentrations (Figure 2, middle). The maximum was not apparent in the case of 3,4-Me$_2$py. In Table 1 is summarized the slope of the plots (k_{app}/[B]) where k_{app} depends linearly on the base concentration.

In Figure 3 (upper), log k_{app} was plotted against log [ImH], and the inhibition process was further examined. At pH 10.0, the log k_{app} values were nearly constant around the concentration range of ImH where the k_{app} was maximum (Figure 2, top). The log k_{app} value began to decrease on increasing the ImH concentration with a slope of -1. A similar tendency was also observed in the case of 1-MeIm and 4-MeImH (Figure 3, lower), although occurrence of turbidity has prevented further addition of 4-MeImH above 100 mM. The base concentrations at the break point ([B]$_{bp}$) are summarized in Table 1. The effect of pH on the inhibition process was examined in the presence of

Table 2. Binding Parameters of Bases to the Ferric Hemin and Spectral Data of the Final Products

bases	pH	[B]$_{mid}$[a]/mM	n	λ_{max}[b]/nm	
1-MeIm	10.0	61.6	5	537	564 (sh)[c]
	8.5	3.60	5	540	563 (sh)
	7.0	5.18	5	539	565 (sh)
4-MeImH	10.0	28.7	5	542	569 (sh)
ImH	10.0	10.5	20	545	567
	8.4	0.747	6	545	568
	6.6	1.63	5	545	569

[a] [B]$_{mid}$ denotes the base concentration for the half-production of the final product (Figure 4). [b] Absorption maxima of the final products in the visible range are indicated. [c] sh, shoulder band.

ImH (Figure 3, upper). It was found that the slope of -1 was independent of pH, but the break point was pH sensitive and the [B]$_{bp}$ was in the order: pH 10.0 > pH 6.7 > pH 8.5 (Table 1). Therefore, the inhibition is not a simple process that depends only on the base concentration, but it also depends on the proton activity.

Base Binding to Ferric Hemin. We have examined the binding property of ImH to the ferric hemin to reveal the contribution of ferriheme in the peroxidase reaction cycle. On addition of bases to the ferric hemin solution, the absorption spectra seemed to change with one set of isosbestic points (data not shown), and the spectra of the final products (Table 2) were very close to those of bis-coordinated iron porphyrin complexes.[25b,26] Therefore, the equilibrium reaction was analyzed[25] by assuming a simple one-step binding of n mol of a base to the ferric hemin (PP)

$$PP + nB \overset{\beta_n}{\rightleftharpoons} PP(B)_2$$

$$\beta_n = [PP][B]^n/[PP(B)_2] \quad (2)$$

where β_n is the apparent equilibrium constant for one step binding of the base. [B]$_{mid}$ is defined as the base concentration where [PP] = [PP(B)$_2$], and eq 2 gives β_n = [B]$_{mid}^n$. Since [E]$_0$ = [PP] + [PP(B)$_2$], eq 2 is rewritten as

$$[B]_{mid}^n = ([E]_0 - [PP(B)_2])[B]^n/[PP(B)_2] \quad (3)$$

Logarithm of eq 3 gives, after rearrangement

$$\log ([E]_0/[PP(B)_2] - 1) = n \log [B]_{mid} - n \log [B] \quad (4)$$

The n and [B]$_{mid}$ values were obtained graphically (Figure 4), and are summarized in Table 2. The n values were pH sensitive in the case of ImH, varying from 5 (pH 6.6) to about 20 (pH 10.0). This indicates that number of ImH molecules which concern in the formation of bis-ligated complex increases on increasing pH. However, the n value was 5 in the case of 1-MeIm and 4-MeImH even at pH 10.0 (Table 2). Therefore, the pH dependency of the n value seems to be characteristic only to the ImH.

On the other hand, the [B]$_{mid}$ value is strongly correlated to the [B]$_{bp}$ value. Namely, both of the [B]$_{bp}$ and [B]$_{mid}$ values at pH 10.0 were in the following order: 1-MeIm > 4-MeImH > ImH (Tables 1 and 2). In addition, the [ImH]$_{mid}$ was in the order: pH 10.0 > pH 6.6 > pH 8.4 (Table 2), and this order was the same with that of the [ImH]$_{bp}$ obtained from the kinetic results (Table 1), although the pH values for [ImH]$_{mid}$ and

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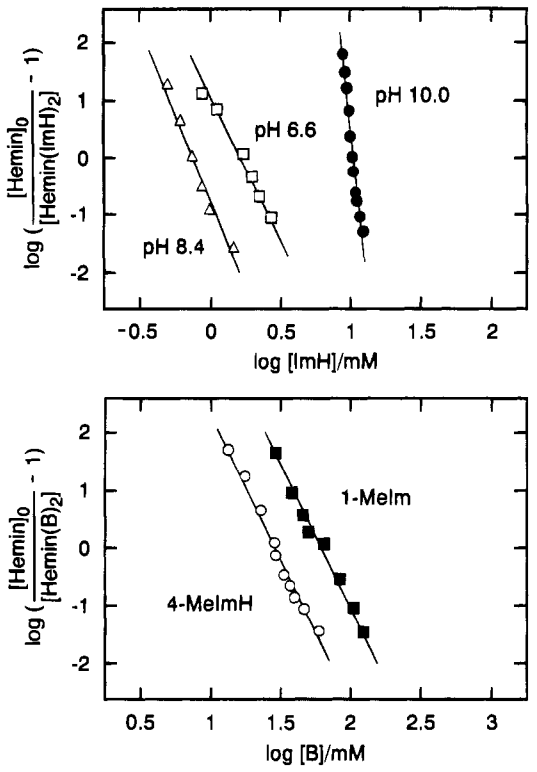


Figure 4. Analysis plots for determining binding parameters of the bases to the ferric hemin. The absorbance data at 542 (4-MeImH), 537 (1-MeIm), and 545 nm (ImH) were used. The added bases and the pH were 4-MeImH at pH 10.0 (open circles), 1-MeIm at pH 10.0 (closed squares), and ImH at pH 10.0 (closed circles), at pH 8.4 (open triangles), and at pH 6.6 (open squares). The buffers employed were sodium borate (pH 10.0), sodium pyrophosphate (pH 8.4), and sodium phosphate (pH 6.6), and all the mixtures contained 10% DMF.

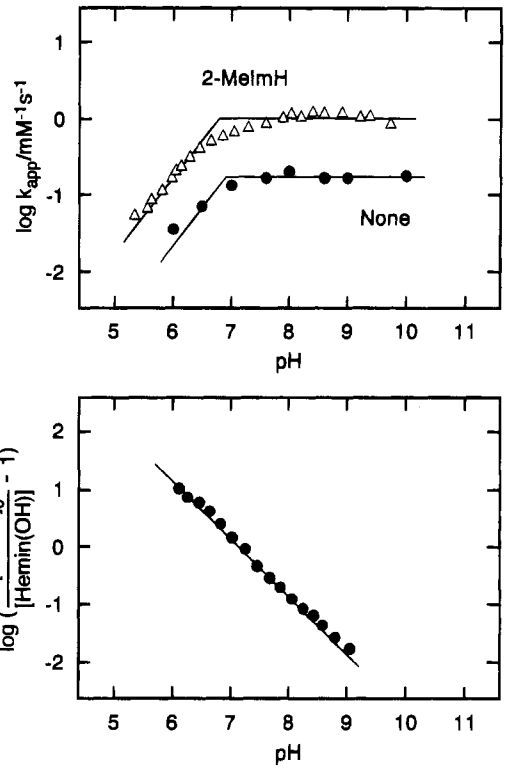


Figure 5. (Upper) Dependencies of log k_{app} on the pH in the presence (open triangles) or absence (closed circles) of 98.3 mM 2-MeImH. Standard reaction mixture was used as in Figure 1. (Lower) Analysis plots for determining the pK_{a1} value of ferric hemin. Absorbance changes of ferric hemin at 596 nm were traced in 50 mM sodium phosphate buffer containing 10% DMF.

$[ImH]_{bp}$ were slightly different. These strongly suggest that the ferric hemin participates in the catalytic cycle, and that the bis-ligation of the bases to the ferric hemin has inhibited the peroxidase activity. The $[B]_{bp}$ values were generally larger than the $[B]_{mid}$ values, which may be attributed to the difference in the DMF content.[26]

pH Dependency. Since hindered 2-MeImH did not inhibit but only promoted the peroxidase activity (Figure 2, bottom), the pH effect was examined in the presence and absence of this base (Figure 5, upper). Hemin was suggested to form μ-oxo dimer species at alkaline pH,[19] and the formation of the dimer was indicated to decrease the peroxidase activity. In the base-free system, however, the log k_{app} values were nearly constant between pH 7 and 10. Therefore, the formation of the dimer species was circumvented in our system. The log k_{app} values were found to decrease by lowering pH below 6.9 with a slope of unity (Figure 5, upper). The addition of 2-MeImH enhanced the k_{app} value by about 10-fold over the pH range examined. This indicates that 2-MeImH has promoted the peroxidase activity of the hemin independent of pH. The pH at the break point was 6.8 in the presence of 2-MeImH.

The pH dependency of the absorption spectra of the ferric hemin was also examined. The equilibrium reaction of the ferric hemin with one mole of proton (H) is expressed as

$$E + H \overset{K_{a1}}{\rightleftharpoons} EH$$

$$K_{a1} = [E][H]/[EH] \qquad (5)$$

where E and EH denote alkaline and acidic forms of the ferric hemin, respectively, and K_{a1} is the acid dissociation constant.

Using the total hemin concentration $[E]_0$ ($[E]_0 = [E] + [EH]$), eq 5 is rewritten as $K_{a1} = [E][H]/([E]_0 - [E])$. Logarithm of this equation gives, after rearrangement

$$\log ([E]_0/[E] - 1) = pK_{a1} - pH \qquad (6)$$

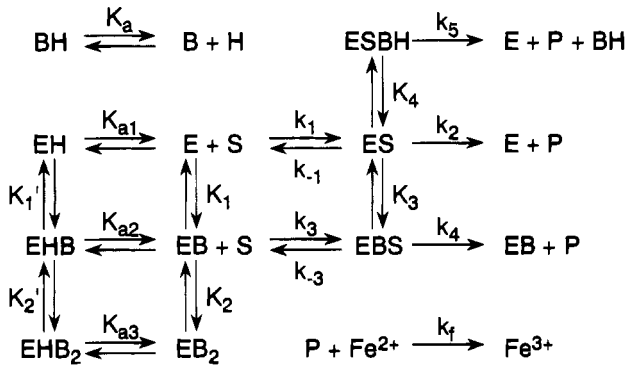
As seen in Figure 5 (lower), the pH dependency was well analyzed by eq 6, and the pK_{a1} was obtained graphically to be 7.1. This value is very close to that of the kinetic break point (pH 6.9). These kinetic and equilibrium studies indicate that the peroxidase activity disappears by the association of one proton equivalent with a pK_{a1} value of about 7. The binding of 2-MeImH to the ferric hemin was also examined between pH 5 and 10, but no spectral change was detected, at least when the concentration of 2-MeImH was increased up to 300 mM (data not shown). Therefore, direct binding of 2-MeImH to the ferric hemin should not be taken into consideration.

Discussion

We have studied the effects of bases and pH on the kinetic and equilibrium properties of the hemin. We now propose the reaction scheme that satisfy the observed results (Scheme 1) and explain the reaction mechanism.

Ferric Complexes. In the absence of bases, ferric hemin was found to be in pH-dependent equilibrium (Figure 5, lower) between acidic and alkaline forms (EH and E in Scheme 1, respectively). The EH and E could be assigned to aquo and hydroxo complexes as shown in Figure 6. The acidic form of the water-soluble ferric porphyrin ($Fe^{3+}P$) was suggested to be diaquo complex, $Fe^{3+}P(H_2O)_2$.[19c,20b] A single deprotonation step to form the hydroxo complex was observed[20b] in a wide pH range (pH 4−14), and the pK_a of the ligating water was

Scheme 1. Proposed Catalytic Cycle of Hemin–Hydrogen Peroxide System[a]



[a] Key: S, hydrogen peroxide; BH and B, acidic and alkaline forms of a base, respectively; Fe^{2+} and Fe^{3+}, ferro- and ferricyanide, respectively; P, product which is responsible for the oxidation of ferrocyanide. For other species, see text. The definitions of equilibrium constants are given in the Appendix.

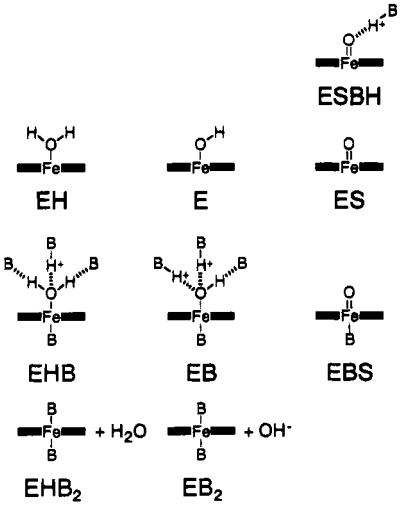


Figure 6. Schematic drawing of the proposed structure of the hemin complexes. The hemin complexes that appear in Scheme 1 are illustrated.

determined to be 7.24.[20b] This pK_a value is close to that obtained in our hemin system ($pK_{a1} = 7.1$). Therefore, the EH complex may also have two aquo ligands, but we have eliminated one of the water molecules for clarity (Figure 6).

In the presence of an unhindered base, bis-ligated ferric hemin (EHB_2 at acidic or EB_2 at alkaline pH) was produced (Table 2). The mono-ligated species (EHB and EB) were not detected spectrophotometrically (Scheme 1). Since the absorption spectra of the bis-complexes were nearly independent of pH (Table 2), EHB_2 and EB_2 should be the same species (Figure 6). In the equilibrium reaction for bis-binding, the $[B]_{mid}$ values for ImH and 1-MeImH were pH dependent (Table 2). This could be well explained by the acid-base equilibria between BH and B, and between EH and E (Scheme 1). At pH 10, the hydroxo ligand is strongly binding to the iron in E, and higher amounts of bases were necessary to displace the hydroxide and to produce the bis-complex (EB_2). At pH about 8.5, EH begins to occur and the weak aquo ligand in EH could be displaced easily by the bases. At neutral to acidic pH, the bases begin to be protonated (BH), and the binding ability is weakened. Therefore, the bases showed the highest affinity to the ferric hemin (and hence the lowest $[B]_{mid}$ value) at pH about 8.5.

On the other hand, the n value was estimated to be 5 to 20 (Table 2), although 2 equiv of bases might be sufficient to produce the bis-complex. Indeed, the n value was usually estimated to be 2 in the binding study of bases to ferric porphyrins in organic solvents.[25b,26] Therefore, the large n value should be attributed to the water solvent in our system. The bases should displace the aquo (in EH) or hydroxo (in E) ligand in a concerted manner, and the cooperativity may be originated from the hydrogen bonding effect in water solvent (Figure 6).

ES Intermediate. In the absence of bases, the peroxidase activity was strongly linked to the hydroxide coordination to the ferric hemin (Figure 5, upper). Since an intermediate species (ES in Scheme 1) was suggested to occur (Figure 1, bottom), this should be produced from E. The reaction cycle assuming the ES intermediate well explains the pH dependency of the activity in the base-free system. By solving the kinetic equations (Appendix eqs A34 and A35), we obtain

$$\log k_{app} = \log\{k_1k_2/(k_2 + k_{-1})\} \text{ for pH} > pK_{a1} \quad (7)$$

$$\log k_{app} = \text{pH} - pK_{a1} + \log\{k_1k_2/(k_2 + k_{-1})\} \text{ for pH} < pK_{a1} \quad (8)$$

At the cross point of eqs 7 and 8, $\text{pH} = pK_{a1}$. The pH at the kinetic break point was in good agreement with the pK_{a1} value of the titration experiments (Figure 5), which supports our reaction scheme. The ES may be a ferryl–oxo complex analogous to compound II in peroxidases (Figure 6) as will be discussed later. This species may oxidize ferrocyanide directly, or indirectly via formation of another oxidant (P in Scheme 1). At present, we tentatively assign this species to hydroxy radical ($HO^{\bullet}$), which was produced by the reaction of ferryl–oxo compound (ES) with nearby water. The hydroxy radical is a potent one-electron oxidant and readily react with ferrocyanide. Therefore, the oxidation step (k_f) should be very fast and may not be rate-determining. A similar mechanism was postulated in the water-soluble iron porphyrin system.[20g]

EBS Intermediate. In the presence of bases at pH 10, the promotion process was observed even in the case of sterically hindered 2-MeImH and 1,2-Me_2Im (Figure 2). Since the binding of 2-MeImH to the ferric hemin was not detected, the ferric hemin does not concern in the promotion process and we have to consider another intermediate, EBS (Scheme 1). In other words, the formation of the EBS species should be responsible for the rate enhancement by bases. By solving the kinetic equations (Appendix eq A41), we could derive the following equation, where $[B]_0 \ll K_1$ and K_2:

$$k_{app} = k_1(k_2 + k_4[B]_0/K_3)/(k_{-1} + k_2) \quad (9)$$

k_{app} linearly depends on base concentration. The observed rate enhancement by bases is consistent with that the bases ligate directly to the iron in the EBS intermediate as discussed below.

The effect of bases on the rate enhancement can be examined with the $k_{app}/[B]$ value in Table 1, which is given by $(k_1k_4/K_3)/(k_{-1} + k_2)$ (eq 9). Comparing the $k_{app}/[B]$ values for ImH vs 2-MeImH and 1-MeIm vs 1,2-Me_2Im at pH 10.0, the 2-methyl substituent is found to reduce the value considerably. On the other hand, the effect of basicity can be examined by comparing the $k_{app}/[B]$ values for ImH vs 4-MeImH (with NH proton) and 3,4-Me_2py vs 1-MeIm (without NH proton). The basicity can be estimated by the pK_a values of the bases, and it is clearly seen that the weaker bases give the lower $k_{app}/[B]$ values. Moreover, the bases with NH proton were found to have enhanced the rate efficiently. The hindered bases ligate to the ferric ion with less affinity, while the unhindered NH bases usually bind with higher affinity.[25b,26] The stronger base should bind with the higher affinity to the *ferryl* ion in EBS as to the

ferric ion, and would activate the postulated *trans* oxo ligand by the stronger electron donation. The structure of EBS is schematically drawn in Figure 6. The 4-Me_2Npy has the largest pK_a value among the bases examined, and hence the k_{app}/[B] value should at least be larger than that of 1-MeIm. The kinetic studies were, however, done at pH 10, and this pH is close to the pK_a value of 4-Me_2Npy. Therefore, a considerable amount of 4-Me_2Npy should be protonated (about 33%) at pH 10.0. The difference in the ring system (pyridine and imidazole) may also be due more to the lower k_{app}/[B] value of 4-Me_2Npy than that of 1-MeIm.

In the presence of higher amounts of bases, on the contrary, the catalytic activity was found to be inhibited (Figure 3) by the bis-coordination of bases to the ferric hemin (Figure 4). By the kinetic analysis (Appendix, eq A43), we obtain

$$\log k_{app} = -\log [B]_0 + \log[k_3k_4K_2/(k_{-3} + k_4)] \quad (10)$$

for $[B]_0 \gg K_3$, which well explains the slope of -1 in Figure 3. Therefore, one equivalent of a base would have bypassed the reaction route from EBS to EB_2 through EB (Scheme 1).

ESBH Intermediate. 2-MeImH showed higher activity than that in base-free system at every pH examined (Figure 5). At alkaline pH, the activation was explained by the formation of EBS intermediate as discussed above. At acidic pH, the enhancement by other bases such as ImH was less apparent (Figure 1), which can be explained that ImH is protonated and can not bind to the ferryl ion to produce EBS. Therefore, we should include another intermediate (ESBH) in the catalytic cycle to explain the activation by 2-MeImH. The 2-MeImH is also protonated at acidic pH (below its pK_a) and should lose the binding ability to produce EBS, but the protonated NH^+ moiety now gets the ability to form hydrogen bonds. Recently, we have established by the use of resonance Raman spectroscopy that sterically hindered imidazoles prefer the formation of hydrogen bonding adduct with the methoxo ligand in Fe(OEP)(OMe) rather than to ligate directly to the ferric ion.[25b,27] Similarly, the protonated 2-MeImH would be able to hydrogen bond to the oxo ligand in the ESBH intermediate (Figure 6). The involvement of ESBH species could well account for the pH dependency of log k_{app} (Appendix, eq A45):

$$\log k_{app} = \mathrm{pH} - pK_{a1} + \log [k_1/\{1 + k_{-1}/(k_2 + k_5[B]_0/K_4)\}] \quad (11)$$

This gives a parallel line at acidic pH to that given by eq 8 for base-free system. The ESBH species may also be formed in the case of unhindered bases. The formation should, however, require a relatively higher amount of bases, and bis-complexes are readily produced. Therefore, the ESBH intermediate would be characteristic for the hindered base.

Mechanism. As discussed above, at least three reaction intermediates should be included in the catalytic cycle of the hemin system to satisfy the observed kinetic results (Scheme 1). At highly alkaline pH, H_2O_2 may dissociate spontaneously[20a,b] to form HOO^-. The k_{app} value, however, was constant between pH 7 and 10 (Figure 5), and the pH examined was far from the pK_a of the H_2O_2 (11.6[15b]). Therefore, the spontaneous formation of HOO^- was not considered in our study. Instead, hemin-catalyzed production of peroxo complex, Fe^{3+}(PP)(OOH), seems to occur. In the base-free system, the coordinating hydroxide in E would subtract a proton from H_2O_2 and promote the formation of the Fe^{3+}(PP)(OOH), since the pK_a value of H_2O (14) is much greater than that of H_2O_2, and the produced HOO^- is neutralized by the ligation to the ferric ion. The importance of the hydroxide ligation in E to the activity should be attributed to the production of this peroxo complex.

A peroxo complex was reported to be produced on reacting Fe^{3+}(TPP)(Cl) with *tert*-butyl hydroperoxide in the presence of alkaline reagents in methylene chloride at 77 K.[28] The peroxo complex was also detected as the reaction intermediate of cytochrome *c* oxidase.[29] Since the iron ion in the hydroxo complex (E) is in the ferric state, the formal valent of the iron in the peroxo complex is +5. This valency is isoelectronic with the ferryl−oxo π-cation radical state of peroxidases (compound I). The peroxo−iron complex is unstable[28] and readily produces the ferryl−oxo complex by homolytic cleavage of the O−O bond on increasing temperature.[30] In the case of cytochrome *c* oxidase, the peroxo intermediate is short-lived (about 10 μs) and a ferryl−oxo complex, an analogous intermediate to the compound II of peroxidases, is reported to be produced.[29] Therefore, the peroxo complex should also be too unstable to be of concern in the oxidation cycle at ambient temperature in our system, and the ferryl−oxo ES intermediate may be readily produced by the collapse of the peroxo complex.

In peroxidases, the proximal histidine ligand is proposed to have imidazolate character,[31] which induces more electron donation to the ferryl ion to activate the *trans* oxo ligand. In addition, the distal histidine in peroxidases is hydrogen bonding to the oxo ligand in the compound II,[32] which was found[32a] to enhance greatly the oxidation activity of peroxidases. This histidine is closely located to the iron to affect the bonding between iron and external ligand such as CO[33] or OH^-.[32c] In our system, the stronger electron donation by the bases in EBS and the hydrogen bonding between the protonated 2-MeImH and the oxo ligand in ESBH was suggested to promote the peroxidase activity. Therefore, the roles of bases in the EBS and ESBH intermediates are closely correlated to the proximal and the distal histidines in the compound II in view of the activation mechanism of the oxo ligand. An unhindered base would be an effective activator at alkaline pH by electron donation, while a hindered base would be so at acidic pH by hydrogen bonding.

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Conclusion

The hemin-catalyzed peroxidase activity was studied in detail in aqueous solution with special interest in the effects of bases and pH. Coordination of hydroxide to the ferric hemin was essential to the activity, which may promote the subsequent formation of ferric−peroxo and ferryl−oxo complexes. Coordination of the bases to the ferryl ion *trans* to the oxo ligand greatly promoted the activity. The bis-coordination of the bases to the ferric hemin has inhibited the activity by occupying the axial coordination sites. Sterically hindered 2-MeImH was a less effective activator, but hydrogen bonding to the oxo ligand at acidic pH was explained to increase the activity. The *trans* and hydrogen-bonding effects of the bases were quite similar to those found in the peroxidase intermediate compound II.

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Appendix

General Solution for the Rates. The dissociation constants in Scheme 1 are defined as below:

$$K_a = [B][H]/[BH],\ K_{a1} = [E][H]/[EH] \quad (A1)$$

$$K_{a2} = [EB][H]/[EHB],\ K_{a3} = [EB_2][H]/[EHB_2] \quad (A2)$$

$$K_1 = [E][B]^{n-1}/[EB],\ K_2 = [EB][B]/[EB_2] \quad (A3)$$

$$K_1' = [EH][B]^{n-1}/[EHB],\ K_2' = [EHB][B]/[EHB_2] \quad (A4)$$

$$K_3 = [ES][B]/[EBS],\ K_4 = [ES][BH]/[ESBH] \quad (A5)$$

By the cyclic nature of the scheme

$$K_1'K_{a1} = K_1K_{a2},\ K_2'K_{a2} = K_2K_{a3} \quad (A6)$$

The initial concentration of the hemin ($[E]_0$) and the total concentrations of ferric hemin ($[E]_t$) and intermediates ($[I]_t$) in the steady-state are given by

$$[E]_0 = [E]_t + [I]_t \quad (A7)$$

$$[E]_t = [EH] + [EHB] + [EHB_2] + [E] + [EB] + [EB_2] \quad (A8)$$

$$[I]_t = [ES] + [EBS] + [ESBH] \quad (A9)$$

With eqs A1−A9, we obtain

$$[EH] = ([H]/K_{a1})([E]_t/\alpha_1) \quad (A10)$$

$$[EHB] = ([H]/K_{a1})([B]^{n-1}/K_1')([E]_t/\alpha_1) \quad (A11)$$

$$[EHB_2] = ([H]/K_{a1})\{[B]^n/(K_1'K_2')\}([E]_t/\alpha_1) \quad (A12)$$

$$[E] = [E]_t/\alpha_1 \quad (A13)$$

$$[EB] = ([B]^{n-1}/K_1)([E]_t/\alpha_1) \quad (A14)$$

$$[EB_2] = \{[B]^n/(K_1K_2)\}([E]_t/\alpha_1) \quad (A15)$$

$$[ES] = [I]_t/\alpha_2 \quad (A16)$$

$$[EBS] = ([B]/K_3)([I]_t/\alpha_2) \quad (A17)$$

$$[ESBH] = ([BH]/K_4)([I]_t/\alpha_2) \quad (A18)$$

where α_1 and α_2 are defined as

$$\alpha_1 = [H]/K_{a1} + ([H]/K_{a1})([B]^{n-1}/K_1')(1 + [B]/K_2') + 1 + ([B]^{n-1}/K_1)(1 + [B]/K_2) \quad (A19)$$

$$\alpha_2 = 1 + [B]/K_3 + [BH]/K_4 \quad (A20)$$

Employing steady-state approximation for $[I]_t$ ($d[I]_t/dt = 0$)

$$k_1[E][S] + k_3[EB][S] = k_{-1}[ES] + k_2[ES] + k_{-3}[EBS] + k_4[EBS] + k_5[ESBH] \quad (A21)$$

Inserting eqs A13, A14, and A16−A18 into eq A21, we obtain

$$[S]([E]_t/\alpha_1)(k_1 + k_3[B]^{n-1}/K_1) = ([I]_t/\alpha_2)\{(k_{-1} + k_2) + (k_{-3} + k_4)[B]/K_3 + k_5[BH]/K_4\} \quad (A22)$$

Defining a, b, and c as below

$$a = (k_1 + k_3[B]^{n-1}/K_1)/\alpha_1 \quad (A23)$$

$$b = (k_2 + k_4[B]/K_3 + k_5[BH]/K_4)/\alpha_2 \quad (A24)$$

$$c = (k_{-1} + k_{-3}[B]/K_3)/\alpha_2 \quad (A25)$$

Eq A22 is now reduced to

$$a[S][E]_t = (b + c)[I]_t \quad (A26)$$

When the oxidation rate of ferrocyanide (k_f) by a product (P) is very fast and this step is not rate-determining, the velocity (V) for the formation of ferricyanide is given by

$$V = k_2[ES] + k_4[EBS] + k_5[ESBH] \quad (A27)$$

Inserting eqs A16-A18 into eq A27 using eq A24, we obtain

$$V = ([I]_t/\alpha_2)(k_2 + k_4[B]/K_3 + k_5[BH]/K_4) = b[I]_t \quad (A28)$$

With eqs A7, A26, and A28, the initial velocity (V_0) is given by

$$V_0 = ab[S]_0[E]_0/(a[S]_0 + b + c) \quad (A29)$$

where $[S]_0$ is the initial concentration of hydrogen peroxide. Since the V_0 was linear to $[S]_0$ (Figure 1), the first term in the denominator in eq A29 should be neglected ($a[S]_0 \ll b + c$) and thus the equation V_0 is simplified to

$$V_0 = a[S]_0[E]_0/(1 + c/b) \quad (A30)$$

The apparent second-order rate constant, k_{app}, is then defined as

$$k_{app} = V_0/([S]_0[E]_0) = a/(1 + c/b) \quad (A31)$$

1. Without Bases. In the absence of bases ([B] = [BH] = 0), the terms a and c/b in eq A31 are simplified to $a = k_1/([H]/K_{a1} + 1)$ and $c/b = k_{-1}/k_2$, using eqs A19 and A23−A25. At alkaline pH above pK_{a1}, $[H]/K_{a1} \ll 1$ and the k_{app} is given by

$$k_{app} = k_1k_2/(k_2 + k_{-1}) \quad (A32)$$

At acidic pH below pK_{a1}, $[H]/K_{a1} \gg 1$ and the k_{app} is given by

$$k_{app} = (K_{a1}/[H])k_1k_2/(k_2 + k_{-1}) \quad (A33)$$

Logarithm of eqs A32 and A33 gives

$$\log k_{app} = \log\{k_1k_2/(k_2 + k_{-1})\} \quad \text{(A34)}$$

$$\log k_{app} = \text{pH} - \text{p}K_{a1} + \log\{k_1k_2/(k_2 + k_{-1})\} \quad \text{(A35)}$$

2. With Bases. Since the initial concentration of a base ($[B]_0$) is much greater than $[E]_0$, the concentration of the iron-bound base is much less than the $[B]_0$. Therefore

$$[B]_0 = [B] + [BH] \quad \text{(A36)}$$

With eqs A1 and A36, we obtain

$$[B] = [B]_0/(1 + [H]/K_a),\ [BH] = [B]_0/(K_a/[H] + 1) \quad \text{(A37)}$$

2.1. Alkaline pH. At alkaline pH, $[H] \ll K_{a1}$ and K_a for all the bases except for 4-Me_2Npy (Table 1), which was discussed in the text. Therefore, eq A37 is reduced to $[B] = [B]_0$ and $[BH] = 0$, and the terms a and c/b in eq A31 are given by

$$a = (k_1 + k_3[B]_0^{n-1}/K_1)/\{1 + ([B]_0^{n-1}/K_1)(1 + [B]_0/K_2)\} \quad \text{(A38)}$$

$$c/b = (k_{-1} + k_{-3}[B]_0/K_3)/(k_2 + k_4[B]_0/K_3) \quad \text{(A39)}$$

using eqs A19 and A23−A25.

In the promotion process, the $[B]_0$ was low and far from $[B]_{mid}$ where the bis-complex was half-produced. Therefore, $[B]_0 \ll K_1$ and K_2, and eq A38 is reduced to $a = k_1$. The k_{app} in eq A31 then is given by

$$k_{app} = k_1(k_2 + k_4[B]_0/K_3)/\{(k_{-1} + k_2) + (k_{-3} + k_4)[B]_0/K_3\} \quad \text{(A40)}$$

If $(k_{-1} + k_2) \gg (k_{-3} + k_4)[B]_0/K_3$, eq A40 is simplified to

$$k_{app} = k_1(k_2 + k_4[B]_0/K_3)/(k_{-1} + k_2) \quad \text{(A41)}$$

When $[B]_0 \ll K_3$, eq A41 equals to eq A32, and the base promotion term is lost. Therefore, at least $[B]_0 \approx K_3$ and hence $K_3 < K_1$ and K_2.

In the inhibition process, on the other hand, $[B]_0 \gg K_1$ and K_2, and eq A38 is reduced to $a = k_3K_2/[B]_0$. Since $K_3 < K_1$ and K_2 as discussed above, $[B]_0 \gg K_3$, and hence eq A39 is reduced to $c/b = k_{-3}/k_4$. k_{app} in eq A31 is then given by

$$k_{app} = k_3k_4(K_2/[B]_0)/(k_{-3} + k_4) \quad \text{(A42)}$$

The logarithm of eq A42 gives

$$\log k_{app} = -\log[B]_0 + \log[k_3k_4K_2/(k_{-3} + k_4)] \quad \text{(A43)}$$

2.2. Acidic pH. At acidic pH, $[H] \gg K_a$, $[BH] = [B]_0$, and $[B] = 0$ by eq A37. At this pH region, $[H] \gg K_{a1}$ ($pK_{a1} = 6.9$), and using eqs A19 and A23−A25, terms a and c/b are given by $a = k_1K_{a1}/[H]$, $c/b = k_{-1}/(k_2 + k_5[B]_0/K_4)$. Therefore, k_{app} in eq A31 is given by

$$k_{app} = (k_1K_{a1}/[H])/\{1 + k_{-1}/(k_2 + k_5[B]_0/K_4)\} \quad \text{(A44)}$$

The logarithm of eq A44 gives

$$\log k_{app} = \text{pH} - \text{p}K_{a1} + \log[k_1/\{1 + k_{-1}/(k_2 + k_5[B]_0/K_4)\}] \quad \text{(A45)}$$

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