New Complexes of Peroxidases with Hydroxamic Acids, Hydrazides, and Amides*

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SUMMARY

Horseradish peroxidase forms spectroscopically distinct, reversible complexes with hydroxamic acids (R-CO-NHOH), hydrazides (RCO-NHNH2), amides (RCONH2), and α-hydroxyketones (RCO-CH2OH). Binding of these compounds to the enzyme depends on the polar and steric character of R and the hydrogen bonding capacity of -CO-X-Y (X-Y = NH-OH, NH-NH2, NH-H, CH2OH). Hydroxamate anions and hydrazide cations do not associate with the enzyme. The dissociation constants (Kd) for the enzyme-RCOXY complexes span seven orders of magnitude (Kd = 0.3 to 2 x 10^-7 M), the greatest affinity being shown by compounds with a planar, aromatic R group. This is attributed to an interaction of the R moiety at an apoprotein hydrophobic crevice.

Spectrophotometric, electron paramagnetic resonance, and magnetic susceptibility measurements indicate that the association of horseradish peroxidase with hydroxamic acids entails a transition from a mixed spin state of the enzyme to a high spin derivative.

The spectroscopic characteristics of enzyme-RCOXY complexes are similar, suggesting that X-Y substituents do not interact directly with the metal ion of the prosthetic group but perturb its environment. Analogous conclusions were drawn from (a) the parallelism between RCOXY affinities for manganic and ferric peroxidases which does not pertain to ligands (e.g. F-) substituting in the first coordination sphere of the metal ion and (b) the lack of pronounced spectroscopic changes in RCOXY-ferroperoxidase complexes.

The association of peroxidase with hydroxamic acids is competitively inhibited by specific enzyme substrates (hydrogen donors), permitting the evaluation of so far unknown enzyme-(donor) substrate binding parameters (Kd). Such a competitive behavior also implies the proximity of RCOXY to the active site.

RCOXY compounds influence heme-linked ionizations and ligand interchange reactions, e.g. they inhibit the formation of alkaline peroxidase and peroxidase-cyanide complex.

With cyanide and RCOXY, ferriperoxidase gives tertiary complexes. Their formation occurs sequentially:

enzyme + HCN → enzyme-cyanide + RCOXY → enzyme-cyanide-RCOXY.

The current work originated with the observation that aromatic peracids are exceptionally efficient oxidants of horseradish peroxidase, the 1:1 reaction resulting in the formation of compound I and release of the parent carboxylic acid. Two interpretations are consonant with these observations: (a) aromatic peracids form a complex with the enzyme which rearranges to compound I concurrently with the scission of the O-O bond, or (b) in its complex with the enzyme, the peracid is rapidly hydrolyzed, giving the physiological oxidant H2O2 at, or near, the active site. In either case, peracids appear to have a greater affinity for the enzyme than hydrogen peroxide or its monoalkyl derivatives.

Hydroxamic acids (Scheme 1A), hydrazides (B), and α-hydroxyketones (C) are structurally related to and isoelectronic with peracids (D). Like peracids, they are weak acids (2-5); they have a planar O---C---X constellation (6-8); and they show a propensity for metal binding (9-13) and H-bond formation (14-16). In view of these similarities, it seemed that Compounds A to C in Scheme 1 might serve as excellent probes of the active site, permitting the assessment of alternatives a and b. This proved to be the case. It has now been established that RCOXY compounds (where R is an aliphatic, alicyclic, or aromatic residue, and X-Y = NH-OH, NH-NH2, CH2-OH, etc.) are not hydrolyzed in the presence of H-peroxidase but form stable, reversible complexes with the enzyme.

* Communicated in part at the 154th Meeting of the American Chemical Society, Chicago (September 1967). This research was supported by grants from the Medical Research Council of Canada (MT-1270) and Life Insurance Medical Research Fund (G-G4-3G).
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‡ The abbreviations used are: H-peroxidase, horseradish peroxidase; EPR, electron paramagnetic resonance.

Scheme 1
In this communication we delineate the spectroscopic and magnetic properties of such RCOXY-peroxidase complexes and present a hypothesis on the nature of enzyme-ligand interactions.

In another communication we shall outline the kinetics of RCOXY oxidation by compounds I and II, showing that hydroxamic acids are extraordinarily efficient reductants of enzyme-peroxide derivatives.

**EXPERIMENTAL PROCEDURE**

**Materials and Assays**—Electrophoretically purified H-peroxidase (Lots HPOFF 61B and SAF) was obtained from Worthington Biochemical Corp. Based on heme assays (17), its absorbivities in 0.01 M phosphate (pH 6.5) were: (mM⁻¹ cm⁻¹) ε₅₈₀ 32.3, ε₆₃₀ 102.2, ε₁₆₀ 11.2, and ε₃₃₀ 2.21. The amino acid composition of these preparations (18) corresponds predominantly, in the classification of Shannon et al., to isoenzyme C (19) or, in Paul and Stigbrand’s classification, to isoenzyme IIIb (20).

**Manganic H-peroxidase**—Manganic H-peroxidase (21) was prepared by recombination of apo H-peroxidase with manganic protoporphyrin in 0.02 M borate (pH 8.3) at 2°C. The former was derived from the Worthington enzyme, either by Theorell’s acid-acetone technique (22) or Teale’s acid-butanol technique (23). Manganic protoporphyrin was synthesized by the method of Taylor (24) and recrystallized from dimethyl sulfoxide-tert-butanol (1:9, v/v).

**Hydroxamic Acids**—Most of the compounds used in this study were synthesized in this laboratory, some by courtesy of Dr. J. Wood, by standard hydroxynaminolysis of the corresponding carboxylic esters or acid chlorides (25). C, H, and N analyses of these derivatives, carried out by Galbraith Laboratories, Knoxville, Tenn., were within ±0.2% of the theoretical values. Some compounds were checked against commercial preparations available from Haynes Chemical Research Corp., Lugoff, S. C. (o-fluorobenz- and p-hydroxybenzhydroxamic acids); and Raylo Chemicals, Edmonton, Alberta, Canada (1-naphtho-, 2-naphtho-, m-iodo-, and cyclohexyl hydroxamic acids). O-Benzoyl hydroxylamine was prepared by Jencks’ method (26). Other highest purity reagents were obtained either from Fisher Scientific Co. or from Aldrich Chemical Co. All solutions were made in water distilled twice, once from alkaline permanganate.

**Solutions**—Stock solutions of benzhydroxamic acids, benzhydrazides, and benzamide were made in water, except in the case of m-iodo-, 1-naphtho-, 2-naphtho-, and cyclohexylhydroxamic acids; the latter were dissolved in aqueous alcohol containing 20% (v/v) methanol. Ethanol was the solvent for α-hydroxycacetophenone. Except for p-hydroxybenzhydroxamic acid, which is autoxidizable, weakly acid solutions of hydroxamic acids and hydrazides may be stored at 4°C for several days without detectable decomposition (27).

**Measurements of Equilibrium Constants**—The formation of enzyme-ligand complexes (HRP-L) was followed spectrophotometrically at 410 nm. At this wave length, the difference in molar absorbivities (Δε₅₈₀) is greatest (Fig. 2), the ligands generally do not absorb and the measurements are most accurate, since 410 nm is near the absorption maxima of H-peroxidase (403 nm) and its hydroxamic acid complexes (408 nm) (Fig. 1).

The titrations (Figs. 5, 7–10) were carried out at 25°C by adding 2- to 40-μl aliquots of the ligand solutions to 2.5 ml of peroxidase (2 to 10 μm), by using calibrated microsyringes. The dependence of the equilibrium constant on hydronium ion concentration was examined in 5 mM acetate, pH 4 to 5; 10 mM 2,2-dimethyl glutarate, pH 4 to 6; and 20 to 80 mM phosphate, pH 5.8 to 8.

Borate (30 mM) was used only in mixtions with benzamidine, since it forms complexes with hydroxamic acids (28). Over the entire pH range, the equilibrium constants were nearly independent of ionic strength when η = 0.05 ± 0.03.

**Molar Absorptivities of H-peroxidase-RCOXY Complexes**—Molar absorptivities of H-peroxidase-RCOXY complexes were evaluated in different buffers at 25°C with saturating amounts of the titrant. Whenever this method was not applicable, either due to weak ligand-enzyme affinity (benzamidine) or to limited titrant solubility (α-hydroxycacetophenone; cyclohexyl hydroxamic acid), the change in molar absorptivities (Δε₅₈₀) was derived from the linearized form (Equation 1) of the standard equilibrium relationship (Equation 2).

\[
\frac{1}{L_T} = \frac{HRP_L}{K_r(v)} \Delta k - \frac{1}{K_r(v)} \quad (1)
\]

\[
K_r = \frac{(HRP_L - HRP_{L}(v)(L_T - HRP_{L}(v))}{HRP_{L}(v)} \quad (2)
\]

For complexes with hydroxylazides, \( r = [1 + (H^+/K_a)] \) (Equation 3); for hydroxamic acids, \( r = [1 + (K_a/K_r)] \) (Equation 4). In the above equations, \( K_r \) is the acid dissociation constant of the ligand; HRP is the total concentration of all ionized forms of H-peroxidase; and \( \Delta k \) represents the observed absorbance change at \( λ \) nm, when the solution light path is 1. From the plot of \( 1/Δk versus 1/L_T \), \( K_r \) and \( \Delta k \) may be evaluated.

**Spectrophotometric Measurements**—These measurements were carried out by using a Cary 14 recording spectrophotometer equipped with a Universal transmission slidewire. Hence, very small absorbance changes could be accurately evaluated.

**Electron Paramagnetic Resonance**—EPR spectra were taken at 77 and 42°K with a Varian X-band spectrometer (V.4509) equipped with 100-kHz field modulation.

**Magnetic Susceptibility**—The experiments were performed with an instrument designed by Tassaki et al. (29). Water was used as calibration standard. The measurements between 0 and 196° were first made by using H-peroxidase itself (Fig. 4A) and then in the presence of equimolar amounts of benzhydroxamic acid (Fig. 4B). The sample volume was the same in both cases (0.7 ml). A nearly complete conversion (~95%) of the enzyme into its benzhydroxamic acid complex was achieved by addition of C₈H₈CONHOH (0.36 mM).

**Potentiometry**—The pH values of the reaction media at 22°C were measured by using a Radiometer model 25 pH meter equipped with a Radiometer type GK 2001 C combined calomel glass electrode.

**RESULTS**

**Optical and Magnetic Properties of H-peroxidase-RCONHY Complexes** (where \( Y = OH, NH_2, H \))—The absorbance of horseradish ferriperoxidase between 220 and 1300 nm is drastically altered on ligation to RCONHY. Fig. 1 illustrates this effect for benzhydroxamic acid (\( R = \text{phenyl}, Y = \text{OH} \)), and a similar pattern is seen with hydrazides (\( Y = \text{NH}_2 \)) and amides (\( Y = \text{H} \)). In all cases, there is a typical hypochromic effect upon the Soret band, coupled to its shift from 403 nm in H-peroxidase to approximately 408 nm in the RCONHY derivatives. In the visible absorption region, the complexes show bands at 503 to 505 nm and 637 to 639 nm, which may be compared to those of the...
enzyme at 497 to 499 nm and 641 nm2 (Table VIII). This distinct absorption pattern remains essentially unaltered from -196 to 40°C and between pH 3 and 9.

In the ultraviolet region, the spectroscopic differences become less striking (Fig. 2); between 220 and 245 nm, the observed absorbance is nearly equivalent to the sum of absorbances contributed by the enzyme and its ligand.

The EPR spectra of H-peroxidase and its benzhydroxamic acid complex point to changes in the “heme-linked” interactions. Thus, at 77 and 4.2°C the unliganded enzyme absorbs near \( g = 6.2 \) and \( g = 5.0 \), respectively (Fig. 3, a and b). On the other hand, at 77°C the absorption derivative of the complex suggests coalescence of transitions giving, near \( g = 5.85 \), an intense but rather broad band (\( \sim 220 \) Oe). The latter is resolved, at 4.2°C, into a doublet with \( g \approx 6.1 \) and \( g \approx 5.4 \). The difference between the splitting factors is substantially smaller in the complex (\( \Delta g = 0.7 \)) than in the free enzyme (\( \Delta g = 1.2 \)) and implies a transition to a higher axial symmetry of the coenzyme (30) or reflects a change in the spin relaxations.

Magnetic susceptibility measurements complement the above data (Fig. 4). For H-peroxidase, the effective magnetic moment \( (n_{eff}) \) is 5.23 Bohr magnetons, which is in excellent agreement with the results of Theorell and Ehrenberg (31). In the presence of benzhydroxamic acid, the paramagnetism increases, giving \( n_{eff} \) of 5.97 Bohr magnetons. This is effectively the same as the theoretically computed value for an ion with five unpaired electrons \( (n_{eff} = 5.92 \) Bohr magnetons). The magnetic susceptibilities of H-peroxidase and its benzhydroxamic acid complex follow Curie’s Law, between -196 and 0°C. Apparently, in contrast to several other hemoproteins (29, 32-34), both free and liganded forms of the H peroxidase retain their high spin character over a wide temperature range.

Dissociation Constants of H-peroxidase-RCOXY Derivatives—
FIG. 4. Temperature dependence of molar paramagnetic susceptibility of 3.73 μM H-peroxidase (A) and its benzhydroxamic acid complex (B). Benzhydroxamic acid, 3.7 mM; 0.01 M potassium phosphate buffer (pH 6.5).

The assay of the enzyme-RCOXY complexes was based on the spectroscopic measurements described in detail under "Experimental Procedure." In all cases, the equilibrium was established within the mixing time of the reagents. Between pH 4 and 9 at 25°, the complexes are stable and no secondary derivatives were detectable. At all levels of enzyme saturation, the spectra pass through a single set of isosbestic points.

A typical experiment for the measurement of benzhydroxamic acid binding is illustrated in Fig. 5. The data and the slope of the line 1.

The effect of pH on the apparent dissociation constants of peroxidase benzhydroxamic acid, benzhydrazide, and benzamide complexes at 25° is shown in Table II. For any given compound then, Kₐ calculated on the basis of the neutral form is virtually invariant from pH 3.5 to 9.0 (Table II). Amidine, which do not ionize or protonate between pH 1 and 14, associate with the enzyme equally well in acid and alkaline solutions (Table II), providing that the conversion of neutral H-peroxidase into its alkaline derivative (characterized by pKₐ ~ 10.9 (35)) is taken into account. This is expressed in Equation 5.

\[
\log \frac{\Delta A}{\Delta A_r - \Delta A} = pH + \log \frac{K_a K_i}{T_r + K_i}
\]

where \(L_T\) = (benzamide) \(\supseteq\) (enzyme). \(\Delta A\) represents the observed change in absorbance at 395 nm, a wave length isosbestic.

### Table I

**Apparent dissociation constants (Kₐ) of peroxidase-hydroxamic acid complexes in 0.02 M potassium phosphate buffer (pH 6.0) at 25°**

<table>
<thead>
<tr>
<th>Hydroxamic acid</th>
<th>10⁶ Kₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphtho-2-OH</td>
<td>2.45</td>
</tr>
<tr>
<td>Benz-2-OH</td>
<td>2.51</td>
</tr>
<tr>
<td>p-Methylbenz-2-H</td>
<td>2.27</td>
</tr>
<tr>
<td>p-Fluorobenz-13</td>
<td>2.35</td>
</tr>
<tr>
<td>p-Fluorobenz-22</td>
<td>2.35</td>
</tr>
<tr>
<td>Isonicotino-100</td>
<td>3.77</td>
</tr>
<tr>
<td>Cyclohexyl-2400</td>
<td>2.10</td>
</tr>
<tr>
<td>Aceto-62,000</td>
<td>1.20</td>
</tr>
<tr>
<td>Form-3 x 10⁴</td>
<td>3.30</td>
</tr>
</tbody>
</table>

### Table II

**Effect of pH on the apparent dissociation constants of peroxidase benzhydroxamic acid, benzhydrazide, and benzamide complexes at 25°**

<table>
<thead>
<tr>
<th>Complex</th>
<th>pH</th>
<th>10⁶ Kₐ</th>
<th>10⁶ Kₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzhydroxamic</td>
<td>4.11</td>
<td>2.35</td>
<td>2.45</td>
</tr>
<tr>
<td>acid (pKₐ = 8.8)</td>
<td>5.05</td>
<td>1.81</td>
<td>1.38</td>
</tr>
<tr>
<td>5.98</td>
<td>1.38</td>
<td>1.98</td>
<td>1.0</td>
</tr>
<tr>
<td>6.82</td>
<td>1.85</td>
<td>1.98</td>
<td>3.4</td>
</tr>
<tr>
<td>8.75</td>
<td>3.3</td>
<td>3.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

a Kₐobs = K₁ × T (Equation 2).

b For the conjugate acid form C₄H₅CONH-—NH₃⁺ (Equation 3).
for the neutral form of H-peroxidase and its benzamide complex; 
\( \Delta A_T \) is the maximum change in absorbance attending conversion of the enzyme into its alkaline derivative.

Analyses of H peroxidase benzamide interaction, shown in Fig. 6a, are consonant with the predictions based on Equation 5. Thus, at different benzamide concentrations, the plots of log
\( \left( A / A_{T} - A \right) / \Delta A_T \) versus pH give lines of slope 1; and the pH at which log
\( \left( A / A_{T} - A \right) / \Delta A_T \) = 0, i.e. pK_{ac} (app), increases with higher concentrations of the ligand. This is illustrated by the secondary plot shown in Fig. 6b, where
\[
pK_{ac(app)} = pK_a + \log \left( 1 + \frac{H_+}{L_T/K_1} \right)
\] (6)

The intercept on the ordinate gives
\( pK_a \sim 10.95 \), in excellent agreement with the value obtained through direct titration of the enzyme (35).

Three conclusions are allowed by the above data. (a) The heme-linked effect leading to the formation of alkaline peroxidase is governed by a single ionization; (b) below pH 12, H-peroxidase-benzamide complex is not subject to such an ionization; and (c) if benzamide binds to the alkaline peroxidase, then its affinity is much weaker than that for the neutral enzyme.

Interaction of H-peroxidase with Cyanide in the Presence of Hydroxamic Acids and Hydrazides—If the enzyme-RCOXY interaction involves a direct coordination of -COXY to the ferric ion, such a binding should be competitively inhibited by typical hemoprotein ligands such as cyanide, fluoride, or azide. Of these, cyanide is the reagent of choice. It reacts readily with H-peroxidase (36, 37), giving a complex whose dissociation constant is nearly pH invariant from pH 4.2 to 7.5 (37) and whose spectrum (38, 39) differs radically from those of H-peroxidase-RCOXY derivatives (Fig. 1).

For a fully competitive ligand interchange in a peroxidase-benzhydroxamic acid-cyanide system, the ratio (HRP-BHA) to (HRP-cyanide) should be
\[ \frac{HRP-BHA}{HRP-cyanide} = \frac{K_1}{K_2} \times \frac{HCN_T}{BHA_T} \] (7)

providing that the concentrations of ligands BHA_T and HCN_T are at least 10-fold greater than the total concentration of the enzyme, HRP_T. K_1 and K_2 are the dissociation constants of peroxidase-RCOXY and peroxidase-cyanide complexes. The above relationship expressed in linear form
\[
\frac{1}{HRP-BHA} = \frac{1}{HRP_T} \times \left( \frac{K_2}{K_1} \right) \times \frac{1}{BHA_T} \times \frac{1}{HCN_T} + \frac{1}{HRP_T}
\] (8)

implies that
\[ m = \frac{1}{HRP_T} \times \frac{K_1}{K_2} \times \left( \frac{1}{BHA_T} + \frac{1}{K_1} \right) \] (9)

the relationship between 1/HRP-BHA and HCN_T should be
\[
\frac{1}{HRP-BHA} = \frac{1}{HRP_T} \times \left( \frac{K_2}{K_1} \right) \times \left( \frac{1}{BHA_T} + \frac{1}{K_1} \right) \frac{HCN_T}{HRP_T} + \frac{1}{HRP_T}
\] (10)

The slope, m, of 1/HRP-BHA versus HCN_T plot now becomes
\[
m = \frac{1}{HRP_T} \times \left( \frac{K_1}{K_2} \right) \times \left( \frac{1}{BHA_T} + \frac{1}{K_1} \right)
\] (11)

Table III

<table>
<thead>
<tr>
<th>Conditions: (H-peroxidase) 8.3 µM; 0.05 M potassium phosphate (pH 6.05) at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-8} BHA</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>97</td>
</tr>
<tr>
<td>194</td>
</tr>
<tr>
<td>385</td>
</tr>
<tr>
<td>580</td>
</tr>
</tbody>
</table>

* K_i/K_{ac(app)} = 1.24.
Hence,

\[ \frac{1}{BHA_T} = HRP_T \times \frac{K_3}{K_1} x - \frac{1}{K_4} \]  

(12)

By using Equation 12 and the data shown in Fig. 7b, \( K_4 \) was found to be 1.5 ± 0.1 \( \times 10^{-4} \) and \( K_1:K_3 = 1.11 \pm 0.05 \), in agreement with the expected \( K_1:K_3 \) value.

The results of similar analyses, using 1- and 2-naphthohydroxamic acid and benzhydrazide are summarized in Table IV. In all cases, the observed partition constant, \( K_1:K_3 \), closely agrees with \( K_1:K_3_{\text{calcd}} \) by using \( K_1 \) and \( K_3 \) constants obtained in independent studies. Furthermore, according to Equation 11, \( m \) should converge to a limiting value with increasing ligand \( (L_T) \) concentration and become equivalent to \( (1/HRP_T) \times (K_1/K_3) \times (1/K_3) \), when \( 1/L_T < 1/K_3 \). Under such conditions, the extent but not the rate of formation of H-peroxidase-cyanide complexes (\( E \cdot CN + E \cdot CN \cdot L \)) should be nearly independent of \( L_T \). And such is the case (Table V).

The data presented in Table V indicates that the initial velocity \( (v_i) \) of formation of the enzyme-cyanide complexes \( (E \cdot CN + E \cdot CN \cdot BZH) \) is proportional to the concentrations of cyanide \( (HCNT) \) and free enzyme \( (E_f) \). The simplest scheme meeting these requirements is

\[ E \leftarrow k(L) \rightarrow E \cdot L \]  

\[ h_0(HCN) \]

\[ E \cdot CN \leftarrow k(L) \rightarrow E \cdot CN \cdot L \]

and

\[ v_i = \frac{d(E \cdot CN)}{dt} + \frac{d(E \cdot CN \cdot L)}{dt} = \frac{h_0}{K_1 + L_T} \times HCN_T \]

(13)

\( k_3 \) evaluated in this manner (Table V) was found to be: 1.3 ± 0.1 \( \times 10^5 \) \( \text{m}^{-1} \text{s}^{-1} \), compared to \( k_0 = 1.0 \pm 0.1 \times 10^4 \) \( \text{s}^{-1} \) obtained by Chance (36) and Ellis et al. (37).

Direct Determination of \( K_T \)—Direct evaluation of \( K_T \) became possible with the observation that the spectrum of the enzyme-cyanide complex is slightly different in the presence and in the absence of RCOXY ligands.

Such changes are independent of cyanide concentration when \( K_1:HCNT >> K_3.L_T \). The maximum differences in molar absorptivities \( (\Delta e_{\text{HRP-HCN-RCOXY}} - \Delta e_{\text{HRP-HCN}}) \) range from approximately \( \Delta e_{\text{abs}} = 3000 \) \( \text{m}^{-1} \text{cm}^{-1} \) when RCOXY = 2-naphthohydroxamic acid to approximately \( \Delta e_{\text{abs}} = 5000 \) \( \text{m}^{-1} \text{cm}^{-1} \) with benzhydrazide (Fig. 8a). Since at 430 nm the absolute molar absorptivity of H-peroxidase-cyanide complex is \( 82 \pm 10^6 \) \( \text{m}^{-1} \text{cm}^{-1} \), it is evident that the increase induced by RCOXY compounds is relatively small. Nonetheless, this is adequate for an accurate measurement of RCOXY binding to the enzyme-cyanide complex.

Accordingly, from the data presented in Fig. 8a, we obtain \( K_4 = 4.4 \times 10^{-4} \) \( \text{m} \) in good agreement with the value derived indirectly (cf. Table IV).

Interaction of H-peroxidase with Hydrogen Donors—Although phenols and aromatic amines have long been recognized as the specific peroxidase substrates (41), the association constants governing their interaction with the enzyme are unknown. Such parameters can be now readily calculated, exploiting the inhibition of RCOXY binding in the presence of hydrogen donor substrates. This is illustrated in Fig. 9A. From these results, we can derive the dissociation constants \( (K_4) \) for H-peroxidase-substrate (HRP-S) complexes, assuming that binding of the specific substrates \( (S) \) and titrants \( (L) \) occurs at the same enzyme site.

With substrate in excess \( (S_T >> HRPT) \) at a pH where \( S \) and \( L \) are largely in the unionized forms, the relevant relationship is:

\[ \frac{\text{HRP-L}}{L_T - \text{HRP-L}} = \frac{\text{HRP_T} \times K_4}{K_s + K_s} - \frac{K_s}{K_s + K_s} \]

(14)

\[ \times \text{HRP-L} \]

from which \( K_s \) may be obtained. Plots based on Equation 14 are shown in Fig. 9B for benzhydrazide, both in the absence of substrates and in the presence of 2.3 mM hydroquinone, 5.2 mM aniline, and 9.1 mM phenol. In all cases, the lines intersect the abscissa at a point corresponding to the total enzyme concentration. Also, as would be expected for competitive binding, the calculated dissociation constants are independent of the initial hydrogen donor concentration or the nature of the titrant (Table VI).

Apparently, in contrast to the association between RCOXY and H-peroxidase-cyanide complex, the binding of hydrogen donors and RCOXY is fully competitive. An independent
Interaction of $RCOXY$ with Manganic Peroxidases and Ferreroxidases—The absorbance pattern of Mn(III) protoporphyrin apoperoxidase is altered on formation of the enzyme-hydroxamic acid derivatives; the greatest differences being associated with $\pi-\pi^*$ transitions of the porphyrin macrocycle (Table VII). The dissociation constants, $K_1$, derived in this manner are $2.1 \pm 0.2 \times 10^{-6}$ M for the benzhydroxamic acid complex and $1.6 \pm 0.2 \times 10^{-7}$ M for the 2-naphthohydroxamic acid derivative. For the corresponding complexes with the ferric enzyme, the dissociation constants are $2.4 \times 10^{-6}$ M and $2 \times 10^{-7}$ M. Clearly, the affinities of peroxidases for the aromatic hydroxamic acids are not governed by the identity of the central metal ion. This is by no means typical of Mn(III)- and Fe(III)-enzyme reactions (21, 42).

On the other hand, $RCOXY$ compounds do not bind equally well to the reduced enzyme and, as shown in Table VII, do not elicit large spectroscopic changes. Such weak heme-linked effects are analogous to those observed with peroxidase-phenol or peroxidase-cyanide-$RCOXY$ complexes (Figs. 8 and 10) and indicate that the $-COXY$ residue does not coordinate to the metal ion. The dissociation constant of ferroperoxidase-benzhydroxamic acid complex is approximately $K_1 = 3 \pm 2 \times 10^{-4}$ M at pH 7.1 (in 5 mM phosphate) and 25°C; i.e. it is of the same order of magnitude as $K_d$ for the ferriperoxidase-cyanide-benzhydroxamic acid complex (Table IV). The uncertainty in $K_1$ is largely due to the autoxidizability of the ferrous enzyme. Thus, the spectrophotometric measurements used in these assays are subject to errors which, so far, we have not been able to eliminate.

**DISCUSSION**

The $RCOXY$ ligands may be divided into two categories. In one subclass we have hydroxamic acids, hydrazides, amides, and $\alpha$-hydroxyacetophenone, all of which associate with H-peroxidase to give spectroscopically distinct derivatives. The second group includes $N$- and $O$-substituted hydroxamic acids, $O$-benzoyl hydroxylamine, $N$-hydroxybenzene sulfonamide, phenacyl halides, or benzaldehyde which, like phenols and aromatic amines, appear to bind to the enzyme but without markedly changing its characteristic spectrum. In some cases, although not with $^{10}$

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*Fig. 8.* a, difference spectra obtained on titrating $4.2 \mu$m peroxidase-cyanide complex with benzhydroxide. HCN$_T$, 3 mM; 0.08 M phosphate (pH 6) at 25°C. b, analysis of the data in Fig. 8a according to Equation 1. HCN$_T$, 3 mM (0); 14 mM (○).

**FIG. 9.** A, titration of $4.1 \times 10^{-4}$ M H-peroxidase with benzhydroxide, in the absence (○) and presence of hydrogen donors: 5.2 mM aniline (○); 2.3 mM hydroquinone (○); and 9.1 mM phenol (●). B, results shown in A, replotted according to Equation 14. BZH, benzhydroxide.

**TABLE VI**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration range</th>
<th>$pH$</th>
<th>$\times 10^6 K_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>2-10</td>
<td>6.8</td>
<td>$4.3 \pm 0.2$</td>
</tr>
<tr>
<td>Phenol</td>
<td>2-10</td>
<td>6.8</td>
<td>$4.2 \pm 0.2$</td>
</tr>
<tr>
<td>Aniline</td>
<td>4-12</td>
<td>6.7</td>
<td>$17.7 \pm 1.3$</td>
</tr>
<tr>
<td>Mesidine</td>
<td>2.5-7.5</td>
<td>6.7</td>
<td>$16.4 \pm 1.0$</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>2-6</td>
<td>6.7</td>
<td>$2.5 \pm 0.3$</td>
</tr>
</tbody>
</table>

---

evaluation of $K_{phenol}$ by analysis of the difference spectrum between H-peroxidase and its phenol complex is shown in Fig. 10a. From the observed hyperbolic increase in absorbance with increasing concentration of phenol, $K_{phenol}$ was computed to be $5.0 \pm 0.5 \times 10^{-4}$ M (Fig. 10c). Furthermore, the results in Fig. 10b demonstrate that phenol associates nearly as well as ferriperoxidase-cyanide complex. Thus, gauged by phenol binding the conversion of h high spin ferric enzyme into a low spin cyanide derivative does not entail a pronounced change in the structure of the apoenzyme.
Effect of benzhydroxamic acid and benzhydrazide on the absorption spectra of manganic peroxidase and ferroperoxidase and ferroperoxidase-carbon monoxide complexes.

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Titrant</th>
<th>Absorption bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese</td>
<td>Benzhydroxamic acid</td>
<td>482, 376</td>
</tr>
<tr>
<td>Manganese</td>
<td></td>
<td>42.8, 80.7</td>
</tr>
<tr>
<td>Ferro-</td>
<td>Benzhydroxamic acid</td>
<td>53.8, 73.8</td>
</tr>
<tr>
<td>Ferro-</td>
<td>Benzhydrazide</td>
<td>557, 437</td>
</tr>
<tr>
<td>Ferro-</td>
<td></td>
<td>13.1, 89</td>
</tr>
<tr>
<td>Ferro-COH</td>
<td>Benzhydrazide</td>
<td>557, 428</td>
</tr>
<tr>
<td>Ferro-COH</td>
<td></td>
<td>12.9, 94.4</td>
</tr>
<tr>
<td>Ferro-COH</td>
<td>Benzhydroxamic acid</td>
<td>572.5, 541.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.1, 13.9, 161</td>
</tr>
<tr>
<td></td>
<td></td>
<td>571, 492.6, 424</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.7, 14.9, 155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>423, 161</td>
</tr>
</tbody>
</table>

* The enzyme was reduced under argon with NaSO₄.
† In the presence of approximately 1 mm CO.
‡ In 5 mm phosphate, at pH 7.1, and 25°C.

benzaldehyde and O-benzoyl hydroxylamine, this behavior may be due to inherent steric demands of a given substituent and may also reflect conformational isomerism, dictated by the repulsion of nonbonding electrons on X-Y and the π-electrons of the carbonyl group (7, 43).

In this context, the following points deserve emphasis: (a) Ionized ligands (benzhydroxamate anion, benzhydrazide cation) do not associate with H-peroxidase (Table II). This is consistent with, but does not establish, the nonpolar nature of the —COXY binding site. (b) The carbonyl group is not the sole determinant in the formation of spectroscopically active enzyme complexes. For example, benzaldehyde is an inert titrant. (c) When X = NH, Y can be —OH, —NH₂, or —H; and when Y = OH, X can be —NH or —CH₃. Hence these groups per se cannot play a dominant role in coordination. (d) Association of the enzyme with the enol tautomers (e.g. R—C(OH) = NOH, R—C(OH) = CHOH) appears unlikely, because (i) α-hydroxyacetophenone enolizes slowly (t₁/₂ ~ 170 hours) (44), whereas its ligation to the enzyme is fast (t₁/₂ < 10 s, when LT = 3 mM); (ii) imidic acids, enol forms of amides, are unknown (45); and (iii) in the 220 to 250 nm range the difference spectrum of the enzyme—benzhydroxamic acid complex and H-peroxidase (Fig. 2) is superimposable on the absolute spectrum of the ligand. Contributions from the enol tautomer, benzhydroximic acid, would be expected to show an altered absorption pattern (cf. the differences in the spectra of O-alkyl benzhydroxamic and benzhydroximic acids (46)).

We infer that spectroscopically distinct complexes are only formed when the ligands contain an acidic residue (X), in a formally uncharged —C—X—Y constellation. This underscores the importance of the functional group as a whole rather than the individual components. Mechanistically, this may imply either chelation of the hemin-iron or polyfunctional H-bonding.

The chelation hypothesis is less likely on at least two counts. First, it is hardly plausible for amides; second, it is difficult to reconcile with the kinetics of RCOXY binding. For instance, with benzhydroxamic acid, the apparent second order rate constant (kₐp ~ 0.4 ± 0.1 x 10⁸ M⁻¹ s⁻¹ at 25°C) is greater than for any other H-peroxidase-ligand reaction.

Polyfunctional hydrogen bonding, resembling the hydrogen bonded interactions of ureido groups with proteins (47, 48), is not open to these objections. It is supported by a correlation between peroxidase-ligand affinity (expressed by Kᵢ) and acid dissociation constant of the ligands, Kₐ (Fig. 11). Such relationships (49–52) rest on an intrinsic property of the hydrogen bond, relating its strength to the tendency of proton transfer (expressed by Kᵢ) from the donor-acid to the acceptor-base.

Accordingly, in a homologous series of compounds (e.g. R = C₆H₅—), we expect the greatest affinity between peroxidase and benzhydroxamic acid (pKₐ 8.8) (2, 3), decreasing with benzhydrazide (pKₐ ~ 11.5 to 12.5) (4), and even more so with benzamide (pKₐ ~ 15) (10). Such a trend is discernible in Fig. 11. For α-hydroxyacetophenone, Kₐ is unknown but, like that of α-bromoacetophenones, it should be 10⁻¹⁴ to 10⁻¹₇ M (38). Hence, the ketol would be a weak ligand and such is the case.

Hydrogen bonding, particularly of the monofunctional type,

To view the spectrograms see page 510.
for the higher affinity of 2-naphthohydroxamic acid of interaction, the hydrophobic binding, which must be responsible for the hydrogen donor substrates. The association of the R-crevice in the prosthetic group with a concomitant change in the relative position of trans-ligands, the central metal ion, and porphyrin would give a configuration more akin to that of metmyoglobin, where the porphyrin ring is not planar but slightly concave towards the sixth coordination position (58).

The lower rhombicity of the coenzyme (Fig. 3) and the higher paramagnetism of the H-peroxidase-benzhydroxamic acid complex (Fig. 4) and its metmyoglobin-like optical spectrum (Fig. 1) support this argument. The inhibition of alkaline peroxidase formation by benzamide (Fig. 6) also agrees with the proposed model, since both the heme-linked ionization of the Fe- L'→HX system and ligand interchange reactions are impaired in the ferriperoxidase-benzamide complex.

Furthermore, the similar affinities of mananganic and ferric peroxidases for hydroxamic acids contrast with those for fluoride and suggest again that the formation of RCONHOH-enzyme complexes does not entail replacement of a ligand in the first coordination sphere of the metal ion. Rather, as outlined previously, CO→X→Y exerts its effect indirectly, through hydrogen bonding, most likely to water coordinated at the distal site of the prosthetic group (L/H=H2O). Such hydrogen bonding should be of lesser importance when cyanide occupies the distal site since the nitride nitrogen is only a very weak proton acceptor (50). Indeed, the differences in affinities of ferriperoxidase-cyanide for benzhydroxamic acid and benzhydrazide (Table IV) are much smaller than those for the corresponding complexes with ferri peroxidase itself (Table I).

Similarly, if in ferriperoxidase the sixth coordination site of the prosthetic group is not occupied by water, as in ferrymyoglobin (60), the dissociation constants for ferriperoxidase-

Table VIII

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Absorption bands λ(cm^-1)/10^4*</th>
<th>Difference in molar absorptivities (A6410)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A6410 M^-1</td>
</tr>
<tr>
<td>Benzamide</td>
<td>638</td>
<td>3.42</td>
</tr>
<tr>
<td>Acetohydroxamic acid</td>
<td>638</td>
<td>3.47</td>
</tr>
<tr>
<td>Benzyldrazide</td>
<td>639</td>
<td>3.47</td>
</tr>
<tr>
<td>Benzamide</td>
<td>639</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

does not provide large binding energy (54) but becomes more significant when polyfunctional interactions can occur (55).

Consider now the following equilibria

$$K = \frac{HRP-L'}{HRP-L}$$

If HRP-L' is the only enzyme-ligand derivative, then ligation of benzamide, benzyldrazide, and benzhydroxamic acid are defined by ΔΔε values ranging from 28 x 10^4 to 60 x 10^4 m^-1 cm^-1 (Table VIII), a gradation of perturbation effects dependent on the —COXY structure.

Alternatively, if a spectroscopically inert HRP-L complex precedes the formation of HRP-L', then Δε will be contingent on K". When K" is large, only small spectroscopic changes may be expected. K' is then analogous to K, as defined for ternary enzyme-ligand complexes. Such comparison strengthens the proposal that binding of the R group of RCOXY is the main driving force in the ligand-enzyme association. It is this type of interaction, the hydrophobic binding, which must be responsible for the higher affinity of 2-naphthohydroxamic acid (K1 ~ 2 x 10^-7 M), compared to that of acetohydroxamic acid (K1 ~ 620,000 x 10^-7 M) and formhydroxamic acid (K1 ~ 0.3 M). The change in free energy of binding from 2-naphtho- to formhydroxamic acid amounts to ~ 8 kcal per mole, i.e. within the range of binding energies (~ 5 to ~ 10 kcal per mole) observed on association of nonpolar compounds with proteins (56, 57).

A plausible hypothesis is that both R and —CO→X→Y residues associate at a heme-linked, extended, largely apolar region of H-peroxidase, in which the R-crevice is also the binding site for the hydrogen donor substrates. The association of the —COXY moiety near the prosthetic group with a concomitant change in the relative position of trans-ligands, the central metal ion, and porphyrin would give a configuration more akin to that of metmyoglobin, where the porphyrin ring is not planar but slightly concave towards the sixth coordination position (58).

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Similarly, if in ferriperoxidase the sixth coordination site of the prosthetic group is not occupied by water, as in ferrymyoglobin (60), the dissociation constants for ferriperoxidase-RCOXY complexes should parallel K1 rather than K" values. The preliminary data on ferriperoxidase-benzhydroxamic acid complex, where K = 3 ± 2 x 10^-4 M supports this proposal.

These results do not preclude some interaction between the metal ion and the COXY moiety. For the observed shift of the ferriperoxidase Soret band from 437 to 428 nm, which is caused by an amine reagent, benzyldrazide, but not by benzhydroxamic acid (Table VII), may indicate a weak contribution from a hemochromogen type of complex. However, its formation cannot be extensive since the changes in the Soret region are not reflected in the pattern of α- and β-bands.

The strongest evidence suggesting the proximity of the RCOXY site to the prosthetic group is the fully competitive binding between RCOXY ligands and the peroxidase H-donors, i.e. phenols and aromatic amines (Table VI, Figs. 9 and 10).
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Gregory R. Schonbaum


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