J. Sci. I. R. Iran

Vol.8 No.4
Autumn 1997

ELUCIDATION OF pK_a VALUES FOR ACTIVE SITE OF HORSERADISH PEROXIDASE AND BINDING STUDY OF INTERACTION WITH N-PHENYL BENZHYDROXAMIC ACID USING A SPECIAL DIFFERENCE SPECTROPHOTOMETRIC TECHNIQUE

B. Farzami*, K. Nazari and A. A. Moosavi-Movahedi

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Islamic Republic of Iran
** Department of Biochemistry, Tehran University of Medical Sciences P.O. Box 14155-5399, Tehran, Islamic Republic of Iran*

Abstract

The binding behavior of a competitive inhibitor, N-phenylbenzhydroxamic acid (BHA) against horseradish peroxidase (HRP) was studied in order to understand and predict the interaction mechanism of hydrogen donors with the enzyme. The dissociation constants of the complexes of HRP-BHA, HRP-donor and HRP-BHA-azide were estimated at specified conditions by difference spectroscopy. The binding site of BHA and that of hydrogen donor were also detected by spectroscopic titration of HRP in the presence of BHA and specific amino acid modifiers. A histidine binding site with $pK_a = 6.40$ was detected for the binding of BHA. On the other hand, activity-pH measurements showed that the maximum interaction of BHA with HRP occurs at a minimum point of activity at pH=6.40. The catalytic behavior of His 42 in the kinetic mechanism of enzyme action was also discussed.

Introduction

Horseradish peroxidase (HRP; E.C. 1.11.1.7, donor: H_2O_2 oxidoreductase) catalyzes the oxidation by peroxide of a wide variety of substances, many of which have similar spectral absorption properties [1-4]. Some reviews about peroxidase biochemistry [5-8] and chemistry are available [9-11].

HRP forms spectroscopically distinct, reversible complexes with hydroxamic acids (R-CO-NHOH), hydrazides (R-CO-NHNH$_2$), amides (R-CO-NH$_2$) and aromatic substances (phenols, aminobenzoic acids, aniline, cresols,...) [12, 13]. Spectrophotometric, electron paramagnetic resonance and magnetic susceptibility revealed that hydroxamic acids bind close to the heam group at the distal side [14]. Also, the results indicate that the mode of inhibition of hydroxamic acids need not be, as generally supposed, by metal chelation. Mechanisms involving either hydrogen bonding at the hydrogen donor binding site or the formation of a charge transfer complex between hydroxamic acid and an electron-accepting group in the enzyme are considered to be more feasible. Furthermore, spectroscopic studies show that the association of HRP with hydroxamic acids entails a transition from a mixed spin state of the enzyme to a high spin derivative [12, 15]. High spin ferric HRP combines with cyanide, thiocyanide, flouride and azide anion,

Keywords: Benzhydroxamic acid; pK_a, Binding constant; Difference spectrophotometry; Horseradish peroxidase

producing a low spin derivative [16].

Interaction of N-phenyl benzhydroxamic acid (BHA) with HRP in the presence of azide has still not been investigated, especially from the binding studies point of view. However, interaction of HRP with the hydrogen donors has already been studied extensively [17-19]. Phenols and aromatic amines as hydrogen donors have long been recognized as the specific peroxidase substrates and dissociation constants governing their interaction with the enzyme have been determined [12]. The minimal distances between the enzyme iron and the protons of the phenol rings are in the range of 8.0-11.0 A° [20]. This means that hydrogen donors bind close to the active site of HRP.

In the present paper, we have reported a study of the interactive behavior of HRP related to N-phenyl benzhydroxamic acid in the presence of azide and hydrogen donors (guaiacol and P-toludine). It is the aim of this study to evaluate the binding and inhibition mechanism for HRP-BHA complexes in order to predict the binding site of BHA on HRP. Considering the same binding site for hydrogen donor compounds, a binding pattern may be introduced for the strength and the position of the binding of these compounds near the acitve site of HRP. These sites of binding of functional groups on HRP to hydroxamic acid were estimated using a special difference spectrophotometric technique devised in this laboratory [21-23].

Experimental Section

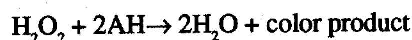
Materials

Horseradish peroxidase Type II and 4-bromophenacyl bromide diethylpyrocarbonate were obtained from Sigma. N-phenyl benzhydroxamic acid and guaiacol were purchased from Fluka Chemical Company. Activity measurements were made in buffer solutions of phosphate salts pH=7.1, 2.5 mM, I=0.0069. Other buffer solutions were prepared with acetate and tris buffer in a concentration of 0.05 M.

Methods

A UV/Vis Milton Roy spectrophotometer Model 3000 was used for kinetic studies and optical density measurements. In the activity or rate measurements, the assay was based on the formation of tetraguaiacol (a red-brown complex) from oxidation of guaiacol by hydrogen peroxide at the wavelength of 470 nm and temperature of 25°C in the time range of 20 seconds. The concentration of enzyme in 1.5 ml cuvette was approximately 10^{-9} M.

The general form of the reaction catalyzed by HRP is as below:



$$H_2O_2 + 2AH \rightarrow 2H_2O + \text{color product}$$

The spectrophotometer gives the rate of reaction as $\frac{\Delta A_{470}}{\Delta t}$ by analyzing the data automatically using an on-line kinetic computer program. This rate is easily converted to $\frac{\Delta x}{\Delta t}$ (rate of decomposition of H_2O_2) considering the $\varepsilon_{470}=1.3\times10^{4}\ M^{-1}.\ cm^{-1}$ for tetraguaiacol. Also, the rate could be written as the enzyme activity $\left(\frac{\text{unit}}{\text{mg, HRP}}\right)$ using the expression of

$$\frac{U}{mg} = \frac{4\times\Delta A_{470}/20\ \text{sec.}}{\varepsilon_{470}\times\frac{\text{mg HRP}}{\text{ml mixture}}}$$

One unit of HRP corresponds to the decomposition of 1μmol H_2O_2 per minute. To a cuvette containing 1.48 ml of guaiacol 90 mM in phosphate buffer with pH=6.4, 10μl of 1.5×10^{-7} M HRP at pH= 6.4 was added. After fixing the temperature at 25°C, 10μl of H_2O_2 11 mM was injected into the cell and the reaction was monitored during 20 seconds. at 470 nm as mentioned above.

The formation of HRP-ligand complexes was followed by difference spectrophotometry at 410 nm. At this wavelength, the difference in molar absorptivity ($\Delta\varepsilon_{410}$) is greatest, the ligands generally do not absorb and the measurements are most accurate, since 410 nm is near the absorption maxima of HRP (403 nm) and its complexes (408-416 nm) [12]. The titrations were carried out at 25°C by adding 10μl aliquots of ligand solution to 1.49 ml of HRP solution. For each sample solution with a particular ligand concentration, absorbance change (ΔA_{410}) due to formation of the related complex, was measured after incubation of the mixture for 10 minutes. The dependence of the equilibrium constant on hydronium ion concentration was examined in 5 mM acetate buffer, pH 4 to 5 and 2.5 mM phosphate buffer pH 5.5-8. Over the entire pH range, the dissociation constants were nearly independent of ionic strength when I≤ 0.05 M. In all calculations, the molecular weight of HRP was taken to be 40, 700 [24].

Also in the pK_a determination, difference spectrophotometric measurements were made by preparing solutions of HRP (0.03%w/v) in buffers ranging in pH from 3 to 9 with 0.1 units of pH intervals. For pHs 3.0-5.5 acetate buffer and for pHs 5.6-9.0 tris buffer was used.

The ionic strength was kept constant at 0.05 M. Two sets of equivalent tubes were prepared containing buffer solutions of identical pH and the enzyme HRP. In one set, 20 μl of a 1 M solution of BHA was added and the absorbance of each tube was read separately in a 1 ml cuvette at 275 nm. This wavelength was chosen for estimation of the pK_a of the groups on enzyme bound to BHA, since the initial observations showed that the

extinction coefficient of the HRP-BHA complex was largest at this wavelength. Thus, the ionization of the specific groups on the enzyme bound to HRP could alter the electronic charges on BHA more significantly in its specific pH range of ionization.

The experiments were repeated in the presence of 4-bromophenacyl bromide (as Asp. and Glu. modifier) at a pH range of 3.0-5.5, and diethylpyrocarbonate (a His modifier) at a pH range of 5.6-9.0 baseline for native HRP (without addition of BHA and modifiers) was obtained by comparing the absorbance of each tube containing native HRP against the absorbance of the buffer (as blank) with the same pH.

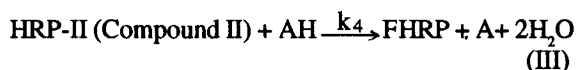
Results and Discussion

In the peroxidase kinetics it is necessary to distinguish between the two types of specific activity that may be measured depending on the experimental conditions: i) the velocity constant for the formation of the enzyme-substrate complex (Compound I):

$$\text{HRP} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{HRP-1 (Compound I)} \quad \text{(I)}$$

and ii) the rate constant for the reaction of the secondary complex with the hydrogen donor molecule:

$$\text{HRP-1} + \text{AH} \xrightarrow{k_7} \text{HRP-II (Compound II)} + \text{A} \quad \text{(II)}$$



$$\text{HRP-II (Compound II)} + \text{AH} \xrightarrow{k_4} \text{FHRP} + \text{A} + 2H_2O \quad \text{(III)}$$

The rate of utilization of peroxide to form the colored reaction product at 470 nm using guaiacol as substrate could be obtained (Equation 1), and k_1 and k_4 could be approximated from Equations (2) and (3) respectively;

$$\frac{dx}{dt} = \frac{[E]}{\frac{1}{k_4[D]_o} + \frac{1}{k_1[X]_o}} \quad (1)$$

where [E], $[D]_o$, and $[x]_o$ represent the concentration of enzyme, the initial concentration of donor, and the initial concentration of peroxide, respectively [2,25]. If the conditions of assay are chosen so that $k_4 [D]_o >> k_1[x]_o$; reaction (I) could represent the rate-limiting step and k_1 is obtained:

$$\frac{dx}{dt} \cong k_1 [x]_o [E] \quad (2)$$

where $\frac{dx}{dt}$ is the rate of substrate decay over the measured time interval. However, if the assay is adjusted for the conditions where $k_1[x]_o >> k_4[D]_o$ then, k_4 could be obtained [25]:

$$\frac{dx}{dt} \cong k_4 [x]_o [E] \quad (3)$$

Based on Equations (2) and (3) and the slopes of the linear plots of Figure 1, the values of k_1 and k_4 were found to be $k_1=0.4\times10^7$ and $k_4=1.3\times10^5$ sec^{-1} (for more details see the legend of Fig. 1).

The absorption spectrum of HRP in the Soret region is slightly altered in the presence of hydrogen donors. This indicates that binding occurs close to the haem group [17]. The binding constants of the ligands to HRP were obtained from spectrophotometric titration using the expression [26]:

$$\frac{1}{\Delta A} = \frac{K_d}{\Delta A_\infty} \times \frac{1}{[S]} + \frac{1}{\Delta A_\infty} \quad (4)$$

where K_d is the dissociation constant of HRP-ligand complex, ΔA is the observed absorbance change, ΔA_∞ is the maximum absorbance change and [S] represents the ligand concentration. Equation (4) indicates that changes of $\frac{1}{\Delta A}$ vs. $\frac{1}{[S]}$ are a line with Y-intercept of $\frac{1}{\Delta A_\infty}$ and slope of $\frac{K_d}{\Delta A_\infty}$. Thus, K_d could be determined from the slope of such a plot by a linear regression. Figure 2 shows a typical titration profile for the HRP-toluidine system, and the dissociation constant of the complex according to Equation (4) and Figure 2 is obtained as 5.3×10^{-7} M. For complexes

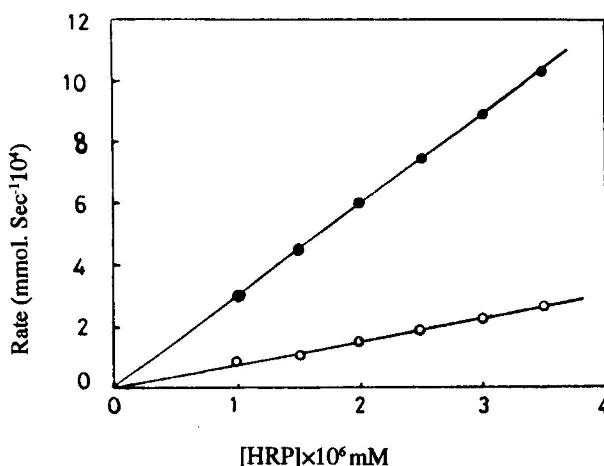


Figure 1. Dependence of the rate of reaction on the enzyme concentration
●, Slope = 294 = $k_1 [H_2O_2]$ from Equation (2), $[H_2O_2]_o = 7.3 \times 10^{-2}$ mM;
○, Slope = 77.8 = k_4 $[\text{guaiacol}]_o$ from Equation (3), $[\text{guaiacol}]_o$ = 0.6 mM

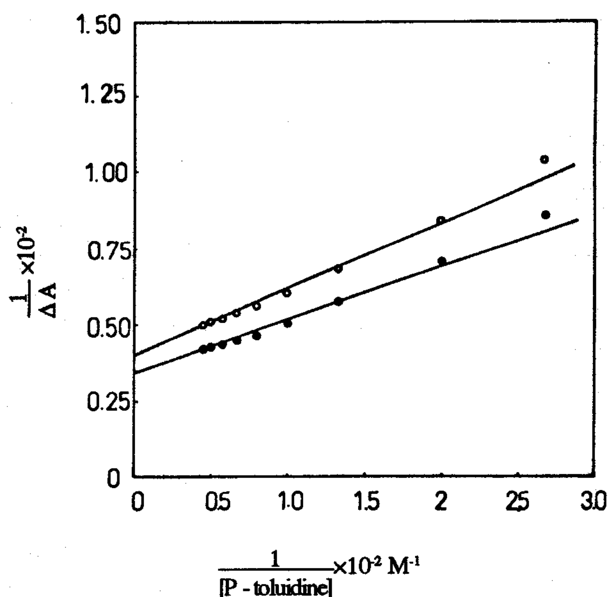


Figure 2. Reciprocal plots based on Equation (4) and estimation of K_d for HRP-toluidine complex in the absence of azide

●, In the presence of azide; ○, In the absence of azide

Slope $= \frac{K_d}{\Delta A_\infty} = 0.215$, Y-intercept $= \frac{1}{\Delta A_\infty} = 0.406\times10^{-2}$

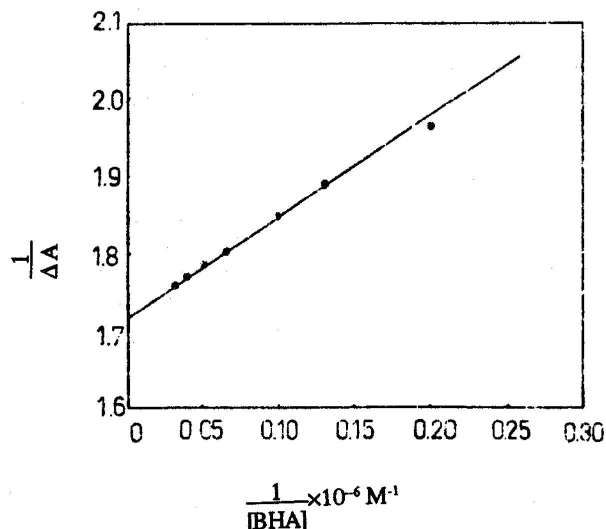


Figure 3. Titration of HRP (4×10^{-3} mM) with BHA and plotting the data based on Equation (5) for estimation of K_d and ε_{410} for HRP-BHA complex

Slope $= \frac{K_d \times r}{[HRP]_{tot} \times \varepsilon_{410}} = 1.322\times10^{-6}$,

Y-int$= \frac{1}{\varepsilon_{410} \times [HRP]_{tot}} = 1.717$, r=1.004 at pH=6.4 phosphate buffer 2.5 mM., 1=1 cm.

with benzhydroxamic acid (BHA), Equation (4) rearranges as below [12]:

$$\frac{1}{\Delta A} = \frac{K_d \times r}{[HRP]_{tot} \times 1 \times \varepsilon} \times \frac{1}{[BHA]_{tot}} + \frac{1}{1 \times \varepsilon \times [HRP]_{tot}} \quad (5)$$

$$r = \left(1 + \frac{K_a}{[H^+]}\right) \quad (6)$$

In the above equation, ε is the extinction coefficient of the complex, "1" is the solution light path, $[HRP]_{tot}$ is the total concentration of all ionized forms of HRP, and K_a is the acid dissociation constant of the ligand. Thus, the plot of

$$\frac{1}{\Delta A_{410}} \text{ vs. } \frac{1}{[BHA]} \text{ has a slope} = \frac{K_d \times r}{[HRP]_{tot} \times 1 \times \varepsilon} \text{ and}$$

Y-intercept $= \frac{1}{1 \times \varepsilon \times [HRP]_{tot}}$. Slope and intercept are obtained by a linear regression, and then by replacing the values of 1, $[HRP]_{tot}$ and r in the slope and intercept relations, the values of ε (extinction coefficient for HRP-BHA complex) and K_d (dissociation constant of the complex) could be obtained. Figure 3 shows a typical plot for the measurement of binding of N-phyenyl benzhydroxamic to HRP. Measurements were followed by recording the absorbance of the complex at 410 nm as explained in the experimental section. Figure 3 represents $\varepsilon_{410}=1.456\times10^{5}\ M^{-1}\ cm^{-1}$ and $K_d=7.65\times10^{-7}$ M (see legend of Figure 3).

In evaluating K_d for the complex under study, the acid-base properties of ligands were duly assessed [27] according to Equation (IV):

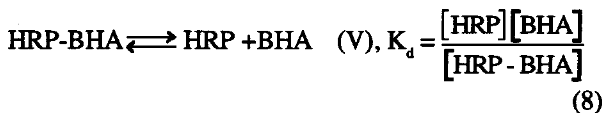
$$\text{R-CONHOH} \rightleftharpoons \text{RCONHO}^- + H^+ \ (pK_a = 8.8) \quad (IV)$$

The calculated K_d value on the basis of the neutral form of BHA was virtually invariant in the range of pH=3.0 to pH=9.0.

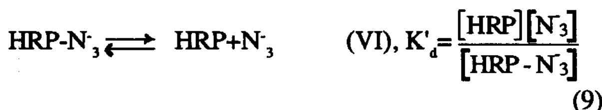
If the enzyme-BHA complex involves a direct coordination of BHA to the ferric ion, such a binding should be competitively inhibited by typical hemoprotein ligands such as azide anion. It reacts readily with HRP, giving a complex whose spectrum differs from that of the HRP-BHA complex [28]. For a fully competitive ligand interchange in a HRP-BHA-azide system, the ratio of $[azide]_{tot}$ to $[BHA]_{tot}$ is according to the following equation:

$$\frac{[HRP\text{-}azide]}{[HRP\text{-}BHA]} = \frac{K_d}{K'_d} \times \frac{[azide]_{tot}}{[BHA]_{tot}} \quad (7)$$

where Equation (7) may be obtained from the following reactions:



$$HRP\text{-}BHA \rightleftharpoons HRP + BHA \quad (V),\ K_d = \frac{[HRP][BHA]}{[HRP\text{-}BHA]} \quad (8)$$



$$HRP\text{-}N_3^- \rightleftharpoons HRP + N_3^- \quad (VI),\ K'_d = \frac{[HRP][N_3^-]}{[HRP\text{-}N_3^-]} \quad (9)$$

K_d's were obtained based on conditions where the concentrations of ligands $[BHA]_{tot}$ and $[azide]_{tot}$ were at least 100 times greater than the total concentration of the enzyme $[HRP]_{tot}$. In this condition, concentration of the complex is negligible related to the concentration of ligand, since we have: $[L]=[L]_{tot} - [HRP\text{-}L] \cong [L]_{tot}$ and then Equation (7) becomes satisfactory. The above relationships could be expressed in a linear form [12]:

$$\frac{1}{[HRP\text{-}BHA]} = \frac{1}{[HRP]_{tot}} \times \frac{K_d}{K'_d} \times \frac{1}{[BHA]_{tot}} \times [azide]_{tot} + \frac{1}{[HRP]_{tot}} \quad (10)$$

Equation (10) implies that the partition constant $\frac{K_d}{K'_d}$ increases with the increasing concentration of BHA. The partition constant shows the distribution of HRP between complexes in the form of HRP-BHA and HRP-azide, and therefore, at a fixed but greater concentration of BHA, more HRP molecules exist in the form of an HRP-BHA complex. This trend reflects the formation of a ternary HRP-BHA azide complex according to Equation (11):

$$HRP\text{-}BHA\text{-}azide \rightleftharpoons HRP\text{-}azide + BHA \quad (VII)$$

$$K''_d = \frac{[HRP\text{-}N_3^-][BHA]}{[HRP\text{-}BHA\text{-}N_3^-]} \quad (11)$$

where K''_d is the dissociation constant of ternary complex. Thus, the relationship between $\frac{1}{[HRP\text{-}BHA]}$ and $[azide]_{tot}$ should be:

$$\frac{1}{[HRP\text{-}BHA]} = \frac{1}{[HRP]_{tot}} \times \frac{K_d}{K'_d} \times \left(\frac{1}{[BHA]_{tot}} + \frac{1}{K''_d}\right) [azide]_{tot} + \frac{1}{[HRP]_{tot}} \quad (12)$$

so that the slope "m" of $\frac{1}{[HRP\text{-}BHA]}$ vs. $[azide]_{tot}$ becomes

$$m = \frac{1}{[HRP]_{tot}} \times \frac{K_d}{K'_d} \times \left(\frac{1}{[BHA]_{tot}} + \frac{1}{K''_d}\right) \quad (13)$$

or

$$\frac{1}{[BHA]_{tot}} = [HRP]_{tot} \times \frac{K'_d}{K_d} \times m - \frac{1}{K''_d} \quad (14)$$

From Equation (12), the reciprocal plot of [HRP-BHA] vs. azide concentration at constant and varied concentrations of BHA gives linear plots (Fig. 4a) from which the slopes could be replotted according to Equation (14) as shown in Figure 4b. Because of the formation of a ternary complex HRP-BHA-N_3^-, a single and real value for this partition ratio must be examined in a range of fixed concentrations of BHA as indicated in Figure 4a and estimated from the plot based on Figure 4b. Equation (14) represents a line with Y-intercept $= -\frac{1}{K''_d}$ and slope $= \frac{K'_d}{K_d} \times [HPP]_{tot}$. Thus, K_d and partition ratio $\frac{K_d}{K'_d}$ were obtained to be 4.13×10^{-4} M and 1.3 respectively (see also the legend of Fig. 4).

Furthermore, according to Equation (13), the values of "m" should converge to a limiting value with increasing

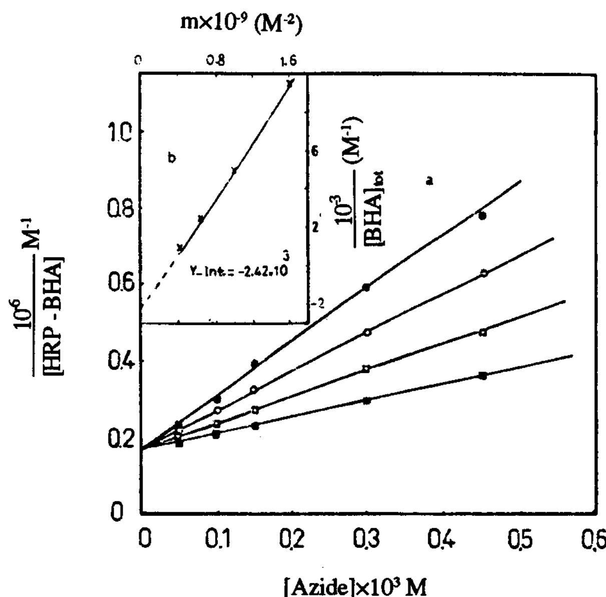


Figure 4. a) Titration of HRP with azide in the presence of different concentrations of BHA
■, 0.1 mM; □, 0.2 mM; ○, 0.4 mM; ●, 0.6 mM
b) plots based on Equation (14) for estimation of K_d and $\frac{K_d}{K_d}$
$[HRP]_{tot} = 6\times10^{-3}$ mM, Y-interecept $= -2.42\times10^{-3} = \frac{q}{K''_d}$,
Slope $= 4.58\times10^{-6} = \frac{K'_d}{K_d} \times 6\times10^{-6}$

ligand concentration and become equivalent to the value of

$(\frac{1}{[HRP]} \times \frac{K_d}{K'_d} \times \frac{1}{K''_d})$.

Such a condition is governed for the experiment, so that $\frac{1}{[BHA]_{tot}} << \frac{1}{K''_d}$ and the extent of reaction remains nearly independent of total concentration of BHA.

The dissociation constant of HRP-hydrogen donor complexes could thus be calculated, assuming that the binding of the specific substrates (S) and titrant (L) occurs at the same site. With the substrate in excess of ($[S]_{tot} >> [HRP]_{tot}$) at a pH where "S" and "L" are largely in the unionized forms, the relevant relationship is:

$$\frac{[HRP-L]}{[L]_{tot}-[HRP-L]} = \frac{[HRP]_{tot}}{K_d} \times \frac{K_S}{[S]_{tot}+K_S} - \frac{K_S}{K_d([S]_{tot}+K_S)} \times [HRP\text{-}L]. \quad (15)$$

From the above equation, K_s may be obtained. Plots based on Equation (15) are shown in Figure 5 for BHA as titrant and in the presence of guaiacol as substrate. Figure 5a shows titration of HRP with BHA in the presence of guaiacol 5 mM. Concentrations of the HRP-BHA complex were estimated using its molar absorptivity (1.46×10^5 M^{-1}. cm^{-1}) and then values for the Y-axis and x-axis, according to equation (15), were calculated and a plot as Figure 5b was obtained. Equation (15) represents a line with a slope of $-\frac{K_S}{K_d([S]_{tot}+K_S)}$ and x-intercept of $[HRP]_{tot}$. By replacing values of $K_d = 7.65 \times 10^{-7}$ M for HRP-BHA complex and $[S]_{tot} = 5 \times 10^{-3}$ M into the slope relation, a value of 6.6×10^{-3} M was obtained for the dissociation constant of HRP-guaiacol complex (for more details see the legend of Fig. 5). 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.

The pK_a values of functional groups bound to the active site of HRP were determined using a novel ultraviolet spectroscopic method devised in this laboratory [21-23]. The assumption used here was based on the fact that if an ionizing species in a protein molecule binds to a ligand i.e., metal ion or a molecule such as a substrate or a coenzyme in a specific manner, the extent of charge on the interacting molecule will be changed as a function of the ionization of the group under study. This effect could be transmitted to an adjacent aromatic group that may be present in the molecule or juxtaposed in a position where it could be affected by it. This effect is in fact similar to the direct titration of the group and could be followed as a function of changing pH [29, 30]. Theoretical studies have also shown that the average distance of an aromatic residue in a protein is about 0.8÷1.0 nm away from any reference point [31]. This distance is close enough for a ligand to mediate the transfer of charges according to our previous observations [29, 30]. Therefore, whether the ligand is in itself chromophore such as BHA in our case, or a chromophoric species located at close proximity, the transfer of a charge could be relayed.

On the basis of the above evidence the plot of changes of difference absorption or emission spectra, when a substrate such as BHA is specifically bonded to a binding site, is plotted against pH, and a peak is produced corresponding to the pK_a value of the group that interacted with that specific ligand. The mode of this trend is discussed and formulated by the dependence of a physical property such as molar absorptivity or the rate of reaction on pH variation [22].

Using the difference spectrophotometric method, two sharp peaks at pH=4.34 and 4.56 which belong to aspartate and/or glutamate carboxyl groups interacting with BHA were obtained. Another sharp peak appeared at pH=6.38 which was assigned to the imidazole group of histidine (Fig. 6). At pH=8.80, a minimum in the titration curve profile was observed that coincides with the pI of HRP obtained in our studies using the titration procedure of

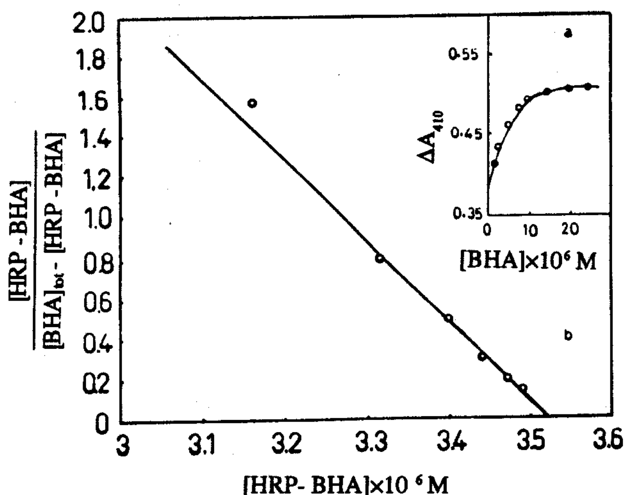


Figure 5. a) Titration of HRP (3.5×10^{-3} mM) with BHA in the presence of guaiacol (5 mM). $\varepsilon_{410} = 1.456 \times 10^5 M^{-1}. cm^{-1}$ (from Figure 3) was used for the calculation of HRP-BHA complex concentrations which were used for plotting the data of Figure 5b. b) Plot based on Equation (15) for estimation of K_s for HRP-guaiacol complex
[guaiacol] = 5 mM, [HRP]= 3.524×10^{-6}M

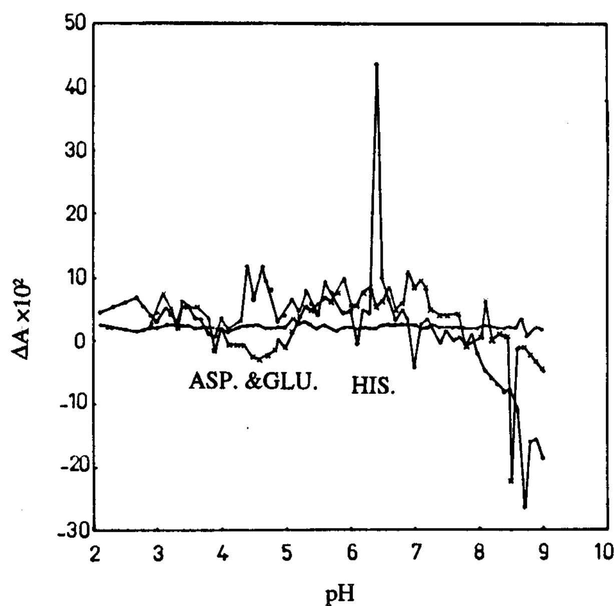


Figure 6. pH profile for estimation of pK_a's values for the interacting functional groups of HRP with BHA.
○, Native baseline; ●, In the presence of BHA;
×,HRP+modifiers in the presence of BHA

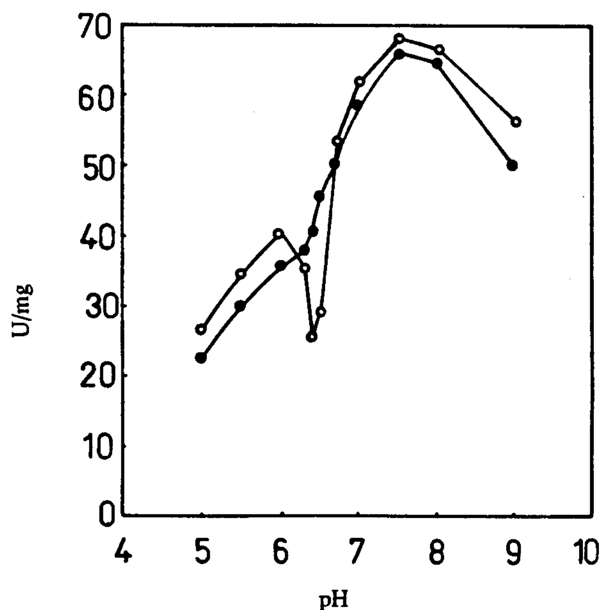


Figure 7. Activity measurements of the enzyme as a function of pH. ○, In the presence of BHA; ●, In the presence of diethylpyrocarbonate; in the absence of diethylpyrocarbonate

Tanford [32]. Figure 7 shows the changes in enzyme activity due to the incremental variation in pH. With BHA present, a maximum at pH (optimum)=7.45 was obtained, although when a specific modifier of histidine, diethylpyrocarbonate was used, a sharp dip in the pH rate profile at pH =6.38 was obtained that coincided with the pK_a obtained from the titration curve of the active site. All the values obtained from the active site titration of functional groups were further substantiated using specfic modifiers. Using 4-bromophenacyl bromide, a modifier for aspartate and glutamate, diminshed the peaks at 4.34 and 4.56. The use of specific histidine modifier diethyl pyrocarbonate removed the peak at pH=6.40 (Fig. 6). The evidence of pH rate profile, together with the results obtained from titration curve regarding the values of pK_a's strongly suggests that at least the three residues of Asp., Glu. and His are interacting with BHA. Binding data clearly indicate that BHA binds strongly to HRP and forms a 1:1 stable complex with the enzyme. Also a comparison of the value of K_d and K'_d shows that the formation of a HRP-azide complex is more probable than HRP-BHA complex, in a mixture of HRP, BHA and azide anion. This may be attributed to the direct coordination of azide to the ferric ion. The value of K_d indicates that the ternary complex dissociates to other binary complexes such as HRP-N_3^- and HRP- BHA.

According to the previous reports, HRP has only three His residues in its primary structure. One of them (His 170) occupies the fifth coordination position on the haem iron (proximal His) [33, 34]. Folding of HRP to an α-helical form led to locating His 42 at the distal side and close to the haem group (distal His) [35]. His 42 is associated with Arg. 38 which participates in the heterolytic cleavage of O-O bond of hydrogen peroxide on the haem iron [34]. We could assume that the same His is involved in binding the enzyme with BHA [25-28]. Therefore, it could be suggested that through such interactions a hydrogen bond between the hydroxyl group of BHA and the imidazole moiety of histidine 42 could be formed which could lead to the competitive inhibition of the enzyme by BHA against the hydrogen donor binding [36].

Apparently, in contrast to the association between BHA and HRP-azide complex, the binding of hydrogen donors and BHA is fully competitive [12]. Figure 2 demonstrates that donors associate nearly as well with ferri-HRP-azide complex. Thus, gauged by donor bindings, the conversion of high spin ferric enzyme to a low spin azide derivative does not entail a pronounced change in the structure of the apoenzyme. Since the association of HRP with BHA is competitively inhibited by specific enzyme substrates (hydrogen donors), such behavior implies the proximity of a hydrogen donor to the active site of HRP and the residue of His 42 could be introduced as a catalytic residue in the kinetic mechanism of HRP action.

Acknowledgements

The financial support given this project by the Research

Council of the University of Tehran is gratefully acknowledged.

References

1. Maehly, A. C. In *Methods of biochemical Analysis*, (ed. Glick), p. 358. Interscience Publishers, New York, (1954).
2. Chance, B. The properties of the enzyme-substrate compounds of HRP and peroxides, part III. *Arch. Biochem. Biophys*, **22**, 224, (1949).
3. Chance, B. The properties of the enzyme-substrated compounds of HRP and peroxides, part IV. *Ibid.*, **41**, 416, (1952).
4. George, P. Mechanism of the catalytic action of peroxidases. *Biochem. J.* **54**, 267, (1953).
5. Williams, R .J. P., Moore, G. R. and Wright, P. E. In *Biochemical aspects of inorganic chemistry*, pp. 369-441. Wiley Interscience, New York, (1977).
6. Morrison, M. and Schonbaum, G.R. Kinetics of oxidation of PABA catalyzed by HRP. *Ann. Rev. Biochem.*, **45**, 681, (1976).
7. Yonetani, T. In *Enzymes*, **13**, 345, (1976).
8. Schonbaum, G. R. and Chance, B. *Ibid.*, **13**, 363, (1976).
9. Paul, K.G. *Ibid.*, (3rd edn, **8**, 227, (1963).
10. Saunders, B. C., Holmes-Siedle, A. G. and Stark, B. P. In *Peroxidases*. London, Butterworths, (1964).
11. Brill, A. S. and Williams, R. J. P. Mechanism of the peroxidase action, *Biochem. J.*, **78**, 246, (1961).
12. Schonbaum, G. R. New complexes of peroxidases with hydrozides and amides. *J. Biol. Chem.*, **248**, 502, (1973).
13. Ator, M. A., David, S. K. and Montellano, P. R. Structure and catalytic mechanism of HRP. *Ibid*, **262**, 14954, (1987).
14. Rich, P. R., Wiegand N. K. and Moore, A. L. Studies on the mechanism of inhibition of redox enzymes by substituted hydroxamic acids, *Biochim. Biophys. Acta*, **525**, 325, (1978).
15. Dunford, B. H. On the function and mechanism of action of peroxidases. *Coord. Chem. Rev.*, **19**, 187-251, (1976).
16. La Mar, G. N., Hernandez, G. and de Ropp, J. S. ^{1}H NMR investigation of the influence of interacting sites on the dynamics and thermodynamics of ligand binding to HRP, *Biochemistry*, **31**, 9158, (1992).
17. Lanir, A. and Schejter, A. Equilibria between HRP and aromatic donors. *Biochem. Biophys. Res*, **62**, 199, (1973).
18. Morishima, I. and Ogawa, S. NMR studies of hemoproteins. *J. Biol. Chem.*, **254**, 2814, (1979).
19. Burns, P. S. and Williams, R. J. P. Interaction of HRP with aromatic peracids. *J. Chem. Soc. Chem. Commun.*, **1975**, 795, (1975).
20. Sakurada, J. and Takahashi, S. NMR studies on the spatial relationship of aromatic donor molecules to HRP. *J. Biol. Chem.*, **261**, 9657, (1986).
21. Farzami, B. and Jordan, F. *Metal ions in biology and medicine*, (ed. P. Collery, L. A. Poirier, M. Manfait and J. C. Etienne), p. 126. John Libbey Eurotext, Paris, (1990).
22. Farzami, B. In *Metal ions in biology and medicine*, (ed. V.Z. Anastussopoulou and P. Collery), p. 102, John Libbey Eurotext, Paris, (1992).
23. Farzami, B., Kuimov, A. M. and Kochetov, G. *Ibid.*, p. 40. (1992).
24. Hay, R. W. *Bio-inorganic chemistry* p. 140., Ellish Harwood Limited, (1991).
25. Maehly, A. C. Plant peroxidases. *Meth. Enzymol.*, **2**, 801, (1955).
26. Schejter, A., Lanir, A. and Epstein, N. Binding of hydrogen donors to HRP. *Arch. Biochem. Biophys.*, **174**, 36, (1976).
27. Wise, W. M. and Brandt, W. W. J. *Amer. Chem. Soc.*, **77**, 1058, (1955).
28. Kelilin, D. and Hartree, F. Purification of HRP and comparison of its properties with those of catalase and methaemoglobin, *Biochemistry*, **49**, 88, (1951).
29. Farzami, B., Kuimov, A. N. and Kochetov, G. A. The calcium binding sites of the baker's yeast transketolase. *J. Sci. I.R. Iran*, **3**, 81, (1992).
30. Farzami, B., Moosavi-Movahedi, A. A. and Naderi, G. A. Elucidation of pK_a values for Ca^{2+} binding sites in calmodulin by spectrofluorometry. *J. Biol. Macromol.*, **16**, 181, (1994).
31. Demchenko, A. P. *Ultraviolet spectroscopy of proteins*, p. 263. Springer Verlag, Heidelberg, (1986).
32. Tanford, C. Protein titration. *Adv. Protein. Chem* .,**17**, **69**, (1962).
33. Welinder, K. G. The covalent structure of HRP. *Eur. J. Biochem*, **96**, 483, (1986).
34. Poulos, T. L. and Kraut, J. The stereochemistry of peroxidase catalysis. *J. Biol. Chem.*, **225**, 8199, (1980).
35. Finzel, B. C., Poulos, T. L. and Kraut, J. Crystal structure of cytochrome C peroxidase. *Ibid.*, **259**, 13027, (1984).
36. Banci, L., Bertini, I., Bini, T., Tien, M. and Turano, P. Binding of horseradish, lignin and manganese peroxidases to their respective substrates. *Biochemistry, 32*, **5825**, (1993).