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Chemical and Kinetic Evidence for an Essential Histidine in Horseradish Peroxidase for Iodide Oxidation*

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Horseradish peroxidase (HRP), when incubated with diethylpyrocarbonate (DEPC), shows a time-dependent loss of iodide oxidation activity. The inactivation follows pseudo-first order kinetics with a second order rate constant of 0.43 min^{-1} M^{-1} at 30 °C and is reversed by neutralized hydroxylamine. The difference absorption spectrum of the modified *versus* native enzyme shows a peak at 244 nm, characteristic of *N*-carbethoxyhistidine, which is diminished by treatment with hydroxylamine. Correlation between the stoichiometry of histidine modification and the extent of inactivation indicates that out of 2 histidine residues modified, one is responsible for inactivation. A plot of the log of the reciprocal half-time of inactivation against log DEPC concentration further suggests that only 1 histidine is involved in catalysis. The rate of inactivation shows a pH dependence with an inflection point at 6.2, indicating histidine derivatization by DEPC. Inactivation due to modification of tyrosine, lysine, or cysteine has been excluded. CD studies reveal no significant change in the protein or heme conformation following DEPC modification. We suggest that a unique histidine residue is required for maximal catalytic activity of HRP for iodide oxidation.

Hemoproteins display diversity in biological function, although they have essentially the same heme prosthetic group containing iron protoporphyrin IX. The structural difference in the folded polypeptide chain around the heme cavity appears to dictate the specificity of the catalytic function. In recent years, much attention has been directed toward a determination of the structural peculiarity around the heme cavity of a number of hemoproteins, including cytochrome P-450, hemoglobin, myoglobin, catalase, and peroxidases. Among the peroxidases, cytochrome *c* peroxidase, chloroperoxidase, and horseradish peroxidase have been viewed as model enzymes, because their primary sequences are known, and they have very similar molecular weights. Although the main function of the peroxidases is to oxidize various electron donors by H_2O_2, characterization of the structural domains of the heme periphery, substrate-binding sites, and the route of electron transport from binding sites to the heme iron is incomplete.

Horseradish peroxidase catalyses the oxidation of a large number of electron donors, including aromatic compounds and iodide (Dunford and Stillman, 1976; Dunford, 1982). The oxidation of aromatic donors occurs by two one electron transfer reactions through intermediate formation of compound I and compound II (Dunford and Stillman, 1976; Morrison and Schonbaum, 1976). However, the oxidation of iodide is mediated by one two-electron transfer reaction to compound I only (Roman and Dunford, 1972; Ohtaki *et al.*, 1981; Ohtaki *et al.*, 1982; Nakamura *et al.*, 1985) as shown below.

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{compound I} \quad (1)$$

$$\text{Compound I} + I^- \rightarrow \text{peroxidase} + I^+ \quad (2)$$

The reaction between compound I and iodide leading to I_3^- formation occurs through the intermediate formation of enzyme-hypoiodous complex (*E*OI) as follows (Morrison and Schonbaum, 1976; Magnusson *et al.*, 1984).

$$E + H_2O_2 \xrightarrow{H_2O} EO \xrightarrow{I^-} [EOI]^- \rightarrow E + IO^- \quad (3)$$

$$IO^- + I^- + H^+ \rightarrow I_2 + OH^- \quad (4)$$

$$I_2 + I^- \rightleftharpoons I_3^- \quad (5)$$

Reaction 3 indicates that the enzyme has one binding site for H_2O_2 to form activated enzyme (*E*O) and another site for its interaction with iodide. Although heme iron binds H_2O_2 to form heme ferryl oxygen (Fe(IV)=O), the site responsible for iodide binding prior to electron flow to the heme ferryl group is not precisely known. On the other hand, aromatic donors have been shown to bind at a hydrophobic site in the vicinity of the heme peripheral 8-methyl group (Paul and Ohlsson, 1978, Sakurada *et al.*, 1986). Early kinetic (Bjorkstein, 1970; Pommier *et al.*, 1973), fluorometric (Ugarova *et al.*, 1981) and NMR studies (Sakurada *et al.*, 1985) suggested that iodide may form a complex near the heme moiety of the HRP.[1] Proton NMR studies by Sakurada *et al.* (1987a) have recently revealed that iodide binds to HRP an almost equal distance from the heme peripheral 1- and 8-methyl groups at the distal pocket of the heme. However, due to the absence of a suitable x-ray crystal structure of HRP, adequate information on the heme distal pocket and the role played by the fine structural features during iodide oxidation is lacking. The heme distal pocket of several peroxidases contains some important and invariant amino acid residues. Horseradish peroxidase, cytochrome *c* peroxidase, ligninase, and four isozymes of turnip peroxidase show a highly conserved sequence at the distal pocket of the heme (Tien and Tu, 1987). The invariant histidine residue in concert with arginine for the peroxidases (Poulos and Kraut, 1980) and asparagine for the catalase (Fita and Rossman, 1985) may play a critical role in the catalytic mechanism. The presence of histidine at the heme distal pocket of the lactoperoxidase has also been proposed by Sakurada *et al.* (1987b). Their ^1H NMR and ^{127}I NMR

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[1] The abbreviations used are: HRP, horseradish peroxidase; DEPC, diethylpyrocarbonate.

studies revealed that iodide binding of lactoperoxidase is facilitated by protonation of an ionizable group with a pK_a value of 6–6.8, which is presumably the distal histidine residue. Very recently, Blanke and Hager (1990) have provided evidence by chemical modification studies on chloroperoxidase that 1 histidine residue is essential for catalytic activity, and this may be ascribed to the distal histidine 38 residue of the enzyme.

In this paper, we present evidence by chemical modification studies that HRP loses its catalytic activity to oxidize iodide when 2 of its histidine residues are modified with the histidine-selective reagent diethylpyrocarbonate, but only one of them is essential for the catalytic activity of the enzyme.

EXPERIMENTAL PROCEDURES

Horseradish peroxidase (A_{403}/A_{278} = 3), diethylpyrocarbonate, and hydroxylamine were purchased from Sigma. All other chemicals used were analytical grade. The concentration of HRP was determined by using ϵ_{403} = 102 cm^{-1} mM^{-1} (Aibara *et al.*, 1982).

All kinetic measurements were made in a Pye-Unicam SP8-100 UV/VIS recording spectrophotometer at 30 ± 2 °C, unless otherwise stated. Peroxidase-catalyzed iodide oxidation was monitored by following the formation of I_3^- at 353 nm (Alexander, 1962), as described earlier (Banerjee *et al.*, 1986). The reaction mixture contained, in a final volume of 3 ml, 50 mM sodium acetate buffer, pH 4.5, 1.7 mM KI, a suitable amount of enzyme, and 0.27 mM H_2O_2 added last to start the reaction. All spectral studies were carried out in a Hitachi U-2000 spectrophotometer at 28 ± 2 °C, using quartz cells of a 1-cm light path.

Modification of Horseradish Peroxidase with DEPC—Just prior to use, fresh solutions of DEPC were prepared by dilution of the original DEPC stock with absolute ethanol. The exact concentration of the stock solution was calculated from the increase in the absorbance at 230 nm when an aliquot of the DEPC solution was added to a solution of 10 mM imidazole in 100 mM potassium phosphate buffer, pH 7.5, using an extinction coefficient of 3200 M^{-1} cm^{-1} (Melchior and Fahrney, 1970). The loss of HRP-catalyzed iodide oxidation after incubation with DEPC was determined by the standard assay method described above (Banerjee *et al.*, 1986). For incubations performed at 30 ± 2 °C, the aliquots were removed and immediately quenched with 500 mM potassium phosphate buffer, pH 4.5, at 4 °C, which prevented further inactivation for at least 1 h. For pK_a determinations, HRP was incubated with an excess of DEPC at various pH values (pH 5–8). The modification reaction is specific for an unprotonated histidine residue between pH 5.5 and 7.5 (Lundblad and Noyes 1984). The stoichiometry of the formation of *N*-carbethoxyhistidine residues was calculated from the increase in absorbance at 244 nm using the extinction coefficient of 3200 M^{-1} cm^{-1}. The reaction was initiated by the addition of DEPC and was terminated when the maximum absorbance at 244 nm had been attained.

Preparation of Hydroxylamine—Hydroxylamine solution was prepared by dissolving the solid reagent in 100 mM potassium phosphate buffer followed by titration to pH 6.8 with potassium hydroxide.

RESULTS

Kinetics of Inactivation of HRP by DEPC—When incubated with DEPC, HRP loses its catalytic activity to oxidize iodide by H_2O_2. Both time- and concentration-dependent inactivation of the enzyme (Fig. 1*A*) were observed with excess DEPC dissolved in 8% ethanol at near neutral pH values. The enzyme was not inactivated by ethanol alone. A plot of $\ln(A/A_0)$ against time was used to estimate the rate of inactivation according to the equation $\ln(A/A_0) = K_{obs} \chi t$ (Blanke and Hager, 1990). The inactivation followed first order kinetics with respect to the concentration of DEPC (Fig. 1*A*). The K_{obs} values can be calculated from the slopes of the curves in Fig. 1*A* and were found to be proportional to the concentration of DEPC (Fig. 1*B*). The linearity of this curve indicates a bimolecular reaction between the enzyme and DEPC without reversible complex formation (Church *et al.*, 1985). From the slope of the curve in Fig. 1*B*, the second order rate constant was calculated to be 0.43 min^{-1} M^{-1}.

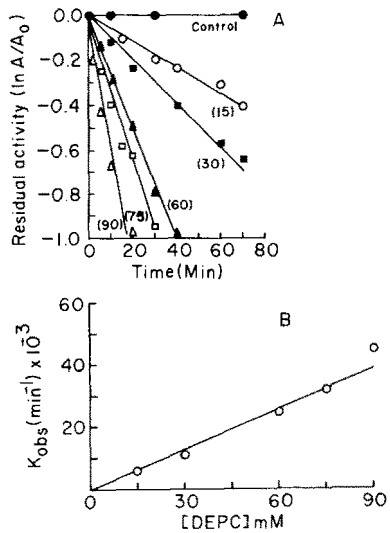

FIG. 1. **Kinetics of the inactivation of HRP with DEPC.** *A*, calculation of the pseudo-first order rate constant of inactivation for iodide oxidation. HRP (2.5 μM) was incubated with different concentrations of DEPC at 30 ± 2 °C in 100 mM potassium phosphate buffer, pH 6.0, and the data were plotted as described in the text. The curves were best fit by least square analysis. The concentrations of DEPC used were indicated in *parentheses*. *B*, determination of the second order rate constant of inactivation of iodide oxidation by HRP. The slopes of the straight lines obtained in *panel A* were plotted against concentrations of DEPC. The slope of this curve indicates the second order rate constant of inactivation, which is 0.43 min^{-1} M^{-1}.

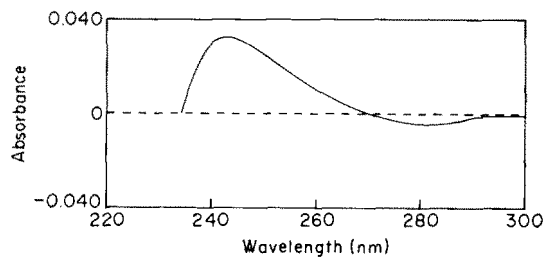

FIG. 2. **UV difference absorption spectrum of native *versus* DEPC-modified HRP.** HRP (5 μM) was incubated with 60 mM DEPC in 100 mM phosphate buffer, pH 6, and the spectrum was taken at the end of 20 min. The reference cuvette contained the enzyme with the same volume of ethanol used in DEPC solution, whereas the experimental cuvette contained the DEPC-modified enzyme solution.

UV Difference Spectrum of DEPC-modified HRP versus Native HRP—The optical difference spectrum of the DEPC-modified HRP *versus* native enzyme is shown in Fig. 2. After 20 min of incubation, the spectrum showed a peak at 244 nm characteristic of the formation of *N*-carbethoxyhistidine. No significant change in absorbance at 280 nm was evident in the spectrum. Thus, the inactivation of HRP by DEPC could not be due to formation of a *O*-carbethoxy derivative of tyrosine residues, which shows a decrease in absorbance at 280 nm (Miles, 1977).

Reaction of DEPC-modified HRP with Hydroxylamine—Hydroxylamine removes the ethoxyformyl group from modified histidine and tyrosine residues but not from the more stable ethoxyformylcysteine and ethoxyformyllysine residues (Miles, 1977). Fig. 3 shows that DEPC-inactivated HRP was completely reactivated on treatment with hydroxylamine, suggesting that the inactivation was due to modification of histidine residues and not due to modification of cysteine or lysine. This result also indicates that the ethoxyformylhistidine did not react further with DEPC, resulting in the cleav-

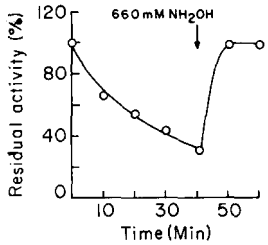

FIG. 3. **Reaction of DEPC-inactivated HRP with hydroxylamine.** HRP (2.5 μM) was incubated with 60 mM DEPC in 100 mM phosphate buffer, pH 6. After 40 min, hydroxylamine was added to a final concentration of 660 mM and incubated for 20 min, and the mixture was dialyzed against a large volume of distilled water for 2 h with changes at 15-min intervals. A control enzyme solution was similarly treated. At the end of dialysis, aliquots were withdrawn from both the control and treated sample for the assay of enzyme activity. Both enzymes showed 80% of the original activity, which is now considered as 100%.

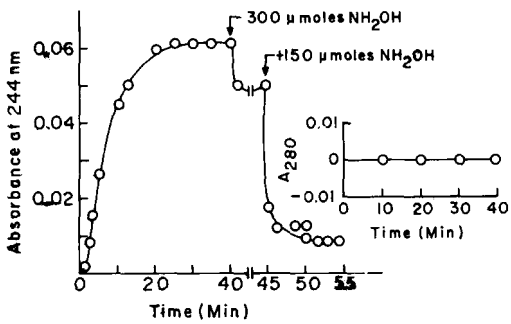

FIG. 4. **Effect of hydroxylamine on optical absorbance at 244 nm.** HRP (7.5 μM) was incubated with 60 mM DEPC in 100 mM phosphate buffer, pH 6, and the change in absorbance against control enzyme was monitored at the time indicated. At the end of 40 min, hydroxylamine was added, and the decrease in absorbance was noted as a function of time. The *inset* of the figure shows no change of absorbance at 280 nm with time as an indication of no tyrosine modification.

age of the imidazole ring (Miles, 1977). Otherwise, the loss of activity would have been irreversible with hydroxylamine. Moreover, the absorbance at 244 nm attained after 40 min falls immediately after addition of hydroxylamine, indicating reversal of the modified histidine residue only (Fig. 4). However, modification of tyrosine residues with DEPC was ruled out by the absence of any significant change in absorbance at 280 nm characteristic to tyrosine modification (Fig. 4, *inset*).

Correlation between the Number of Histidines Modified and the Loss of Catalytic Activity—The number of histidine residues modified can be estimated from the absorbance change at 240 nm (ϵ_{240} = 3200 M^{-1} cm^{-1}) upon addition of DEPC (Ovadi *et al.*, 1967). Fig. 5 represents the correlation between the extent of enzyme inactivation and the extent of histidine modification over an incubation period of 40 min with DEPC. The increase in absorbance at 244 nm represents the extent of modification, whereas inactivation is expressed as the percent of residual activity. The figure shows that after 5 min of incubation with DEPC when 50% of the total histidine is modified, there was no significant loss of activity (activity remaining was 95%). On subsequent modification of the second histidine residue, the activity declined gradually. When 70% of the activity was lost, the modification of the second histidine is almost complete. This suggests that HRP contains two types of histidine residues. The first one, which reacts with DEPC at a faster rate, is not involved in activity, whereas the second one, which reacts relatively slowly, is probably pertinent to activity. This has further been clarified in Fig. 6,

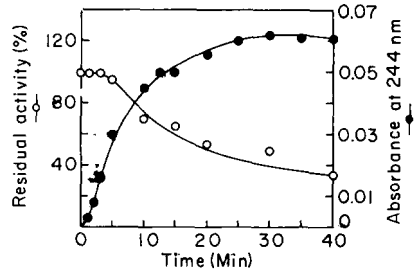

FIG. 5. **Correlation between the extent of inactivation and the extent of modification.** HRP (7.5 μM) was incubated with 60 mM DEPC in 100 mM phosphate buffer, pH 6. The percent of the residual activity and the increase in the absorbance at 244 nm as a measure of modification were plotted as a function of time as indicated.

FIG. 6. **Correlation between the number of histidine residues modified per HRP molecule and the extent of residual activity.** The amount of enzyme and other conditions are the same as described in the legend to Fig. 5. The residual activity was plotted against the number of histidine residues modified per enzyme molecule, and the curve was extended to the x axis to determine the total number of histidine residues modified/HRP molecule.

where the number of histidine residues modified per HRP is plotted against the residual activity. The data clearly indicate that there is one nonessential histidine residue, the modification of which is not associated with the loss of activity. However, when the next 1.5 histidine residues are modified, the enzyme loses 30% of its original activity. When 2 histidine residues are modified, 70% of the activity is lost. The enzyme is completely inactivated with modification of 2.2 histidine residues (extrapolation of the figure), suggesting that the second histidine facilitates the catalytic activity of the enzyme.

Kinetic Analysis of the Determination of the Number of Essential Histidine Residues—In order to determine the number of essential histidine residues in HRP for iodide oxidation, a kinetic analysis was made (Levy *et al.*, 1963; Takeuchi *et al.*, 1986). The inactivation process may be represented as follows,

$$\text{HRP} + n\text{DEPC} \rightarrow \text{HRP-DEPC}_n \quad (6)$$

where HRP-DEPC$_n$ is the inactivated modified enzyme and n represents the number of moles of DEPC required to react with the essential histidine residues to inactivate HRP. The rate of inactivation may be expressed as follows.

$$-\text{d[HRP]}/dt = K[\text{HRP}][\text{DEPC}]^n \quad (7)$$

If [DEPC] $\gg$ [HRP], Equation 7 can be integrated to the following form.

$$\log K_{obs} = n\log[\text{DEPC}] + \log K \quad (8)$$

A plot of log K_{obs} against log[DEPC] will have a slope equal to n. The order of the inactivation was determined experimentally by plotting the K_{obs} values as a function of DEPC concentration. For convenience, the K_{obs} value in Equation 8 may be replaced by the reciprocal of the half-life. This intro-

duces a constant having no effect on the slope (Levy *et al.*, 1963). Hence, the above equation may now be modified as follows.

$$\log 10^3/t_{1/2} = n\log[\text{DEPC}] + \log K \quad (9)$$

The plot of log $10^3/t_{1/2}$ against log[DEPC] (Fig. 7) gives rise to a straight line with the slope $n = 0.97$, which is near unity. This suggests that only one histidine residue is needed for the catalytic activity of HRP.

pH Dependence of the Inactivation of HRP by DEPC—As the unprotonated amino acid residues of a protein are modified by DEPC, valuable information on the nature of the modified group may be obtained from the studies of pH dependence of inactivation of HRP by DEPC. The extent of inactivation of the enzyme by DEPC is dependent on the pH, as shown in Fig. 8*A*. The data may be expressed by

$$K_{obs} = \frac{(K_{obs})_{max}}{1 + \frac{[H^+]}{K_a}} \quad (10)$$

which in linear form may be represented as

$$K_{obs}(H^+) = K_a(K_{obs})_{max} - K_aK_{obs} \quad (11)$$

where K_a is the dissociation constant of the reacting group and $(K_{obs})_{max}$ is the pseudo-first order rate constant of the unprotonated reacting group (Takeuchi *et al.*, 1986). The pseudo-first order rate constant was determined and plotted against pH (Fig. 8*A*). The *open circles* indicate experimental data, and the *solid line* indicates the theoretical curve. By plotting K_{obs} (H^+) against K_{obs} (values taken from Fig. 8*A*), a straight line is obtained (Fig. 8*B*), from which the values of $(K_{obs})_{max} = 0.05$ min^{-1} and $pK_a = 6.22$ were calculated from the ordinate intercept and the slope of the line, respectively. The apparent pK_a value of 6.22 offers additional evidence that the inactivation of HRP is due to modification of the histidine residue.

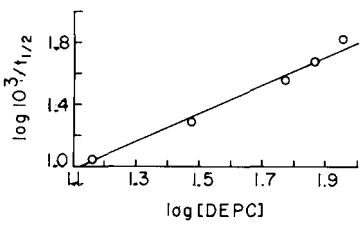

FIG. 7. **The apparent inactivation order of HRP in relation to DEPC concentrations.** The conditions are the same as indicated in the legend to Fig. 1. The slope of this line, 0.97, indicates that modification of a single histidine residue is necessary for inactivation of the HRP.

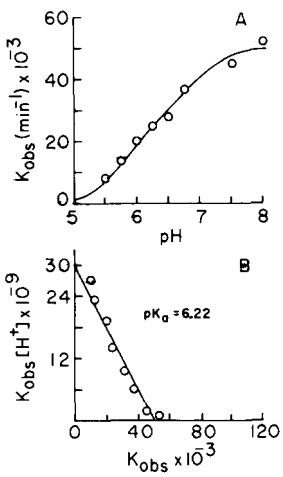

FIG. 8. **The pH dependence of the inactivation rate of HRP with DEPC.** *A*, HRP (2.5 μM) was incubated with 60 mM DEPC in 100 mM phosphate buffer, pH 5–8. The pseudo-first order rate constants obtained were plotted against pH. The curve obtained was a theoretical one. *B*, the experimentally obtained pseudo-first order rate constants were plotted according to Equation 10. The pK_a value obtained from the slope was 6.22 and the $(K_{obs})_{max}$ value was 0.05 min^{-1}.

CD Spectra of the Native and DEPC-modified HRP—Circular dichroism spectra of the native HRP was compared with that of the DEPC-modified HRP (data not shown). The heme CD peak at 400 nm and protein troughs at the UV region are not altered after modification. Moreover, no significant change of the Soret spectrum at 403 nm was evident after modification of the enzyme (data not shown). Thus, the inactivation of HRP by DEPC does not appear to result from nonspecific effects on protein and heme structure.

DISCUSSION

Chemical modification is one of the versatile tools used to delineate requirements for catalytic activity of an enzyme. The identity of the functional amino acid(s) in HRP responsible for iodide oxidation has therefore been explored by kinetic and chemical modification studies using DEPC, a selective reagent for histidine (Miles, 1977; Lundblad and Noyes, 1984; Topham and Dalziel, 1986; Takeuchi *et al.*, 1986; Chang and Nowak, 1989; Burstein *et al.*, 1974; Sams and Mathews, 1988; Konpka and Waskell, 1988; Dumas and Raushel, 1990; Blanke and Hager, 1990). The inactivation of HRP by DEPC requires a large molar excess of the reagent in order to counteract its rapid hydrolysis in aqueous medium. The increase in absorption at 244 nm following incubation with DEPC and its reversal by hydroxylamine offer strong evidence for the formation of carbethoxyhistidine. The possible disubstitution of the imidazole can be excluded, because the inactivation is reversed by hydroxylamine. The formation of dicarboxyethylated imidazole derivative due to ring cleavage is also excluded, as this derivatization would not be reversed by hydroxylamine (Miles, 1977). The potential inactivation of HRP due to modification of lysyl, tyrosine, and cysteine residues with DEPC has also been ruled out. Moreover, all the cysteine residues in HRP are present in disulfide linkage (Welinder, 1979) and thus are not available for modification by DEPC. The inactivation of HRP by DEPC is a time-dependent bimolecular reaction, as evidenced in kinetic studies. The rate of inactivation shows a pH dependence, indicating that the inactivation is due to modification of a titratable residue with a pK_a value of 6.22. This is also strong evidence for histidine derivatization by DEPC, and the value is consistent with the pK_a values for histidine residues modified with DEPC in other enzymes (Takeuchi *et al.*, 1986, Cousineau and Meighen, 1976; Blanke and Hager, 1990).

The primary amino acid sequence of HRP revealed the presence of 3 histidine residues, namely His^{40}, His^{42}, and His^{170} at the heme periphery (Welinder, 1979). The spatial arrangement of the histidine molecules around the heme cavity has been depicted by computer modeling (Sakurada *et al.*, 1986). His^{42} and His^{40} are located at the distal pocket of the heme, whereas His^{170} is ligated with the heme iron at the fifth coordination position at the heme proximal side. Our studies on the stoichiometry of the histidine modified by DEPC indicate the modification of 2 histidine residues. Out of these 2 residues, the first one immediately reacts with DEPC but does not contribute to the catalytic activity. Although the modifi-

cation of the second residue is rather slow, it is needed for the catalytic activity of the enzyme (Figs. 5 and 6). The third histidine is not modified during the reaction. This residue is probably His^{170}, as it may resist modification due to its ligation with the heme iron through one of its nitrogens. No significant changes in the heme CD spectra and Soret absorption spectra provide further support for this deduction. These findings also eliminate the possibility of major alteration of the heme conformation or helicity of the protein structure resulting from extensive nonspecific modification by DEPC. As 2 of the 3 histidine residues of HRP are modified by DEPC and modification of the proximal His^{170} is ruled out, His^{40} and His^{42} are therefore the likely candidates for modification by DEPC. The x-ray crystallographic structural analysis of yeast cytochrome *c* peroxidase (Poulos and Kraut, 1980) and beef liver catalase (Fita and Rossman, 1985) has revealed the presence of a histidine residue at the heme distal pocket. From computer modeling by Sakurada *et al.* (1986), it is evident that HRP contains a distal histidine residue which is His^{42}. Sequence analysis of cytochrome *c* peroxidase (Kaput *et al.*, 1982) and HRP (Welinder, 1979) shows a strong homology in the amino acid sequence of the two enzymes near the heme distal pocket. Amino acid residues 48–52, *i.e.* Arg^{48}-Leu-Ala-Trp-His^{52}, of cytochrome *c* peroxidase are comparable with similar residues 38–42, *i.e.* Arg^{38}-Leu-His-Phe-His^{42}, of HRP. His^{52} in cytochrome *c* peroxidase and His^{42} in HRP appear to be the same residue. His^{40} in HRP is replaced by Ala^{50} in cytochrome *c* peroxidase and is not conserved in any of the peroxidases known so far (Tien and Tu, 1987). Moreover, Sakurada *et al.* (1986) showed that His^{42} and Arg^{38} are very close to the heme edge at the distal pocket of the HRP. They proposed that the distal His^{42} is perpendicular to the heme iron and may play an important role in electron transfer from iodide to heme ferryl group (Sakurada *et al.*, 1987a). That iodide may interact with the distal histidine before it donates its electrons to the heme iron of lactoperoxidase has also been proposed (Sakurada *et al.*, 1987a). Hence, it is more likely that modification of this distal histidine will affect iodide oxidation. His^{40} in HRP appears to be more exposed and away from the heme plane. This explains its faster reactivity with DEPC without affecting the enzyme activity. His^{42} is hanging in the heme cavity perpendicular to the heme plane (Sakurada *et al.*, 1986) and is not easily approachable by DEPC. This explains its slower reactivity even though it may be essential for the catalytic activity of the HRP. Recently, Blanke and Hager (1990) have suggested from their chemical modification studies on chloroperoxidase that a single histidine residue is essential for the catalytic activity of the enzyme. Based on secondary structure prediction by Kenigsberg *et al.* (1987), they have modeled the active site peptide (Blanke and Hager, 1990) and indicated that residues 30–38, *i.e.* Pro^{30}-Ala-Leu-Asn-Ala-Leu-Ala-Asn-His^{38}, show homology with residues 44–52 of cytochrome *c* peroxidase, where residue 52 is the distal histidine. Based on this homology, they have identified histidine 38 as the distal residue and assigned it to play an important role in the chlorination reaction. Similarly, the sequence homology of HRP with cytochrome *c* peroxidase at the distal pocket and the evidence from our chemical modification studies suggest that a unique histidine residue is required for the catalytic activity of HRP in relation to iodide oxidation. The most likely candidate is histidine 42.

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